Partial Purification and Characterization of Epidermal Plasminogen Activator and Their Inhibitor

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Plasminogen activator (PA) and PA inhibitor were partially purified from 2-d-old rat epidermis and characterized in order to elucidate the enzyme–inhibitor interaction in epidermis. PA extracted with buffer containing KSCN was first purified by Blue-Sepharose affinity chromatography. Separation of two PAs, with relative molecular mass (M_r) of 66000 and 44000, was accomplished by Con A-Sepharose column chromatography. The M_r 66000 enzyme had the properties of tissue-type PA (t-PA), while the M_r 44000 enzyme showed those of urokinase-type PA (u-PA) as determined by immunological and fibrin-binding studies. PA inhibitor was extracted in 1,4-piperazinediethanesulphonic acid buffer and purified by $(NH_4)_2SO_4$ precipitation, gel filtration followed by a Mono Q column in an FPLC system. This inhibitor showed M_r 60000 and inhibited human u-PA activity in a dose- and time-dependent manner, but did not inhibit the activity of t-PA from human and murine melanoma cells or plasmin. It inhibited epidermal PA, M_r 44000, more effectively than it did the M_r 66000 epidermal PA. It was stable at 60 °C for 60 min or between pH 5 and 11. This study indicates that both u-PA and t-PA function in normal rat epidermis. On the other hand, an inhibitor which preferentially acts against u-PA exists, but inhibitor to t-PA does not appear to operate under normal epidermal functions.

Keywords plasminogen activator; purification; fibrin binding study; plasminogen activator inhibitor; inhibitor specificity; rat epidermis

Two different genes for plasminogen activator (PA), one for tissue-type (t-PA) and the other for urokinase-type (u-PA) exist,[1] although many types of cells seem to express only one type of PA. Human uterine tissue,[2] endothelial cells[3] and melanoma cells[4] have been used as cell sources for t-PA, whereas kidney cells[5] and cultured human monocyte-macrophages[6] have been regarded as tissue sources for u-PA. However, recent experimental findings have indicated that one cell type may alternate expression of the PA genes, depending upon its functional state. Examples are seen in colon and breast glandular tissue during malignant transformation,[7] and estradiol-treated MCF-7, a stable cell line of human breast cancer origin.[8] The relative molecular mass M_r of PA from normal epidermal keratinocytes varies considerably among the reports. PAs of M_r 35000 to 62000 were found from human[9] and PAs of M_r 48000 to 95000 from rodents.[10] It seems possible that the M_r heterogeneity may reflect certain stages of epidermal cell maturation.

PA inhibitor is also expressed by a group of proteins.[11] Representative PA inhibitors were purified from placenta, endothelial cells, plasma and platelets, and fibroblasts. Epidermal PA inhibitor, M_r 43000 was purified from human cornified cells by Hibino *et al.*[12] and was reported to inhibit u-PA but not t-PA or plasmin activity.

In this study we purified PAs and PA inhibitor from 2-d-old rat epidermis. Immunological and biochemical properties of PAs and their interaction with PA inhibitor were investigated to better understand the relationship between the enzymes and inhibitor in epidermal cells.

Materials and Methods

Materials Bovine fibrinogen (70% clottable) was from Miles Laboratories, Inc. (Elkhart, ID), human urokinase (30000 International units (IU)/vial) was from Green Cross Co. (Osaka, Japan), S-2251 (H-D-Val–Leu–Lys–*p*-nitroanilide) was from Kabi Diagnostica (Studsvik, Sweden), Sephadex G-25, G-100, G-150, Sepharose-4B, epoxy-activated Sepharose 6B, lysine-Sepharose 4B, diethylaminoethyl (DEAE)-Sepharose CL-6B, Con A-Sepharose, Mono Q HR5/5 and the FPLC system were from Pharmacia Fine Chemicals (Piscataway, NJ), bovine serum albumin, bovine fibrinogen (plasminogen, thrombin-free) were from Calbiochem-Behring Corp. (La Jolla, CA), α-chymotrypsin type II (bovine pancreas), pepstatin A, *p*-chloromercuribenzoic acid (PCMB), diisopropyl fluorophosphate (DFP), 1,4-piperazinediethanesulphonic acid (Pipes), α-casein, were from Sigma Chemical Co. (St. Louis, MO), Cibacron blue F3GA was from Ciba-Geigy (Basel, Switzerland), and goat anti-human t-PA immunoglobulin G (IgG) and goat anti-human-urokinase IgG were from American Diagnostica, Inc. (Greenwich, CT).

Rat plasminogen was prepared from citrated plasma by affinity chromatography on lysine-Sepharose followed by gel filtration on Sephadex G-150[13] and DEAE-Sepharose column chromatography.[14] It was used to generate rat plasmin after incubation with urokinase in 20 mM Tris–HCl (pH 7.4) containing 0.14 M NaCl and 0.1% bovine serum albumin at 37 °C for 15 min. Human plasminogen was prepared from outdated human plasma by affinity chromatography on lysine-Sepharose.[15] The concentration of purified plasminogen was determined by measuring 280 nm ($E^{1\%}=16.1$), and molarity was calculated based on a M_r of 93000.

Fibrinogen fragments were prepared from bovine fibrinogen (plasminogen, thrombin-free) at room temperature by the method of Verheijen *et al.*[16]

Melanoma PAs were purified from serum-free medium of cultured B16 cells (HGH18, provided by Dr. F. Hu, University of Oregon) and of cultured human melanoma cells (Bowes, provided by Dr. D. B. Rifkin, New York University Medical Center) on a zinc-chelate column according to Rijken and Collen.[4]

PA Activity Assay Spectrophotometric assay using S-2251 by the method of Verheijen *et al.*[16] was used. Samples were incubated in 60 mM Tris–HCl (pH 7.5) containing 0.1% (v/v) Tween 80, 0.3 mM S-2251 and 0.13 μM human or rat plasminogen with 120 μg/ml fibrinogen fragments. After adequate incubation, the reaction was stopped by adding 50 μl of 50% (v/v) acetic acid. Absorbance at 405 nm was measured against suitable blanks. Enzymatic activity was determined by comparing the absorbance at 405 nm with those after incubation with various concentrations of human urokinase.

PA Inhibitor Assay Fibrin plate assay with plasminogen-rich bovine fibrinogen[17] was used for quantitation of inhibitor activity during purification. Samples or 0.1% bovine serum albumin in 20 mM Tris–HCl (pH 7.5) containing 0.14 M NaCl were preincubated with an equal volume of 10 IU/ml urokinase at 37 °C for 30 min. The reaction mixture, 30 μl, was applied to the fibrin plate and residual activity was determined by comparing the lysed area with those produced after application of 5 and 2.5 IU/ml urokinase mixed with the bovine serum albumin solution. One inhibitor unit (INU) was designated as the amount of protein that inhibits 50% of 2 IU of urokinase.

Spectrophotometric assay was used for characterization of PA inhibitor. As a standard, 50 mIU/ml of urokinase (50 μl) was incubated with 50 μl of various concentrations of PA inhibitor at 37 °C for 30 min. Residual urokinase activity was assayed by the method described above and expressed as a function of the concentration of PA inhibitor. To determine

inhibitory effects for PAs from purified rat epidermis, B16 melanoma cells and Bowes melanoma cells the PAs were first adjusted to approximately 50 mIU/ml and incubated with the serially diluted PA inhibitor in equal volumes, and residual PA activity was calculated as described above.

Plasmin Inhibitor Assay Fifty microliters of rat plasmin (0.05 μM) was first incubated with 50 μl of PA inhibitor at 37 °C for 30 min, then incubated in 0.5 ml of 20 mM Tris–HCl (pH 7.5) containing 0.08% bovine serum albumin, 0.14 M NaCl and 0.3 mM S-2251 at 37 °C. After 1 h the reaction was stopped by the addition of 50 μl of 50% (v/v) acetic acid. Absorbance at 405 nm was measured against suitable blanks and residual plasmin activity was expressed as a function of the concentration of PA inhibitor.

Extraction and Purification of PAs and PA inhibitor from Rat Epidermis All procedures were performed at 4 °C except FPLC fractionation which was conducted at 22 °C. Spinous and granular cells were separated from the epidermis of 2-d-old Sprague-Dawley rats according to the method of Ito *et al.*[18] The cells were homogenized in a glass homogenizer in 20 mM Pipes (pH 6.8) containing 0.14 M NaCl. The homogenate was centrifuged at 100000 × *g* for 30 min and the supernatant (Pipes-extract) was separated. The precipitate was further extracted with 10 volumes of 0.1 M Tris–HCl (pH 8.0) containing 2 M KSCN by stirring for 2 h. The extract (KSCN extract) was separated by centrifugation at 100000 × *g* for 30 min, and dialyzed against 20 volumes of 20 mM Tris–HCl (pH 7.4) containing 1 M NaCl and 0.01% Tween 80 for 36 h with 3 changes.

PAs were purified from dialyzed KSCN extract diluted 1 : 1 with 20 mM Tris–HCl (pH 7.4) containing 0.01% Tween 80. The sample was applied to a Blue-Sepharose column (2.6 × 15 cm) prepared by the method of Heyns and de Moor[19] and equilibrated with 20 mM Tris–HCl (pH 7.4) containing 0.5 M NaCl and 0.01% Tween 80. The column was washed with the equilibration buffer and the equilibration buffer containing 0.1 M L-arginine until the absorbance at 280 nm was zero. The concentration of L-arginine was increased and the active fraction eluted with 0.5 M L-arginine was applied to a Sephadex G-25 column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 1 M NaCl and 0.01% Tween 80 to remove L-arginine. The PA fraction was then applied to a Con A-Sepharose column equilibrate with the same buffer. The column was first washed with the same buffer and the PA fraction eluted with 20 mM Tris–HCl (pH 7.5) containing 2 M KSCN, 0.4 M α-D-methylmannoside and 0.01% Tween 80 was pooled, concentrated (Amicon YM-10) and desalted through Sephadex G-25.

PA inhibitor was purified from Pipes-extract to which solid $(NH_4)_2SO_4$ was added until 50% saturation was achieved. The suspension was incubated for 1.5 h then centrifuged at 13000 × *g* for 20 min. The supernate was removed, adjusted to 90% saturation with $(NH_4)_2SO_4$, and centrifuged as before. The pellet was dissolved in, and dialyzed against, 20 mM Tris–HCl (pH 7.4) cotaining 0.14 M NaCl. The dialyzate was applied to a Sephadex G-100 column (1.6 × 90 cm) equilibrated with the same buffer. Active fractions were pooled and concentrated by ultrafiltration using an Amicon YM-10 membrane. After removal of NaCl by Sephadex G-25 gel filtration, the sample was applied to Mono Q column equilibrated with 20 mM Tris–HCl (pH 7.4) and run in an FPLC system. PA inhibitor was eluted by a gradient with addition of 1 M NaCl to the equilibration buffer. Active fractions were pooled and concentrated by Amicon YM-10.

Characterization of PAs Molecular weight was determined by zymography performed by the method of Heussen and Dowdle[9a] using slab gels (50 × 85 × 1 mm). The separating gel contained 0.1% sodium dodecyl sulfate (SDS), 0.1% α-casein and 11% polyacrylamide (pH 8.8) with or without rat plasminogen (13 μg/ml) and the stacking gel contained 0.1% SDS and 3% polyacrylamide (pH 6.8). After electrophoresis of samples the gels were washed with 2.5% Triton X-100 for 2 h with 2 changes to remove SDS and incubated in 0.1 M glycine–NaOH (pH 8.3) at 37 °C for 12 h for casein digestion. They were stained with 0.2% Coomassie Brilliant Blue R and unstained zones (indicating the mobility of proteinases) were recorded. Urokinase (M_r = 55000 and M_r = 33000) and chymotrypsin (M_r = 25000) were used as M_r markers.

Adsorption of epidermal PA on fibrin clots was determined according to method of Rijken and Collen.[4] Enzyme activity in the clot extract and clot supernatant was spectrophotometrically measured. The activity in the clot extract or supernatant was expressed as a percentage of total activity added.

Goat anti-human t-PA IgG or goat anti-urokinase IgG was coupled to Sepharose by the method of Nielsen *et al.*[20] The enzyme (about 0.5 IU/ml) was applied to a column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 1 mM $ZnCl_2$, 1 M NaCl and 0.5% Tween 80. The column was washed with 0.1 M Tris–HCl (pH 10.0) containing 1 M NaCl and 0.5% Tween 80 then the enzyme was eluted by 0.1 M glycine (pH 2.5) containing 1 mM $ZnCl_2$, 1 M NaCl and 0.5% Tween 80. The eluate was immediately adjusted to pH 7.5 and concentrated. The eluate was analyzed by zymography.

Goat anti-human t-PA IgG was diluted to 1, 2 and 4 μg/ml in 0.14 M NaCl and 50 μl of each or 0.14 M NaCl was mixed with 50 μl of PA purified from rat epidermis, B16 melanoma cells and Bowes melanoma cells and urokinases, all diluted to 50 mIU/ml. After 1.5 h at room temperature residual enzyme activity was measured spectrophotometrically and expressed as a function of the concentration of IgG.

The effects of chemical inhibitors were examined by incubation of PAs (50 mIU/ml) with 0.01, 0.1 or 1.0 mM DFP at 22 °C for 1 h, and other inhibitors (5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PCMB and 10 μM pepstatin A) at 37 °C for 10 min. DFP was removed from the reaction mixture by Sephadex G-25 column chromatography. Percent inhibition was determined by measuring the residual PA activity spectrophotometrically.

Inhibition of PA Activity by PA Inhibitors PAs purified from rat epidermis, B16 melanoma cells and Bowes melanoma cells were first adjusted to approximately 50 mIU/ml and incubated with an equal volume of serially diluted PA inhibitor purified from rat epidermis, and the residual PA activity was calculated.

Protein Determination Protein in the column effluents was monitored at 280 nm; the method of Lowry *et al.*[21] was used with bovine serum albumin as the standard.

Results

Partial Purification of PAs The results of purification of PA are summarized in Table I. Epidermal PAs effectively bound to a Blue-Sepharose column and the activity was eluted with 0.5 M L-arginine. The recovery was about 360% and a more than 42-fold increase in specific activity was achieved in a typical experiment. On Con A-Sepharose affinity column chromatography, 1% of the original activity appeared in the pass-through fraction (fraction I) and the major activity was eluted with 0.4 M α-D-methylmannoside (fraction II). Zymographic analysis of fractions I and II showed the enzymes to gave M_r 44000 and 66000, respectively (Fig. 1).

Inhibition of PA Activity by Chemical Inhibitor No effect was seen with 0.01 mM DFP, but 1 mM DFP effectively inhibited both the M_r 44000 and 66000 enzymes. The inhibition rate of the M_r 66000 enzyme was similar to that of human uterine t-PA.[2] PCMB, EDTA and pepstatin A had no effects.

Binding of PAs to Fibrin Clots, Anti-human t-PA IgG and Anti-urokinase IgG Human urokinase did not bind significantly to fibrin. In contrast, approximately 70% of the M_r 66000 PA was found to bind fibrin. The activity of M_r

TABLE I. Purification of Plasminogen Activators from 2-d-Old Rat Epidermis

Step	Protein (mg)	Total activity (mIU)	Specific activity (mIU/mg)	Recovery (%)
Crude extract	255	1180	4.6	100
Blue-Sepharose				
Non-adsorbed	64.6	93	1.4	7
Adsorbed ·				
0.1 M L-Arg eluate	28.5	7.8	0.27	0.7
0.5 M L-Arg eluate	21.3	4210	198	360
Con A-Sepharose				
Non-adsorbed (Fraction I)	12.0	23	1.9	1
Adsorbed (Fraction II)	5.0	2390	478	200

The enzyme activity was measured spectrophotometrically as described in the text.

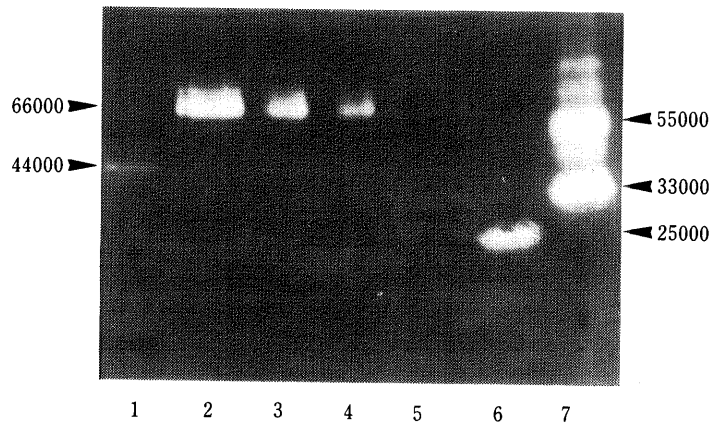

Fig. 1. Zymographic Analysis of Epidermal PA

Purified epidermal PA from Con A-Sepharose was electrophoresed in SDS 11% polyacrylamide slab gels. Lane 1, fraction I (48 μg of protein); lanes 2, 3, 4 and 5, fraction II (15, 7, 4, 1 μg of protein, respectively); lane 6, α-chymotrypsin (2 ng); lane 7, human urokinase (0.25 mIU). The gels were treated as described in the text.

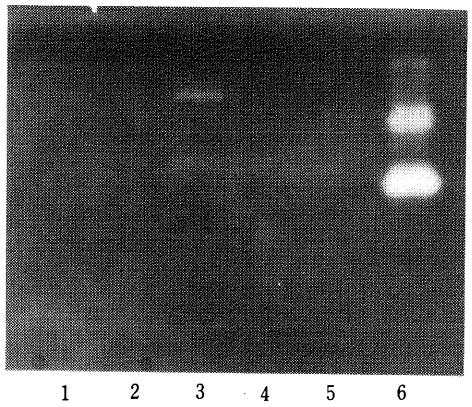

Fig. 2. The Binding of PAs to Goat Anti-human-t-PA and Anti-urokinase IgG

Fraction I (lanes 1, 2), fraction II (lanes 3, 4) and human urokinase (lanes 5, 6) were applied to a column of Sepharose-bound IgG and electrophoresis was carried out in SDS 11% polyacrylamide slab gels. Lanes 1, 3, 5, eluate from Sepharose-bound anti-human-t-PA IgG; lanes 2, 4, 6, eluate from Sepharose-bound anti-human-urokinase IgG. The gels were treated as described in the text.

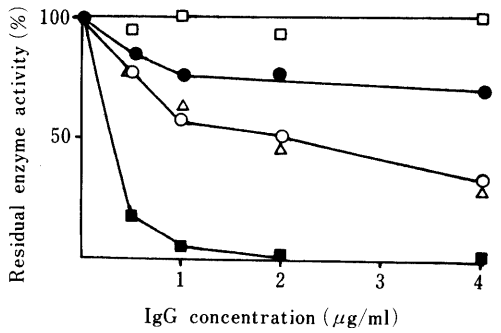

Fig. 3. The Neutralizing Effect of Goat Anti-human-t-PA IgG on the Activities of t-PA, Urokinase and Epidermal PAs

The activities were adjusted to approximately 50 mIU/ml in terms of *p*-nitroaniline liberation. Various concentrations of anti-t-PA IgG were mixed with urokinase (□—□), fraction I (●—●), fraction II (○—○), PA from Bowes melanoma cells (■—■), and B16 melanoma cells (△—△), and the residual enzyme activity was measured spectrophotometrically.

44000 PA was too low for use in the binding tests. Zymographic analysis of the IgG binding test is shown in Fig. 2. The urokinase was adsorbed on anti-urokinase IgG, but not anti-t-PA IgG, bound on Sepharose. The M_r 66000 PA bound to anti-t-PA IgG, but not to anti-urokinase IgG. The M_r 44000 PA was not detectable in the eluates from either IgG column.

Effects of Goat Anti-human t-PA IgG on PAs from Various Cell Sources The IgG inhibited PA activity from Bowes melanoma cells, but not human urokinase even at 4 μg/ml (Fig. 3). This concentration of IgG caused 25% and 75% decreases in the activity of M_r 44000 PA and M_r 66000 PA, respectively. The degree of inhibition of M_r 66000 PA activity resembled that of PA prepared from B16 melanoma cells and porcine t-PA calculated from manufacturer's data (provided by American Diagnostica, Inc.).

Partial Purification of PA Inhibitor Table II summarizes the results of representative experiment. The activity was recovered primarily in the 50—90% $(NH_4)_2SO_4$ precipitate and corresponded to about 118% of the total in the crude Pipes extract. The activity was eluted at the position of about M_r 60000 on a Sephadex G-100 column and was separated from the main protein peak. Specific activity was increased 16.5-fold, but the recovery was only about half of the original activity. In the final purification step, using a Mono Q column in an FPLC system, the activity was adsorbed on the column and eluted at 0.1—0.3 M NaCl with a further increase in specific activity.

TABLE II. Partial Purification of Epidermal PA Inhibitor

Step	Protein (mg)	Total activity (INU)	Specific activity (INU/mg)	Recovery (%)
Crude extract	81	704	8.7	100
$(NH_4)_2SO_4$ 50—90%	16	835	53	118
Sephadex G-100	2.6	366	144	52
Mono Q	0.45	260	578	37

Inhibitor activity was measured by the fibrin plate method as described in the text.

Specificity of PA Inhibitor Epidermal inhibitor suppressed human urokinase activity most effectively (Fig. 4). Urokinase inhibition was seen without preincubation. At up to 30 min of preincubation, a linear relationship existed between the inhibitory activity and time (Fig. 5a). A dose dependent inhibition was also seen with the M_r 44000 enzyme; 80 INU/ml caused 100% inhibition. PA from human melanoma cells was inhibited by 50% by 20

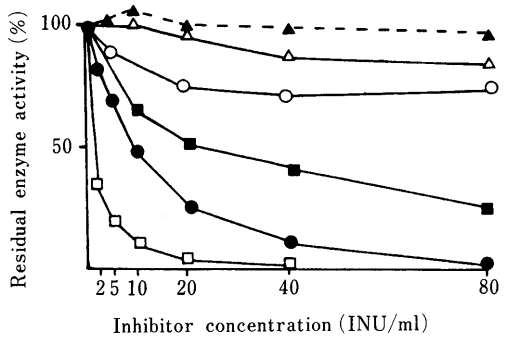

Fig. 4. Specificity of Epidermal PA Inhibitor

PA activity was adjusted to approximately 50 mIU/ml in terms of *p*-nitroaniline liberation. Various concentrations of PA inhibitor purified from 2-d-old rat epidermis were mixed with urokinase (□—□), fraction I (●—●), fraction II (○—○), PA from Bowes melanoma cells (■—■), PA from B16 melanoma cells (△—△) and rat plasmin (▲---▲), and the residual enzyme activity was measured spectrophotometrically. Plasmin used was generated by incubation of 0.05 μM rat plasminogen with 400 IU/ml of urokinase as described in the text.

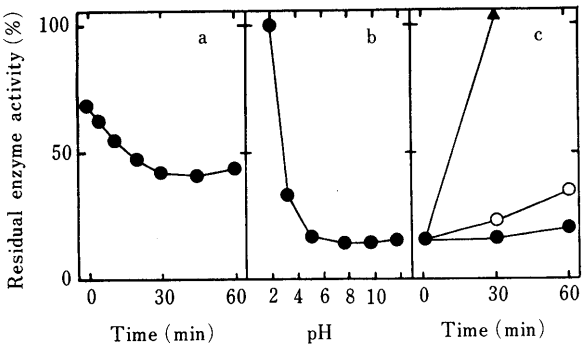

Fig. 5. Properties of Epidermal PA Inhibitor

(a) Time dependency. Inhibitor (2 INU/ml) was preincubated with 50 mIU/ml of urokinase at 37 C for various time periods and the residual enzyme activity was measured spectrophotometrically. (b) Effects of pH. Inhibitor (10 INU/ml) was preincubated in 20 mM KCl–HCl (pH 1.7), 20 mM glycine–NaOH (pH 3.2), 20 mM sodium acetate (pH 4.7), 20 mM Tris–HCl (pH 7.6), 20 mM glycine–NaOH (pH 9.3) or 20 mM KCl–NaOH (pH 11.5) containing 25 μg/ml bovine serum albumin at 37 C for 1 h. Then 0.1 N HCl or NaOH was added to adjust the pH to 7.5. The inhibitor was then mixed with 50 mIU/ml of urokinase and incubated in 10 mM Tris–HCl (pH 7.5) containing 0.05% bovine serum albumin at 37 C for 30 min and residual enzyme activity was measured spectrophotometrically. (c) Heat stability. Inhibitor (10 INU/ml) was preincubated at 37 °C (●—●), 60 °C (○—○) and 100 °C (▲—▲) for 30 and 60 min. The inhibitor was then incubated with 50 mIU/ml of urokinase at 37 C for 30 min and residual enzyme activity was measured spectrophotometrically.

INU/ml, which completely inhibited human urokinase activity. Both B16 melanoma PA and M_r 66000 PA were slightly (up to 20%) inhibited but plasmin inhibition was not seen under the conditions used (Fig. 4).

Temperature and pH Stability of PA Inhibitor The inhibitor was stable at pH values between 5 and 11 but lost activity as the pH decreased. No activity was observed below pH 2 (Fig. 5b). The inhibitor was stable for 1 h at 37 °C and 60 °C but lost activity at 100 °C within 30 min (Fig. 5c).

Discussion

PA was extracted from 2-d-old rat epidermis and two enzymes with different molecular weights were separated from each other. Binding of epidermal PAs to Blue-Sepharose was much greater than that of neuroblastoma strain SK-N-SH PA reported by Gilbert and Wachsman.[22] The increase in PA activity after this purification step suggests that an inhibitor was removed from the crude extract, although we did not detect any inhibitor for human urokinase in the DFP treated KSCN extract (data not shown). In separate experiments we tested *p*-aminobenzamidine-Sepharose column chromatography[23] and zinc-chelate column,[4] as alternative methodologies. They were not suitable for epidermal PA purification, as recovery of the activity was reduced considerably. On the other hand, zinc-chelate column chromatography was a convenient method for partial purification of PA from serum-free medium collected from cultured B16 and Bowes melanoma cells. Con A-Sepharose[24] allowed good separation of M_r 66000 PA and M_r 44000 PA, establishing the presence of two PAs with different properties in rat epidermis. However recovery of the M_r 44000 PA was extremely low. It is most probable that the activity of this enzyme was reduced in 1 M NaCl during Con A-Sepharose chromatography, as observed with a salt sensitive monocyte PA[25] (data not shown).

The M_r of epidermal PAs is compatible with those of PAs detected by Horie *et al.*[26] from KSCN extract of rat epidermis and 2 of 3 PAs detected in culture medium of rat tongue keratinocytes by Birkedal-Hansen and Taylor.[10a] PAs from other keratinocytes include M_r 55000 PA secreted from cultured human epidermal cells[9b] and M_r 62000 from cultured guinea-pig keratinocytes.[10b] Electricwala and Atkinson[10b] presented immunological evidence that the M_r 62000 PA from guinea pig keratinocytes was most likely t-PA and not u-PA. They also showed that the PA bound tightly to a fibrin clot, and the activity was enhanced in the presence of cyanogen-bromide-digested fibrinogen fragments, as seen with t-PA from Bowes melanoma cells.[4] The M_r 66000 PA from rat epidermis has the immunological and functional characteristics of t-PA. In contrast, M_r 44000 PA is immunologically unrelated to t-PA and reacts similar to urokinase. We propose that the epidermis of 2-d-old rats exhibits both u-PA and t-PA gene activities.

The inhibitor purified from rat epidermis showed a somewhat greater M_r than that of the M_r 43000 PA inhibitor from human cornified cells,[12] but resembled monocyte/macrophage inhibitor, M_r 65000,[6] Minactivin, M_r 66000,[27] and rat adenocarcinoma cell PA inhibitor, M_r 66000.[28] The inhibitor showed dose-dependent inhibition of urokinase, but it had no effect on mouse t-PA and suppressed, at least to some degree, human t-PA activity. It did not inhibit plasmin, differing from the protease nexin. PA inhibitor activity was not detected by reverse fibrin autography after SDS-polyacrylamide gel electrophoresis (data not shown), suggesting that the epidermal inhibitor is SDS sensitive. The heat stability and SDS sensitivity of the epidermal inhibitor are similar to those of monocyte/macrophage inhibitor.[6] The inhibitor is more labile to heat as compared to bovine endothelial cells,[29] but is less labile than inhibitors purified from human endothelial cells,[30] human leucocytes,[31] and human cornified cells.[12] A paradoxical points is that t-PA inhibitor was not detected in the present study despite the fact that t-PA activity was greater than u-PA activity as expressed in epidermal extracts. An explanation of the regulating roles of the enzymes and inhibitor should be possible after completion of further studies to elucidate the reason for the increase in PA activities during the first purification step. However, the findings suggest that t-PA activity is not con-

trolled by an endogenous inhibitor in epidermal cells during normal differentiation stages.

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