Purification of epidermal plasminogen activator inhibitor

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A plasminogen activator inhibitor was purified from human cornified cell extract by DEAE-Sepharose, Sephacryl S-200, and high-performance liquid chromatographies on hydroxyapatite HPHT and anion-exchanger Mono Q at pH 7.2 and 8.0. The purified inhibitor showed M_r 43000 and p*I* 5.2. 50% inhibition of fibrinolytic activity (1.5 IU) of urokinase and tissue-type plasminogen activator was attained by 0.60 ng and 11.0 ng purified inhibitor, respectively. Synthetic substrate assay demonstrated slow tight-binding inhibition to both urokinase and tissue-type plasminogen activator. The inhibitor did not inactivate plasmin, thrombin, glandular kallikrein or trypsin.

Plasminogen activator *Enzyme inhibitor* *Urokinase* *Tissue-type plasminogen activator* *Binding inhibition*

1. INTRODUCTION

Plasminogen activator (PA) which converts fibrinolytic proenzyme, plasminogen, to plasmin has been demonstrated with epidermal disorders, such as psoriasis [1,2] and bullous diseases [3]. In normally keratinized cells, however, PA activity was not detectable as reported by Haustein [1] and Ryan and co-workers [4]. In our screening test for serine proteinase inhibitors [5], we demonstrated a possible regulating factor for PA in human cornified cell extract. The present study reports purification of the epidermal PA inhibitor.

2. MATERIALS AND METHODS

2.1. *Enzymes and substrates*

Purified high molecular mass urokinase (55 kDa) was kindly given by Kowa, Tokyo. Tissue-type plasminogen activator (t-PA) from human melanoma cells (>90% two chain) (Bioscot Limited, Edinburgh), plasmin and thrombin (Green Cross, Osaka), trypsin (type III) and glandular kallikrein (Sigma, St. Louis) were purchased. PyroGlu-Gly-Arg-*p*-nitroanilide (pNA) (S-2444), Ile-Pro-Arg-pNA (S-2288), Phe-pipecolyl-Arg-pNA (S-2238), Val-Leu-Lys-pNA (S-2251), and Val-Leu-Arg-pNA (S-2266) were the products of Kabi Diagnostica, Stockholm. Benzoyl-Arg-pNA (BApNA) was from Peptide Institute, Osaka.

2.2. *Extraction and purification of epidermal PA inhibitor*

Human cornified cells (30 g) scraped from heels of 30 healthy individuals (age 23–35) were extracted in 100 mM Tris-HCl (pH 8.0) containing 140 mM NaCl for 2 h at 4°C. Supernatant was concentrated with Amicon using YM 10 membrane and applied to a DEAE-Sepharose column equilibrated with 20 mM phosphate buffer (pH 7.5). Inhibitor fraction eluted with increasing NaCl concentration up to 0.6 M was fractionated by Sephacryl S-200 gel chromatography equilibrated with 20 mM phosphate buffer + 0.14 M NaCl. The inhibitor fraction was then applied on hydroxyapatite HPHT column (Bio-Rad) equilibrated 10 mM phosphate buffer (pH 6.8) + 0.3 M $CaCl_2$ and eluted with increasing phosphate concentration up to 0.4 M. This was followed by high-performance anion-exchange chromatography on Mono Q (Pharmacia) twice. Firstly, Mono Q column was equilibrated with 20 mM phosphate buffer (pH 7.2) and eluted with increasing NaCl concentration up to 0.9 M. Secondly, the obtained

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inhibitor fraction was applied on Mono Q column equilibrated with 20 mM Tris-HCl (pH 8.0).

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [6].

In order to detect the inhibitor activity of the separated protein band, reverse fibrin autography [7] was performed after SDS-polyacrylamide gel electrophoresis with modifications. Urokinase (final concentration, 0.13 IU/ml) was incorporated into the fibrin-agarose indicator gels in the presence of the purified dog plasminogen (final concentration, 15 μg/ml). The gels were incubated at 37°C for 3 h.

Protein concentration was determined by the method of Lowry et al. [8] using bovine serum albumin as standard.

Isoelectric point of the inhibitor was studied by chromatofocusing on Mono P (Pharmacia) using an inhibitor fraction obtained from first Mono Q chromatography. Elution was done with polybuffer with pH interval 7 and 4.

2.3. *Colorimetric inhibitor assay*

Substrates used were S-2444 for urokinase; S-2288, t-PA; S-2251, plasmin; S-2238, thrombin; S-2266, glandular kallikrein; and BApNA, trypsin. Enzyme concentration was standardized to liberate 1.4 nmol *p*-nitroaniline per min in each enzyme-substrate combination in the following assay system. Inhibitor sample (0.01–0.05 ml) or buffer (control) was preincubated with 0.1 ml enzyme in 0.80–0.84 ml Tris-HCl (pH 8.5) (totally, 0.95 ml) at 25°C for 30 min (60 min for t-PA) or specified intervals. Reaction was started by the addition of 0.05 ml of 4 mM substrate at 37°C and absorbance at 405 nm was monitored. In this condition, molar concentration of enzymes determined by active site titration method with *p*-nitrophenyl *p'*-guanidinobenzoate (Sigma) [9] was 2.09 nM for urokinase; 3.86 nM, t-PA; 0.14 nM, plasmin; 0.03 nM, thrombin; and 2.4 nM, trypsin. Molar concentrations of glandular kallikrein were not determined. Inhibitor activity was expressed by the enzyme activity (nmol/min = unit) that was inhibited.

2.4. *Fibrin plate assay*

Mixtures containing 10 μl enzyme (1.5 IU/ml) and 10 μl inhibitor sample or buffer were placed on plasminogen-rich fibrin plates [10] and incubated for 18 h at 37°C. Squared diameter of lysis zone was plotted versus concentrations (IU) of urokinase or t-PA for standard curves. Inhibitor activity (percent inhibition) was calculated from the standard curve.

3. RESULTS

3.1. *Purification of epidermal PA inhibitor*

DEAE-Sepharose chromatography of Tris-buffered saline extract of human cornified cells demonstrated a peak of urokinase inhibitor activity which was eluted at approx. 0.15 M NaCl. The Sephacryl S-200 gel chromatography showed the inhibitor activity for urokinase at approx. M_r 40000. In the following hydroxyapatite HPHT column, inhibitor activity was detected at the fractions eluted with 0.35–0.40 M phosphate. In the first Mono Q chromatography at pH 7.2 inhibition for urokinase was detected in the fraction eluted at approx. 0.12 M NaCl. The second Mono Q chromatography at pH 8.0 demonstrated only one protein peak in which inhibitor activity coincided (fig.1). SDS-polyacrylamide gel electrophoresis of the final preparation showed a single protein band with an M_r of 43000 (fig.2). Reverse fibrin autography demonstrated that the isolated protein with M_r 43000 possesses the inhibitor activity

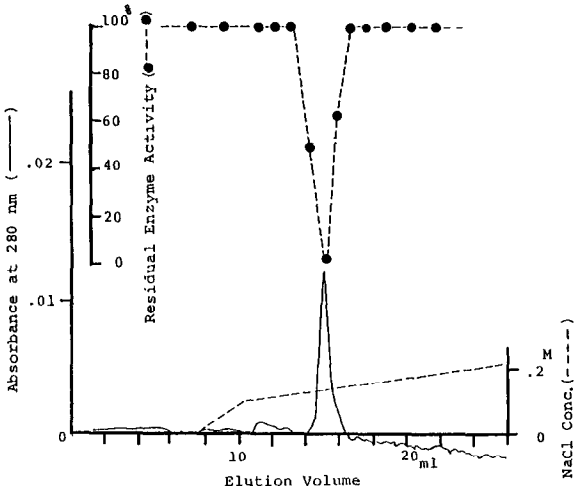

Fig.1. The second Mono Q chromatography at pH 8.0. Solid line shows absorbance at 280 nm. Inhibition for urokinase (●---●) was measured by colorimetric assay using S-2444 as substrate.

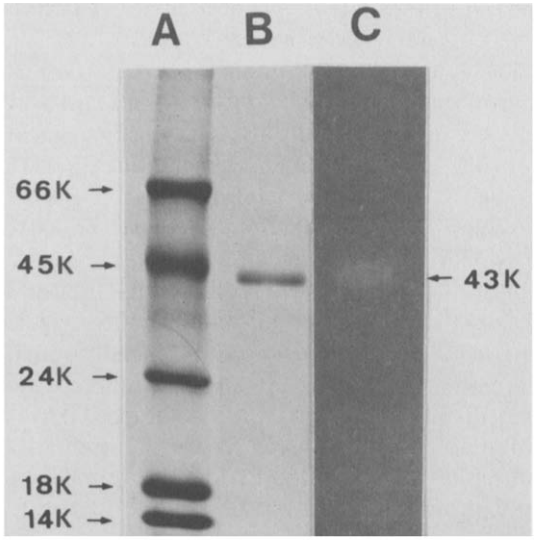

Fig.2. Analysis of the purified inhibitor. Approx. 7 μg of the inhibitor from the second Mono Q chromatography was applied on SDS-polyacrylamide slab gels. After electrophoresis, the gel was stained with Coomassie brilliant blue (lane A, molecular mass markers and B, inhibitor fraction), or analyzed for inhibitor activity by reverse fibrin autography (lane C). Molecular mass of the inhibitor was estimated to be 43 kDa.

toward urokinase (fig.2). Isoelectric point of the PA inhibitor was estimated to be 5.2 by chromatofocusing on Mono P (not shown).

Table 1 summarizes the purification steps of epidermal PA inhibitor. Specific activity increased 1745-fold with a yield of 5.1%.

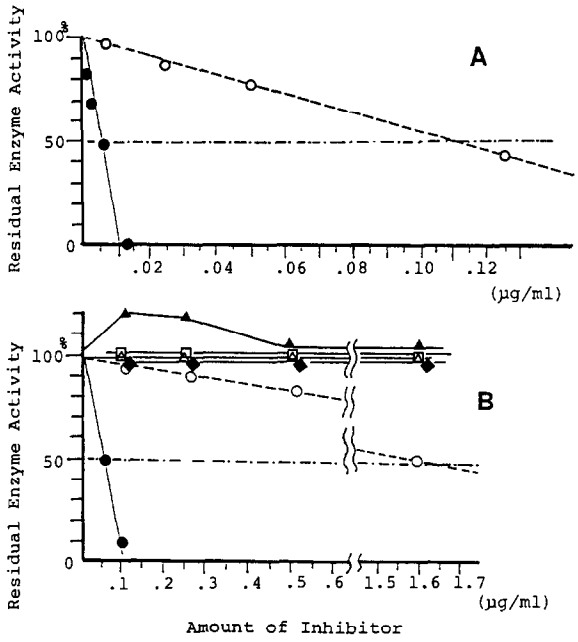

Fig.3. Inhibitor activity of the purified epidermal PA inhibitor on serine proteinases. Residual enzyme activity (%) was plotted versus various amounts of purified inhibitor. (A) Fibrin plate assay for urokinase (●—●) and t-PA (○---○). (B) Colorimetric assay for urokinase (●—●), t-PA (○---○), plasmin (▲—▲), thrombin (□—□), glandular kallikrein (△—△), and trypsin (◆—◆).

3.2. *Inhibitor activity*

Purified inhibitor inactivated both urokinase and t-PA in a dose-dependent manner in fibrin plate assays (fig.3A). It was calculated that 0.60 ng

Table 1

Summary of purification

Step	Volume (ml)	Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification factor
Crude extract	513	1508.0	17.3	0.011	100	1
DEAE-Sepharose	46.5	46.2	16.8	0.364	97	33
Sephacryl S-200	15.8	16.6	12.3	0.741	71	67
HPHT	6.8	1.36	2.43	1.79	14	163
Mono Q at pH 7.2	3.5	0.234	1.09	4.65	6.3	423
Mono Q at pH 8.0	2.5	0.046	0.89	19.2	5.1	1745

of the inhibitor inactivated 50% activity of 1.5 IU of urokinase, whereas 11.0 ng was needed for 1.5 IU of t-PA, showing 18-times less potency in inhibiting t-PA in fibrinolysis. Fig.3B shows the effect of the purified inhibitor in the colorimetric inhibitor assay. The purified inhibitor (50 ng) inactivated 50% of 115 ng of urokinase. In molar concentration, 1.2 nM of the inhibitor showed 50% inhibition for 2.09 nM urokinase, revealing 1:1 stoichiometric interaction between the inhibitor and urokinase. However, 1.65 μg (39.6 nM) of the inhibitor was required for 50% inhibition for 3.86 nM t-PA, showing that 20-times as much inhibitor is needed to inactivate t-PA on a molar basis. The purified inhibitor did not show any inhibitory effect on plasmin, thrombin, glandular kallikrein or trypsin.

3.3. *Time course of inhibition*

The time course of inhibition by the epidermal PA inhibitor demonstrated a slow, tight-binding characteristic toward urokinase reaching the maximum inhibition level at 30 min (fig.4). Similar time dependency was observed for t-PA, although it required 60 min for the maximum inhibition.

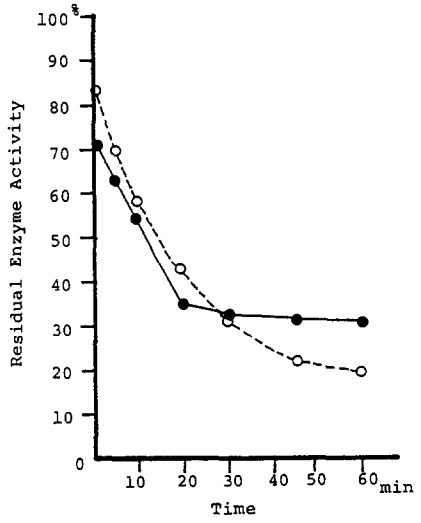

Fig.4. Time course of inhibition. Urokinase (2.09 nM) and t-PA (3.86 nM) were preincubated with 0.06 μg and 1.8 μg of the purified inhibitor, respectively. After appropriate intervals of preincubation at 25°C S-2444 (urokinase assay) (●——●) or S-2288 (t-PA assay) (○---○) were added and residual enzyme activity (%) was plotted.

4. DISCUSSION

The epidermal PA inhibitor is a slow-acting inhibitor with an apparent M_r of 43000 and a p*I* of 5.2. A slow-acting PA inhibitor was also purified from rat mammary adenocarcinoma cells [11]. It showed less potent inhibition on t-PA than urokinase, as well, although its size (M_r 66000) and p*I* (4.5) were different from the epidermal PA inhibitor. In addition, the epidermal inhibitor was distinct from fast-acting PA inhibitors recently purified from other tissues. Partially purified placenta inhibitor (M_r 48000) instantaneously inactivated human ovarian carcinoma PA and urokinase [12]. The leucocyte urokinase inhibitor (M_r 68000, p*I* 4.4–4.5) also showed immediate inactivation of urokinase [13]. Unusually stable PA inhibitor (M_r 55000, p*I* 4.5–5.0) from cultured endothelial cells rapidly inactivated vascular PA and urokinase [14]. The epidermal PA inhibitor was clearly different from protease nexin (M_r 51000 and p*I* 7.5–7.8) [15] which inactivated not only PA but also other serine proteinases, or from minactivin (M_r 66000) [16], a specific urokinase inhibitor of human monocyte product. In addition, α_2-plasmin inhibitor [17], the major antifibrinolytic inhibitor in plasma, showed slow inhibition to urokinase, however it possessed strong inhibitor activity on plasmin which is not inhibited by the epidermal inhibitor.

The epidermal inhibitor showed stronger inhibitory effect on urokinase than t-PA in both fibrinolytic and peptide hydrolytic assays. However, no inhibitory effect was observed for other serine proteinases. From table 1, the content of the epidermal PA inhibitor in the starting extract was calculated as approx. 0.9 mg/1500 mg of extractable protein. This concentration is lower than that of the cysteine proteinase inhibitor (31.6 mg/1500 mg; unpublished) contained in the same starting extract. Although epidermal cells immuno-histochemically showed urokinase antigen in granular layer of normal epidermis [18], activity of urokinase is not detectable in normal epidermis [1,4,5] but appeared to be enhanced in psoriatic conditions (Hibino et al., submitted). Therefore, the function of epidermal PA inhibitor is postulated in normally differentiated epidermal cells.

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