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A HIGHLY SENSITIVE CHROMOGENIC MICROTITER PLATE ASSAY FOR PLASMINOGEN ACTIVATORS WHICH QUANTITATIVELY DISCRIMINATES BETWEEN THE UROKINASE AND TISSUE-TYPE ACTIVATORS

Beth Y. Karlan[1], Ann S. Clark[1,2], and Bruce A. Littlefield[1,3,*]

[1]Department of Obstetrics and Gynecology, [2]Section of Neuroanatomy, and [3]Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510

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A simple and highly sensitive chromogenic microtiter plate assay for plasminogen activators is described. The assay is based on plasmin cleavage of the synthetic tripeptide plasmin substrate H-D-norleucyl-hexahydrotyrosyl-lysine p-nitroaniline, which yields the yellow chromophore p-nitroanilide. Production of the latter compound is then quantitated spectrophotometrically at 405 nm on an ELISA plate reader. Linearity of the assay can be achieved over at least four orders of magnitude in a single experiment (0.01-100 milliPloug units) with appropriate incubation times. Capitalizing on tissue-type plasminogen activator's dependence on fibrin for enzymatic activity, the selective use of soluble fibrin products allows discrimination between urokinase and tissue-type activator. The utility of this aspect of the assay for the analysis of complex samples containing both types of plasminogen activitors is demonstrated.

PAs are highly specific serine proteases which play roles in a number of biological processes (reviewed in refs. 1 and 2). Named for their ability to cleave the inactive zymogen plasminogen to the active general protease plasmin, PAs play an essential role in the process of fibrinolysis. More recently, PAs have been associated with a wide variety of other biological processes, incuding ovulation, trophoblast implantation, mammary gland

* To whom correspondence and requests for reprints should be sent.

Abbreviations: EDTA, *ethylenediaminetetraacetic acid (disodium salt);* ELISA, *enzyme-linked immunosorbent assay;* (m)PU, *(milli)Ploug units;* NA, *p-nitroaniline;* NHLNA, *H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanalide;* PA(s), *plasminogen activator(s);* SDS-PAGE, *sodium dodecyl sulfate polyacrylamide gel electrophoresis;* SFP, *soluble fibrin products;* tPA, *tissue-type plasminogen activator;* UK, *urokinase.*

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involution, cellular differentiation, and maturation of peptide hormones, to name but a few (1,2). PA production has also been demonstrated in many tumor cell types and has frequently been correlated with tumor cell growth rates and metastatic potential (1,2). UK and tPA are the two major, and possibly only, mammalian PA types.

In the past, most investigators including ourselves have measured PA activity using assays based on plasmin-dependent cleavage of radioiodinated fibrin or fibrinogen (3-5). Although sensitive, the utility and convenience of such assays are limited by the requirement for radioiodination, the need to dispose of radioactive wastes, the loss of specific activity of radiolabeled substrates with time, and variabilities between replicate data points. Chromogenic protease substrates consisting of NA derivatives of amino acids or small synthetic peptides, first developed by Erlanger et al (6), offer convenient and sensitive alternatives to the use of radiolabeled substrates (7-9). Using this approach, we have employed the chromogenic tripeptide plasmin substrate NHLNA to develop a simple and highly sensitive chromogenic PA assay adapted for 96-well microtiter plates. Here we describe the assay using conditions wherein linearity can be obtained over at least four orders of magnitude. In addition, we show that the selective use of SFP allows quantitative discrimination between UK and tPA activities, and permits accurate measurement of each PA type in complex mixtures such as conditioned media.

MATERIALS AND METHODS

Preparation of Assay Components: The chromogenic plasmin substrate NHLNA (American Diagnostica, Greenwich, CT) was dissolved in water to a concentration of 5 mM; a working solution was then prepared by dilution to 1.6 mM in Buffer Z (50 mM Tris, 0.1 M NaCl, 1 mM EDTA, 0.01% Tween 80, pH 7.5). This method was employed since direct dissolution of NHLNA in Buffer Z proved difficult. SFP (American Diagnostica) was dissolved directly in Buffer Z to a concentration of 4 mg/ml, followed by dilution in the same buffer to 100 µg/ml. Unused portions of both stock and working solutions of NHLNA and SFP were stored at 4°C and were found to be stable for at least 2 weeks. The SFP used in the studies reported here was a commercially available preparation of fibrin solubilized by digestion with reptilase, a thrombin-like protease present in the venom of the South American viper Bothrops atrox (10,11). However, SFP prepared by other procedures, such as cyanogen bromide cleavage (7,12), would probably be equally effective in this assay.

Plasminogen was prepared according to the method of Deutsch and Mertz (13), except that aprotinin (Sigma) was added to both the diluted plasma starting material and subsequent washing buffers (final concentration, 0.1 TIU/ml), and ε-aminocaproic acid was removed from the column eluate by dialysis against several changes of Buffer Z (24 h at 4°C) rather than Sephadex G-25 chromatography. The plasminogen preparation (judged 95% pure by SDS-PAGE) was adjusted to 1 mg/ml following measurement by the method of Bradford (14), and fractioned into 0.5 ml aliquots which were then frozen at -80°C until use.

UK (Calbiochem) and single-chain tPA (American Diagnostica) standards were prepared at 100 and 1000 PU/ml, respectively, in Buffer Z and frozen in small aliquots at -80°C until use.

Plasminogen Activator Assay: Incubations were carried out in 100 ul volumes in 96-well flat-bottomed microtiter plates. Incubation volumes consisted of 25 µl of sample or PA standard, 25 µl of 100 µg/ml SFP, 25 µl of 1.6 mM NHLNA, and 25 µl of 1 mg/ml plasminogen, added in that order. Details of the SFP, NHLNA, and plasminogen working solutions are presented immediately above. Buffer Z was used instead of SFP or plasminogen when appropriate (see below). Time zero represented the time of addition of plasminogen. Incubation of the covered plates was at 37°C in a small oven. Hydrolysis of the colorless NHLNA by plasmin results in production of NA, whose yellow color was read periodically at 405 nm using a Titertek Multiskan Plus ELISA plate reader.

All samples and standards were assayed both in the presence and absence of plasminogen, with the difference in NA production between these two groups (i.e., plasminogen-dependent NA production) representing PA activity. PA activity in all samples was referenced to human UK standard curves run in each experiment. In experiments where samples were analyzed both with and without SFP, two separate UK standard curves, one with and one without SFP, were run to allow samples to be compared to appropriate UK standard curves.

Cells and Cell Culture Conditions: LICR-LON-HMy2 human lymphoblasts (hereafter referred to as HMy2 cells) were grown as previously described (5). OVCA 433 ovarian cancer cells, originally derived from a human epithelial ovarian cancer (15), were generously provided by Drs. Vimla Band, Vincent Zurawski Jr., and Robert Knapp at Harvard Medical School. These cells were grown in Eagle's Minimum Essential Medium with Earl's salts, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were plated at 1.5×10^6 cells per 75 cm^2 culture flask, incubated in a humidified atmosphere containing 5% CO_2, and passaged every 3-4 days. Cell counts were performed using a hemocytometer and viabilities determined using standard trypan blue exclusion techniques. Viabilities under these growth conditions were generally greater than 95%.

Preparation of Serum-Free Concentrated Conditioned Media: Concentrated conditioned medium from HMy2 cells was prepared as previously described (5), except that the dialysis period was shortened to 24 h with two buffer changes. Concentrated conditioned medium from OVCA 433 cells was prepared in a similar manner, except that the incubation in serum-free medium was for one hour rather than 24 h due to the relatively large amount of PA produced by these cells.

SDS-PAGE Zymography: SDS-PAGE zymography of PAs was performed using previously described modifications (5) of the methods of Roche et al (16).

RESULTS

In the presence of SFP, plasminogen-dependent NA production was a linear function of the known amounts of UK and tPA activities added to the assay (Fig. 1). Moreover, equal amounts of the two PA types led to the generation of approximately equal amounts of NA. In contrast, without SFP, only UK showed appreciable activity, while tPA activity was virtually eliminated. This observation reflects the well-known major stimulatory effect of fibrin on tPA activity (17,18). The slight stimulation of UK by SFP seen in Figure 1 is consistent with previous reports of a relatively minor stimulation of UK activity by fibrin (19). As will be discussed below, this differential sensitivity to SFP can be exploited to discriminate between UK and tPA activities in complex mixtures or biological samples.

To determine the useful range of sensitivity of the PA assay, a single standard curve of UK spanning the range of 0.01-100 mPU was prepared on a single 96-well microtiter plate. As illustrated in Figure 2, four orders of magnitude of PA activity could be accurately assayed simply by allowing the

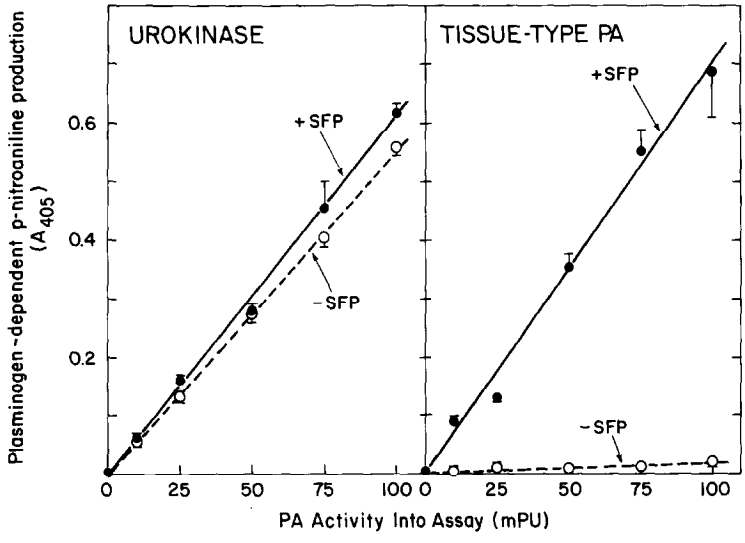

Figure 1. Linearity of plasminogen-dependent NA production as a function of UK and tPA concentration. The indicated amounts of UK (left) and tPA (right) standards were assayed for plasminogen-dependent NA production as described in Material and Methods. The incubation time for this experiment was 40 min. Standard curves were determined in the presence (●) and absence (○) of 25 µg/ml SFP for both UK and tPA. Each point represents the mean ± range of duplicate determinations.

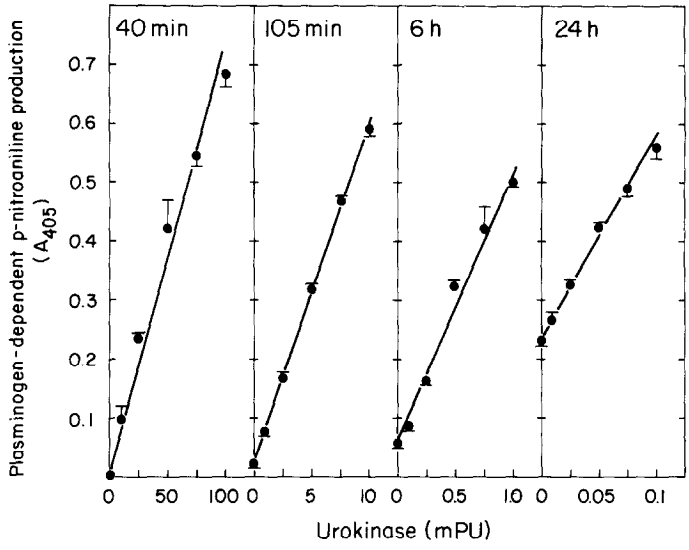

Figure 2. Sensitivity ranges of the PA assay as a function of incubation time. A single UK standard curve spanning four orders of magnitude was prepared (using SFP) on a single 96-well microtiter plate. Plasminogen-dependent NA production was assessed spectrophotometrically at various times, and those times which produced linearity in the four PA activity ranges shown on the x-axis were selected and plotted. Each point represents the mean ± range of duplicate determinations.

plate to incubate for increasing amounts of time. Absorbance readings at 40 min yielded a useful linear range between 0 and 100 mPU of activity; at 105 min, the 0-10 mPU scale was linear, and after 6 h of incubation, useful linearity was achieved between 0 and 1 mPU. By allowing the incubation to continue for a full 24 h, linearity between 0 and 0.1 mPU was demonstrated. The upward shift of the zero value with increasing time probably resulted from small amounts of plasmin contaminating the plasminogen preparation. Thus, the utility of this assay at very low PA levels is limited primarily by the purity of the particular plasminogen preparation used.

The dependence of tPA on SFP for enzymatic activity (Fig. 1) suggested that SFP could be used to quantitatively discriminate between UK and tPA activities in samples containing mixtures of these two enzymes. This was tested by mixing known quantities of UK and tPA standards and assaying the mixtures for PA activity in the presence and absence of SFP. Mixtures assayed in the presence or absence of SFP were compared to separate UK standard curves run in the presence or absence of SFP, respectively. UK

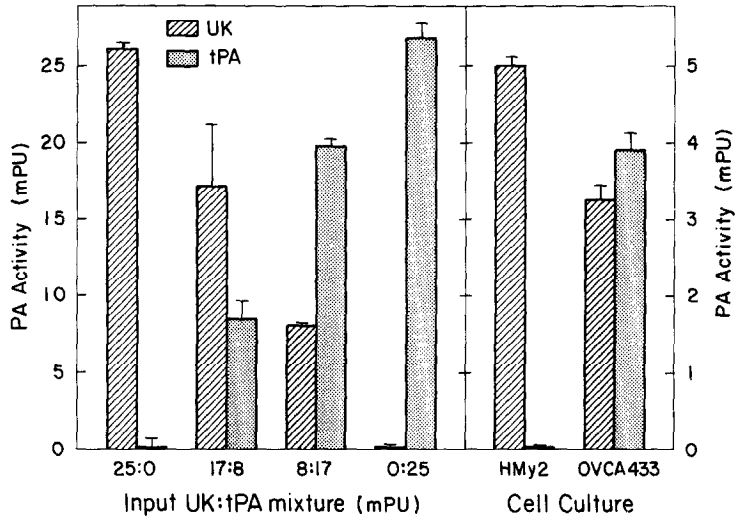

Figure 3. Discrimination between UK and tPA activities using SFP. Known mixtures of UK and tPA standards (left panel), and serum-free concentrated conditioned media from HMy2 and OVCA 433 cells (right panel) were assayed in both the presence and absence of SFP, and compared to UK standard curves run in the presence and absence of SFP, respectively. UK and tPA activities were calculated as described in the text. Values represent means ± standard error of triplicate determinations.

activity was considered to be that activity measurable without SFP, while tPA activity was considered to be the difference between activities measured in the presence and absence of SFP. As shown in the left panel of Figure 3, the calculated UK and tPA activities were remarkably close to the known input UK and tPA levels. Thus, the selective use of SFP allowed accurate measurement of UK and tPA standards in known mixtures.

To demonstrate the assay's ability to discriminate between UK and tPA in biological samples other than known mixtures of standards, serum-free conditioned media from the human lymphoblast cell line HMy2 and the human ovarian carcinoma line OVCA 433 were assayed with and without SFP. UK and tPA activities were then calculated as described above. The right panel of Figure 3 demonstrates that conditioned medium from HMy2 cells contained only UK activity, while medium from OVCA 433 cells had both UK and tPA activities. These results were then substantiated by SDS-PAGE zymography. As seen in Figure 4, the PA in conditioned medium from HMy2 cells (Lane A) comigrated only with the 55,000 dalton high molecular weight form of UK (Lane C), while

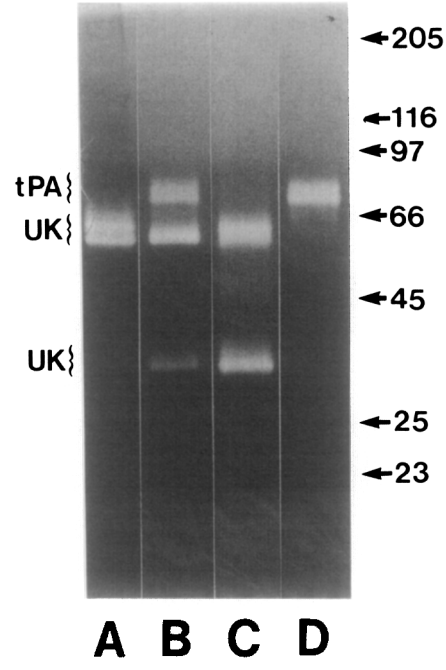

Figure 4. SDS-PAGE zymography of concentrated conditioned media from HMy2 and OVCA 433 cells. Serum-free concentrated conditioned media from HMy2 and OVCA 433 cells were analyzed by SDS-PAGE zymography as described in Materials and Methods. Lanes A and B, concentrated conditioned media from HMy2 and OVCA 433 cells, respectively; Lane C, human UK standard (55,000 dalton native form and 33,000 dalton enzymatically-active proteolysis product); Lane D, human tPA standard. The positions and sizes (in kilodaltons) of molecular weight markers run in a parallel lane are indicated on the right margin. All of the enzymatic activities seen on this gel are plasminogen-dependent (i.e., true PA activities), since no direct caseinolytic activity was observed in a parallel gel polymerized in the absence of plasminogen (data not shown).

conditioned medium from OVCA 433 cells (Lane B) contained PA species which comigrated with both UK (Lane C) and tPA (Lane D). Other studies in our laboratory have immunologically verified the HMy2 PA as UK, and the two OVCA 433 PAs as UK and tPA (data not shown). Thus, the selective use of SFP in the PA assay allows quantitative discrimination between UK and tPA activities in unknown biological samples.

DISCUSSION

The chromogenic PA assay just described provides a simple and highly sensitive method for measuring and discriminating between UK and tPA activities in biological samples. By periodically monitoring the color

development, one can easily choose appropriate incubation times to measure PA activities over a wide range of PA levels. The ability to perform this assay in 96-well microtiter plates which can be read in an ELISA plate reader permits the simultaneous analysis of many samples with minimal effort. Finally, although discrimination between UK and tPA activities on the basis of fibrin dependence cannot replace the need for immunologic identification of unknown PA species, it can provide a fairly reliable preliminary identification of these two PA types. Thus, the simple assay described here should facilitate the study of UK and tPA involvement in the diverse biological processes in which they now appear to be involved.

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