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Comparative Studies on Two Active Enzyme Forms of Human Urinary Urokinase. II. pH- and Heat-stabilities[1] of Plasminogen Activator Activity

Nobuhiko Miwa, Yuji Obata, and Akira Suzuki*

Pharmaceuticals Research Center, Kanebo, Ltd., Tomobuchi-cho, Miyakojima-ku, Osaka 534, Japan.

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pH-stability curves and heat inactivation of the MW 55000 form (H-UK) and MW 36000 form (L-UK) of human urinary urokinase [EC 3.4.21.31] were investigated by measurements of plasminogen activator activity. L-UK was distinctly more stable than H-UK (in terms of activity) on incubation at pH 6.8—9.6 or on heat treatment at temperatures above 60°. The ranges of pH 8.2—9.0 and 5—37°, were favorable for both H-UK and L-UK.

Keywords——urokinase; plaminogen activator; PH-stability of enzyme; heat inactivation of enzyme; gradient gel electrophoresis

Little work has been done to determine the conditions under which the activity of highly purified urokinase (UK) preparations is stable. Prior to studies on the enzymological and physicochemical properties of a protein, such conditions should be determined (*e.g.*, suitable values pH, temperature, ionic strength, and a suitable buffer composition, for maintenance of activity).

We reported in the previous paper[2] the preparation of two highly purified active forms of UK, *i.e.* the heavy form (H-UK) with a molecular weight of 5.5×10^4 and the light form (L-UK) with a molecular weight of 3.6×10^4, both of which were confirmed to be homogeneous in terms of molecular weight and immunogenicity, and to exhibit high specific activity.

Using the highly purified H- and L-UK, we determined conditions under which the activity is stable.

Materials and Method

Materials——The highly purified UK preparations were obtained by serial column chromatography as described in the previous paper.[2] The specific activities of highly purified H- and L-UK were 1.20×10^5 IU/mg protein and 1.52×10^5 IU/mg protein, respectively. Thrombin and fibrinogen used for the determination of plasminogen activator activity were obtained from Mochida Pharmaceuticals Ltd., Tokyo and Daiichi Pure Chemicals, Co. Ltd., Tokyo, respectively.

Plasminogen Activator Activity——Using the National Standard preparation for calibration, we determined the plasminogen activator activity of H- and L-UK by measuring the fibrinolytic rate by the fibrin tube method of Ploug and Kjeldgaard.[3]

pH-Stability Curves——H- and L-UK were dissolved in Britton-Robinson buffer for wide pH range[4] which was modified by adding KCl to adjust the ionic strength of UK solution to 0.15 (buffer I).

The protein solution was sterilized by filtration through a Sartorius Co. cellulose acetate membrane filter with a pore size of 0.2 μ. Protein concentration was 0.30 mg/ml, and the pH was adjusted to one of 17 values between pH 1.0 and 13.0. UK solutions were allowed to stand at 25°. Aliquots were pipetted out from the enzyme stock solutions 1, 3, 7, and 14 days after adjustment of the pH and subjected to the determination of plasminogen activator activity after 200-fold dilution with buffer I, pH 7.8.

The pH of the Stock solution was readjusted to the initial value every two days by gradually adding small amounts of sterile 2 N HCl or 2 N KOH. All the operations except for the activity assay were conducted under sterile conditions.

Dependence of the Stability of Enzymic Activity on Temperature——H- and L-UK were each dissolved at a concentration of 0.30 mg/ml in 50 mM Tris-HCl buffer, pH 8.6 (ouffer II), which had been preincubated at 5°, 25°, 37°, 60°, 80°, and 100°. Aliquots of the enzyme solution were taken several times during the incubation period of 48 hr in a water bath controlled at the appropriate temperature. The aliquot was immersed in an incubator maintained at 37° for 10 min and subjected to the determination of plasminogen activator activity.

When the enzyme solution became turbid and produced precipitates on incubation at 80° and 100°, the aliquot was taken from the suspension after rapid agitation.

Gradient Gel Electrophoresis——PAA4/30, gradient gel plates purchased from Pharmacia Fine Chemicals Co. were used. Electrophoresis was conducted for eight hr at 10°. After the electrophoresis each gel plate was stained with Coomassie Brilliant Blue R250. Other conditions are described in detail in the previous paper.[2]

Results and Discussion

Dependence of the Stability of Plasminogen Activator Activity on pH

The pH-stability curves of H-UK differed in several respects from those of L-UK (Fig. 1): (1) H-UK was inactivated slightly (to a relative activity of approximately 90%) at neutral pH after 1 day without any added stabilizers, *e.g.*, human serum albumin, gelatin, mannitol, *etc.*, while L-UK was hardly inactivated even after 14 days at pH 6.8—9.6 without a stabilizer. (2) The pH-stability curve of H-UK was the same trapezoid form as that of general enzymes, whereas L-UK exceptionally showed a trianguloid stability curve. The pH range over which more than 80% of the maximal activity was exhibited at each standing period was wider for H-UK than for L-UK, *e.g.* 1 day after pH adjustment: 9.3 pH units for H-UK and 5.4 pH units for L-UK. (3) The activity of H-UK decreased gradually at pH 2.0 to 9.5 but decreased rapidly at above pH 10.0. Up to 3 days, H-UK maintained a slightly higher activity level at neutral pH (6.8—7.5) than at slightly alkaline pH (8.0—9.0). From 7 to 14 days, conversely, the activity of H-UK was better maintained at pH 8.0—9.0. The preferred pH for activity maintenance of H-UK, therefore, shifted from neutral pH to alkaline pH with the lapse of time. On the other hand, the activity of L-UK hardly decreased but, if anything, increased slightly to a relative activity of approximately 105% between pH 8.2 and 8.6.

It was indicated that the pH range of 8.2 to 9.0 is preferable to neutral pH (between 6.5 and 7.8) for activity maintenance of L-UK. The most favorable pH for activity maintenance of both UK forms was between pH 8.2 and 9.0, especially pH 8.6.

Dependence of Stability of Plasminogen Activator Activity on Temperature

Although most enzymes generally maintain their activity better at low temperatures (0—5°) than at high temperatures, some exceptions have been reported.[5-7]

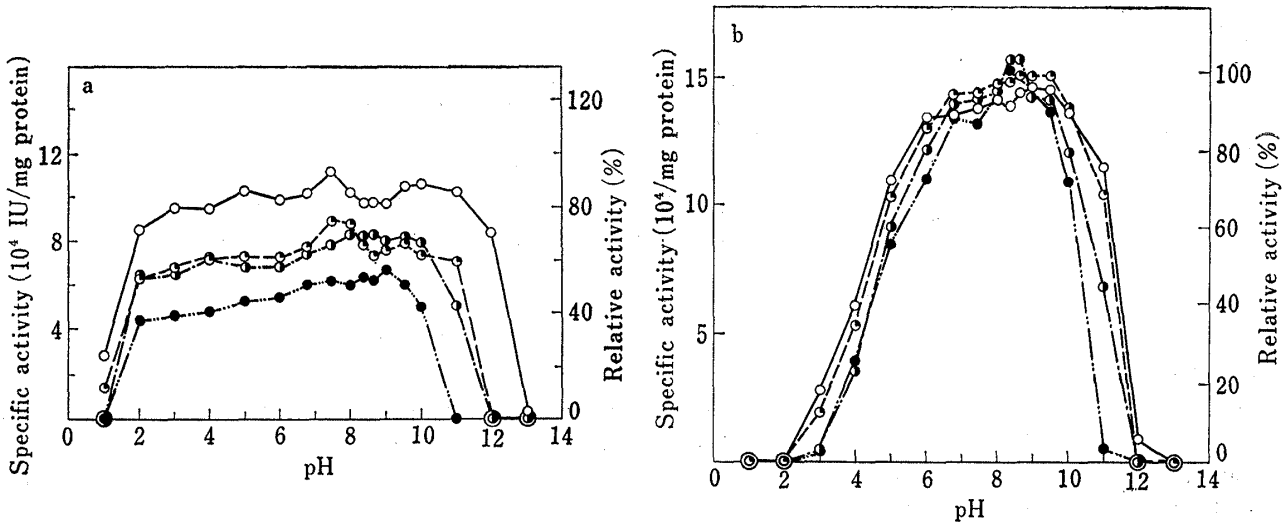

Fig. 1. pH-Stability Curve for Plasminogen Activator Activity

H- and L-UK were allowed to stand at various pH's and 25°, and subjected to periodic determination of plasminogen activator activity until 14 days after dissolving the protein. Relative activity is expressed by taking the initial activity as 100%.
——○——, 1 day; ----◔----, 3 days; ---◑---, 7 days; ----●----, 14 days.
a) H-UK; b) L-UK.

The activity of highly purified UK preparations was therefore determined on standing at six temperatures between 5° and 100° and at pH 8.6 (which was the optimal pH for stability of the activity) (Fig. 1). Some striking differences were found between H- and L-UK (Fig. 2): (1) After an incubation period of 48 hr, L-UK retained almost all of its initial activity at below 60°, while H-UK lost a significant amount of activity even at 37° and most of it at 60° (Fig. 2d).

In spite of the inverse correlation in general between enzyme purity and heat stability, the present highly purified H-UK was not markedly inactivated even when incubated at 60° as compared to partially purified H-UK with a low specific activity (10000—15000 IU/mg protein) prepared by Higashi *et al.*[8]

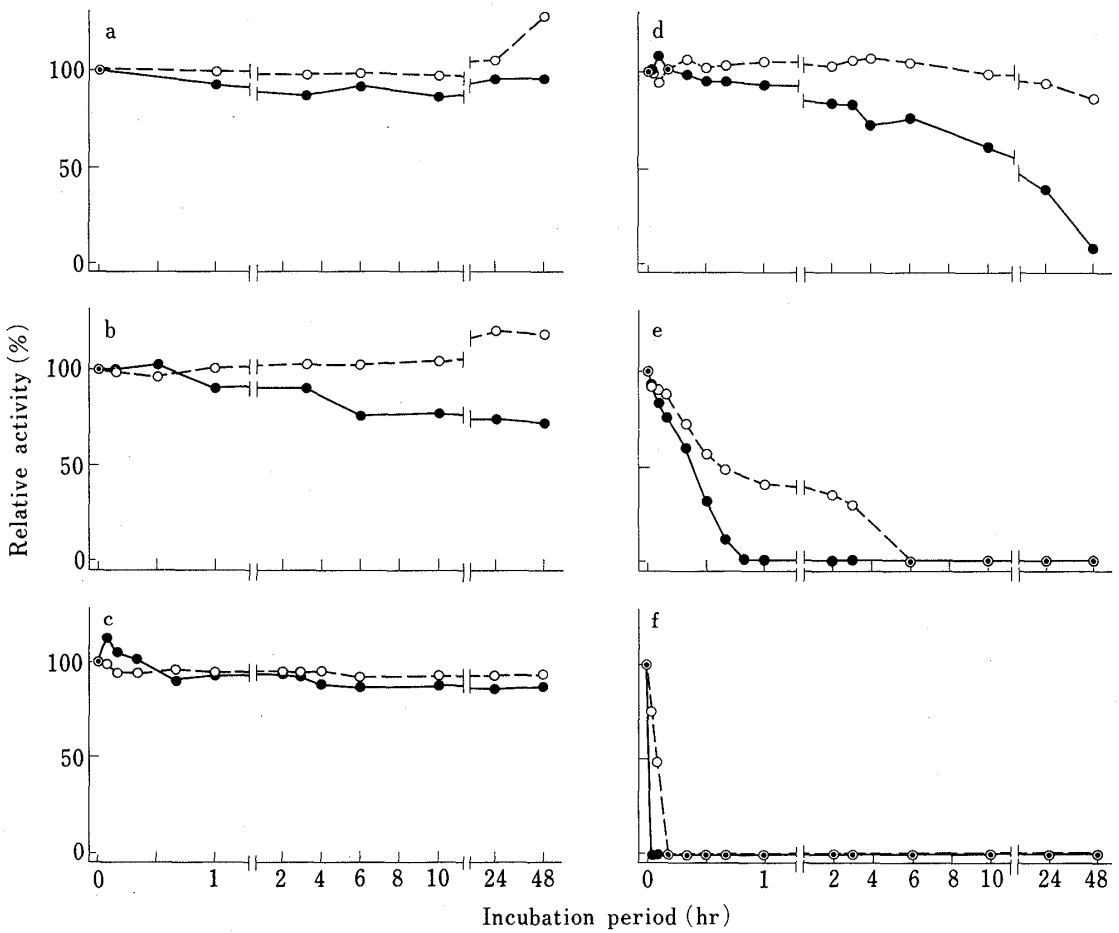

Fig. 2. Changes of Plasminogen Activator Activity during Incubation at Various Temperatures

Both UK forms were incubated at various temperatures at pH 8.6 and subjected to perdic determination of plasminogen activator activity. Relative activity is expressed by taking the initial activity as 100%.
—●— H-UK; ----○---- L-UK; incubation temperatures(°): a) 5; b) 25; c) 37; d) 60; e) 80; f) 100.

Their crude H-UK (which is assumed to contain about 90 mole% H-UK and 10 mole% L-UK) retained 69—83% of its initial activity on incubation at 60° for 10 hr, whereas our highly purified H-UK retained approximately 61% of its initial activity under the same conditions. (2) At 80° and 100° both UK forms were inactivated completely and rapidly, but the inactivation rate of H-UK was greater than that of L-UK (Figs. 2e and f). (3) H- and L-UK retained almost all their initial activities during 48 hr at 37°, although a feeble and transient activation was observed for H-UK at the onset of incubation. At 5° and 25°, L-UK was moderately activated at 24—48 hr. The activation of L-UK was also observed in the determination of the pH-stability curve (Fig. 1b).

In contrast to L-UK, H-UK was slightly inactivated at 5° and considerably at 25°. It is assumed that the conformation of both UK forms may be subtly affected by the changes

of the microenvironment following incubation below 37°, the temperature at which UK acts *in vivo*.

By means of gradient gel electrophoresis (which is less likely to redissolve protein aggregates than SDS-polyacrylamide gel electrophoresis[9]).

We attempted to correlate the polymerization or decomposition of UK molecules with the heat inactivation (Fig. 3). At 60°, the threshould temperature at which H-UK was inactivated markedly, H-UK was observed to aggregate to approximately MW 260000 molecules. It was split partially into MW 36000 form, MW 16000 form, and some other fragments during inactivation to the extent of approximately 10% by incubation at 60° for 2 hr. The MW 36000 form obtained by the heat inactivation of H-UK was inferred to be similar to an active heavy chain, judging from the high remaining relative activity (*e.g.* 86% at 60° for 2 hr) of the heat-treated H-UK in comparison with the high ratio of aggregation and decomposition products. The existence of an active heavy chain is supported by the fact that H-UK reduced with β-mercaptoethanol produced an active heavy chain (MW 3.3×10^4) which was assumed to resemble L-UK.[10] On the other hand L-UK was shown neither to aggregate nor to split at 60°, even after 48 hr, though incubation above 80° aggregated L-UK promptly. These findings, accordingly, suggest that even slight heat inactivation was accompanied by irreversible aggregation and/or splitting of the polypeptide chain in the case of both UK forms. L-UK showed a higher threshold temperature of inactivation and was more resistant to heat inactivation than H-UK.

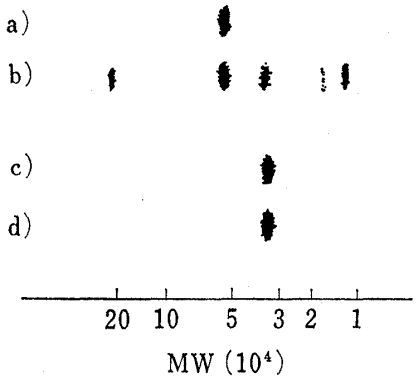

Fig. 3. Gradient Gel Electrophoregrams of Native and Heat-treated UK

a) native H-UK,
b) heat-treated H-UK (60° for 2 hr),
c) native L-UK,
d) heat-treated L-UK (60° for 48 hr).

Based in these findings, we consider that studies of the enzymological and physicochemical properties of UK in solution should be carried out at between 5° and 37° and within 3 hr after dissolving UK.

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References and Notes

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