

# Isolation from Human Plasma of a Plasminogen Activator Identical to Urinary High Molecular Weight Urokinase

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**ABSTRACT** Two different plasmatic plasminogen activators (PA) can be demonstrated after sodium dodecyl sulfate polyacrylamide gel electrophoresis of plasma freshly collected from resting volunteers, followed by transfer of the gels onto plasminogen-rich fibrin-agarose plates. These two PA are also present in plasmas deficient in coagulation Factor XI, Factor XII, prekallikrein, or high molecular weight-kininogen. The slower-moving PA has an apparent 85,000  $M_r$  and is immunologically unrelated to urokinase (UK). The faster moving PA was isolated by immunoadsorption of plasma on anti-UK IgG coupled to Sepharose 4B and appears to be identical to urinary high molecular weight-UK.

## INTRODUCTION

Kallikrein and activated coagulation Factor XI are able to convert plasminogen to plasmin in purified systems (1, 2), but these two enzymes are not effective plasminogen activators (PA)<sup>1</sup> in whole human plasma. As of now only two effective PA have been isolated and well characterized. The first, tissue plasminogen activator (t-PA), is found in most human and animal tissues (3) and was also isolated as "vascular PA" from blood vessel perfusates (4, 5). The second, UK or urokinase-type PA (u-PA), may be isolated from human urine (6) or cell culture fluid (7). t-PA and u-PA are immunologically unrelated (8, 9).

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<sup>1</sup>Abbreviations used in this paper: BSA, bovine serum albumin; CRM, cross-reacting material;  $HM_r$ -UK, high molecular weight-urokinase; PAGE, polyacrylamide gel electrophoresis; t-PA, tissue plasminogen activator; UK, urokinase.

For many years it was believed that urokinase (UK) does not exist in plasma (8, 9), but recently Wijngaards et al. (10) were able to quench PA activity in dextran-activated plasma using specific anti-UK IgG and Wun et al. (11) have isolated u-PA from human plasma Cohn fraction IV-1. Using a zymographic gel procedure (12), these investigators did not detect activity in fresh human plasma near the molecular weight regions characteristic of urinary UK. The demonstration of u-PA in, and its isolation from, fresh human plasma is the subject of this report.

## METHODS

### Material

Imidazole of analytical grade, Tris, bovine serum albumin (Fraction V; BSA), and Triton X-100 were purchased from Fluka AG (Buchs, Switzerland); thrombin (Topostasin, 3,000 National Institutes of Health [NIH] units per vial) from Roche (Basel, Switzerland); fibrinogen (bovine, fraction I, plasminogen-rich, 70% clottable and plasminogen-free, 68% clottable) from Poviet Producten (Bostel, Holland); acrylamide, bis-acrylamide,  $N,N,N',N'$ -tetra-methylethylenediamine, ammonium persulfate and sodium dodecyl sulfate (SDS) from Bio-Rad (München, West Germany); agarose L from Behringwerke (Marburg, West Germany); Coomassie Brilliant Blue R 250 from Merck AG (Darmstadt, West Germany); bromophenol blue from Sigma Chemical Co. (St. Louis, MO); CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden). Pure high molecular-weight urokinase ( $HM_r$ -UK) (single band of 54,000  $M_r$  on SDS polyacrylamide gel electrophoresis (PAGE); sp act: 100,000 IU/mg) was a gift from Hypolab (Coinsins, Switzerland). The first World Health Organization International Reference preparation of human UK was kindly supplied by Dr. P. Gaffney from the National Institute for Biological Standards and Control, London.

### Procedure

**SDS-PAGE-fibrin-agarose.** SDS-PAGE was done on 12.7 × 14 × 0.15 cm slabs according to Laemmli (13), with a 4% stacking and a 10% separating gel. The sample buffer contained no  $\beta$ -mercaptoethanol. 15  $\mu$ l of plasma mixed with 15  $\mu$ l of sample buffer were electrophoresed at 25 mA per

gel until the dye front reached the bottom of the gel. The gels were washed for 1 h in 2.5% Triton X-100 to remove SDS, placed on fibrin-agarose underlays and incubated at 37°C until lysis bands were clearly developed (usually from 6 to 16 h). These underlays were prepared as follows: 24 ml of fibrinogen (10 mg/ml) in 50 mM imidazole-140 mM NaCl, pH 7.35, were mixed on 22.5 × 22.5 cm plastic plates with 15 ml of 2.5% agarose in deionized water and 3 ml of thrombin (1 NIH U/ml) in imidazole-NaCl (the latter two solutions were kept at 54°C; the plastic plates were preheated to 68°C). Molecular weight calibration was performed using proteins of known molecular weight, run under the same conditions. For photography, the underlays were washed in 0.1 M NaCl, dried, and stained with Coomassie Blue.

**Anti-UK IgG and anti-UK columns.** Anti-UK serum was obtained from a goat, immunized by three subcutaneous injections of 100 µg pure HM<sub>r</sub>-UK in complete Freund's adjuvant at 1-mo intervals. Anti-UK immunoglobulins were purified by adsorption on UK-Sepharose; 2.3 mg of pure HM<sub>r</sub>-UK in 0.1 M borate-0.5 M NaCl, pH 8.3, were coupled to 1 ml CNBr-activated Sepharose 4B according to the manufacturer's recommendation (14). After extensive washing, the columns were eluted with 0.1 M acetic acid-0.15 M NaCl, pH 3. The eluted protein showed a single large band of 150,000 M<sub>r</sub> on unreduced and two bands of 50,000 and 25,000 M<sub>r</sub> on reduced SDS-PAGE, and one precipitation line in double diffusion with rabbit anti-goat IgG, indicating that it was IgG. 1 mg of purified anti-UK IgG quenched 5,000 IU of UK (50 µg) in a fibrin plate assay but did not cross-react with partially purified human t-PA (isolated from colon, 68,000 M<sub>r</sub>), kallikrein, or Factor XII. Anti-UK IgG was coupled to Sepharose (12.5 mg of IgG in 0.1 borate-0.5 M NaCl, pH 8.3, per ml CNBr-Sepharose 4B (14); IgG concentrations were measured by optical density, taking E<sub>280</sub><sup>1%</sup> = 14).

**Immunoabsorption of plasma.** 4 ml of fresh normal human plasma were passed over 0.2 ml of anti-UK Sepharose. The column was washed with 20 ml 0.5 M NaCl-0.1 M phosphate, pH 7, followed by 7 ml of 0.1 M phosphate, pH 7, containing 0.1% BSA. Elution was performed with 0.1 M sodium phosphate/phosphoric acid, pH 3, containing 0.1% BSA. The pH of each 1 ml-fraction was immediately adjusted to 7 with 1 M Tris. Aliquots of 50-µl eluate fractions were mixed with 50 µl sample buffer and run on SDS-PAGE.

## RESULTS

After SDS-PAGE of fresh citrated plasma collected from resting volunteers, two major lysis bands with apparent molecular weight of 85,000 and 43,000 (Fig. 1, A, B, C) were detected on plasminogen-rich fibrin underlays. Lysis was not observed on plasminogen-free underlays. Both bands were present in plasma-deficient in Factor XI or HM<sub>r</sub>-kininogen (both cross-reacting material, negative [CRM-] obtained from Dr. R. W. Coleman, Temple University, Philadelphia), Factor XII, or prekallikrein (plasma GK 1701, lot 96, CRM-; purchased from George King Inc., Overland Park, KA). Fig. 1, a, b, c, shows that incubation of normal plasma with anti-UK IgG suppressed the 43,000-M<sub>r</sub> lysis band, but not the 85,000-M<sub>r</sub> band. The 43,000-M<sub>r</sub> band was likewise missing, when plasma was passed over an anti-UK column (Fig. 1, a', b', c').

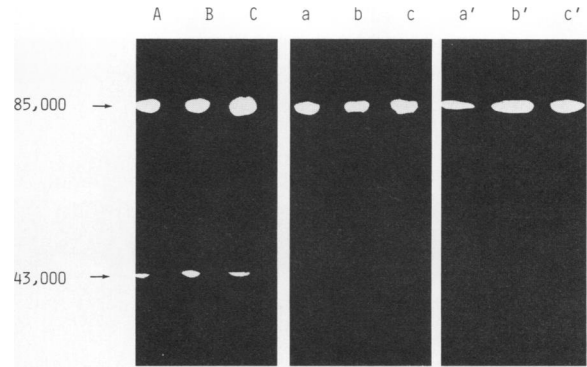


FIGURE 1 Determination of the apparent molecular weight of plasminogen activators in normal human plasma after SDS-PAGE and transfer of proteins onto plasminogen-rich fibrin-agarose. A, B, C: zymograms of three different fresh plasmas; a, b, c: same plasmas after 1 h incubation at 37°C with anti-UK IgG (0.01 mg IgG/ml plasma); a', b', c': same plasmas after adsorption on anti-UK columns.

From this column, a 52,000 D PA was eluted in acid buffer (Fig. 2). Highly purified HM<sub>r</sub>-UK, diluted in the same buffer, also produced a lysis band at 52,000 D.

When HM<sub>r</sub>-UK (M<sub>r</sub>: 54,000) and, as a marker, purified pig heart t-PA (M<sub>r</sub>: 68,000) were added to various dilutions of human plasma, the apparent M<sub>r</sub> of HM<sub>r</sub>-UK increased with decreasing plasma concentrations (from 44,000 to 52,000 M<sub>r</sub>) but that of the marker t-PA did not change (Fig. 3.1). BSA added to solutions containing HM<sub>r</sub>-UK produced the same phenomenon (Fig. 3.2); at 3.2% BSA, the apparent molecular weight of UK was identical to that found in undiluted plasma (44,000 D). This explains why the plasmatic u-PA moved as a protein of 43,000 M<sub>r</sub> in undiluted plasma

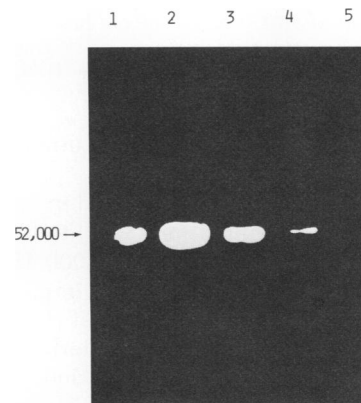


FIGURE 2 Apparent molecular weight of the plasmatic PA eluted from an anti-UK column. 1-5: zymograms of the first five eluted 1 ml-fractions.

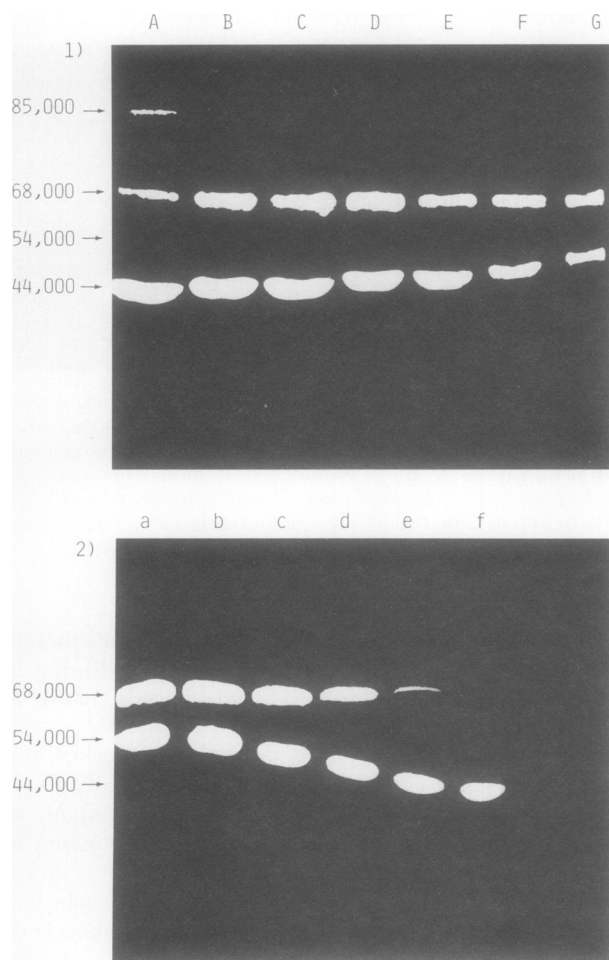


FIGURE 3 Effect of the albumin concentration on the apparent molecular weight of  $HM_r$ -UK and of purified pig heart t-PA activator. 3.1. Zymograms of  $HM_r$ -UK and of pig heart t-PA added to plasma diluted in imidazole-NaCl buffer. Each sample contained 0.2 U of each PA/15  $\mu$ l. (A: undiluted; B-C: 1/2; D-E: 1/4; F: 1/8; G: 1/16 diluted). 3.2. Zymograms of  $HM_r$ -UK and of pig heart t-PA added as above to buffer containing various concentrations of BSA (a: 0%; b: 0.2%; c: 0.4%; d: 0.8%; e: 1.6%; f: 3.2%).

(Fig. 1) and of 52,000  $M_r$  after its elution in a buffer containing 0.1% BSA (Fig. 2).

The 43,000- $M_r$  band was also found in serum. Thus, plasma UK does not bind appreciably to fibrin during coagulation. On the contrary, the 85,000  $M_r$  band was not detectable in serum; this PA is immunologically related to t-PA (preliminary data; not shown).

The SDS-PAGE fibrin-agarose underlay method allows a semi-quantitative determination of  $HM_r$ -UK activity. A good correlation ( $r > 0.95$ ) was observed between the logarithm of the  $HM_r$ -UK concentrations and the width of the lysis zone in 50- $\mu$ l samples con-



FIGURE 4 Semiquantitative determination of UK-related fibrinolytic activity of normal human plasma. A-E: lysis bands obtained with  $HM_r$ -UK standards (A: 0.4; B: 0.2; C: 0.1; D: 0.05; E: 0.025 IU/ml). a-e: pooled first 4 ml eluate from anti-UK columns after immunoadsorption of five different normal plasmas.

taining 0.2% or less albumin (Fig. 4; A-E). Therefore, to determine plasmatic u-PA activity in fresh normal human plasma, 4 ml of plasma were passed over an anti-UK column. After extensive washing of the column, the u-PA activity was eluted and aliquots of the pooled first 4 ml were run on SDS-PAGE. The width of the lysis band on the fibrin-agarose underlays was compared to simultaneously run UK-standards. In 10 healthy volunteers, u-PA activity ranged between 0.03 and 0.3 IU/ml of plasma (Fig. 4; a-e), corresponding to 0.3-3 ng/ml of  $HM_r$ -UK.

## DISCUSSION

While this work was in progress, two other groups of investigators also reported on the presence of u-PA in human plasma. Wun et al. (11) could not demonstrate u-PA activity in fresh human plasma using a slightly different SDS-PAGE zymographic assay, but convincingly purified u-PA from plasma Cohn fraction IV-1. Wijngaards et al. (10) quenched u-PA activity in dextran activated plasma euglobulin fractions. The concentration of active enzyme we have measured (0.3-3 ng/ml) corresponds to that reported by Wijngaards et al. (10), but is lower than the concentration of the UK-antigen (12 ng/ml) determined with a radioimmunoassay by Wun et al. (11). The finding of a UK-antigen concentration higher than that of u-PA activity in normal plasma is in all likelihood due to the presence of several inhibitors that form complexes with UK, such as  $\alpha_2$ -antiplasmin,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -antitrypsin, and antithrombin III. Since the plasmatic u-PA appears not to bind to fibrin, its physiological role in thrombolysis remains to be determined.

## ACKNOWLEDGMENTS

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