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Isoelectric Points of Two Molecular Forms of Human Urinary Urokinase determined by Density-gradient Isoelectric Focusing and Isotachoelectrophoresis

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The isoelectric points (pI's) of MW 55000 form (H-UK) and MW 36000 form (L-UK) of human urinary urokinase (UK) were determined by sucrose density-gradient isoelectric focusing. These values were compared with the pI's which had been estimated previously by isotachoelectrophoresis.

All five subforms fractionated from H- or L-UK by isoelectric focusing were confirmed to exhibit plasminogen activator activity and to be homogeneous in terms of molecular weight.

The pI's evaluated by isoelectric focusing were in accordance with those by isotachoelectrophoresis, for all five subforms of H-UK (pI 8.70, 8.86, 9.05, 9.20 and 9.44) and for three subforms of L-UK (pI 7.54, 8.26 and 8.84). The pI's of the two most alkaline subforms of L-UK (pI 9.17 and 9.43) were underestimated by up to 0.3 pH unit by isoelectric focusing as compared with the values obtained by isotachoelectrophoresis (pI 9.4 and 9.7, respectively).

Thus, the differences between the pI's determined by the two kinds of electrophoresis were slight and restricted to two of the ten subforms.

Keywords——urokinase; density-gradient isoelectric focusing; isotachoelectrophoresis; plasminogen activator activity; SDS-polyacrylamide gel electrophoresis

We reported, in a previous paper,[1] pI determination by isotachoelectrophoresis using Ampholine as a carrier ampholyte for two molecular forms of human urinary urokinase (UK) [EC 3.4.21.31], whose enzymological[2] and conformational[3] properties were studied. These pI values determined by isotachoelectrophoresis for ten subforms of the two molecular forms of UK were compared with those estimated by density-gradient isoelectric focusing in the present work. The subforms which were fractionated from the MW 55000 form (H-UK) and MW 36000 form (L-UK) of UK by isoelectric focusing were checked for homogeneity by SDS-polyacrylamide gel electrophoresis and determination of activity pattern.

Experimental

Materials——Highly purified H- and L-UK were obtained from human urine by serial column chromatography.[1] The specific activities of H- and L-UK were 1.20×10^5 and 1.52×10^5 IU/mg protein, respectively. Thrombin and fibrinogen used for assay of plasminogen activator activity were purchased from Mochida Pharmaceuticals Ltd., Tokyo and Daiichi Pure Chemicals, Co., Tokyo, respectively.

Density-gradient Isoelectric Focusing——Isoelectric focusing was performed essentially according to the method of Vesterberg *et al.*,[4] using an LKB isoelectric separation apparatus with a 110 ml column (LKB 8101). The column was filled, from the bottom up with cathode solution, dense and light gradient solutions and anode solution, which contained 60, 50, 5 and 0% sucrose, respectively. A 1:1 mixture of Ampholine for pH 3.5—10.0 and pH 9.0—11.0 was contained in both the dense and light gradient solutions. H- or L-UK (total activities: 9—11×10^4 IU) was added to the dense gradient solution. Electrophoresis was conducted at 5°C for 18 h at 500 V and then for 48 h at 1000 V using a Toyo solid-state power supply, model 1510. The solution was then taken out (fraction volume: 1 ml) and immediately subjected to the following manipulations: (1) pH measurement using a Hitachi-Horiba Co. pH meter, model F-7; (2) assay of plasminogen activator activity after 20-fold dilution; (3) SDS-polyacrylamide gel electrophoresis.

Plasminogen Activator Activity——By using the International Standard preparation of UK for calibration, the activity was determined according to the fibrin tube method of Ploug and Kjeldgaard.[5]

SDS-Polyacrylamide Gel Electrophoresis——The electrophoresis was performed essentially as described by Weber and Osborn,[6] after concentrating each subform of H- or L-UK fractionated by isoelectric focusing. Other conditions were as described in a previous paper.[1]

Results and Discussion

Comparison between pI's determined by Isoelectric Focusing and Isotachoelectrophoresis

The electrophoregrams of isoelectric focusing show four peaks and one shoulder for H-UK and five peaks for L-UK in terms of plasminogen activator activity (Fig. 1). The pI's of the five subforms of H-UK were within a narrower range than those of L-UK. The subforms of L-UK with the lowest (pI 7.54) and the second lowest (pI 8.26) pI's were not as alkaline as the subform of H-UK with the lowest pI (pI 8.70). In terms of the pI of the most alkaline subform, H-UK and L-UK were similar.

Isoelectric focusing gave the same pI values as isotachoelectrophoresis for all five subforms of H-UK and three subforms of L-UK (Table I). As judged by isotachoelectrophoresis,[1] the two more alkaline subforms of L-UK showed slightly higher pI's than those obtained by isoelectric focusing.

This slight deviation between the results of isotachoelectrophoresis and isoelectric focusing above pI 9 is assumed to be due to one of the following factors. (1) Some disturbance may have arisen in the alkaline region of the pH gradient which was formed with the carrier ampholyte for isotachoelectrophoresis. From the standpoint of precision in pI determination, isotachoelectrophoresis is less reliable than isoelectric focusing, because the pI value in the

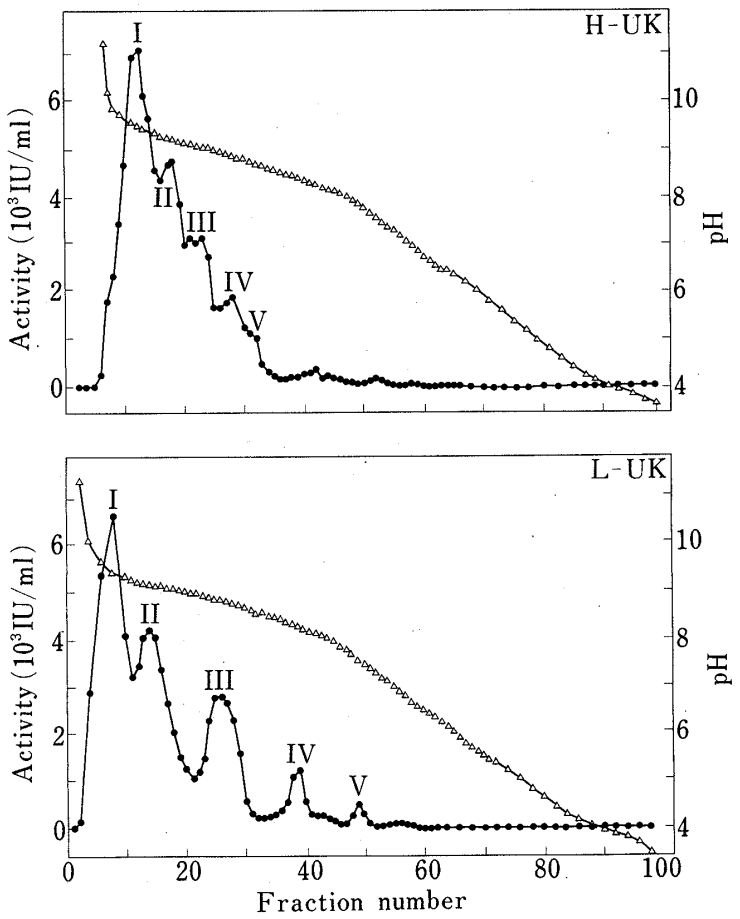

Fig. 1. Electrophoregrams of Density-gradient Isoelectric Focusing for H- and L-UK

—●—, plasminogen activator activity;
—△—, pH; I—V, subforms fractionated from H- and L-UK.

case of isotachoelectrophoresis is indirectly read based on the electrophoretic positions of pI markers.[1] In contrast the pI for isoelectric focusing is directly measured by means of a pH meter; (2) The pI's determined by means of a pH meter with a glass electrode might be underestimated in isoelectric focusing because of "alkaline errors,"[7] which are often observed for a solution with a high concentration of sodium ions.

The results from both kinds of electrophoresis confirm our previous pI determination, taking into consideration that the differences in pI's were restricted to two of the ten subforms and were quite small (subform I of L-UK, 0.3 pH unit; subform II of L-UK, 0.2 pH unit).

Until our pI determination, the pI's of H-UK (MW 54000—55000 form), which is accepted as a pharmacologically preferable and native form,[8] had never been simultaneously determined in comparison with those of L-UK (MW 32000—36000 form), although other investigators have determined the pI's of the MW 33000[9] or 31500 form[10] alone and those of molecular types (MW 47000 and 33000 forms)[11] other than H-UK.

Table I. Comparison between pI's determined by Isoelectric Focusing and by Isotachoelectrophoresis

	Subform	H-UK					L-UK				
		I	II	III	IV	V	I	II	III	IV	V
pI	Isoelectric focusing	9.44	9.20	9.05	8.86	8.70	9.43	9.17	8.84	8.26	7.54
	Isotacho-electrophoresis	9.4	9.2	9.1	8.9	8.7	9.7	9.4	8.8	8.3	7.5

Activities of Subforms of H- and L-UK fractionated by Isoelectric Focusing

It was observed in both UK forms that the most alkaline subform was predominant in terms of plasminogen activator activity (Fig. 1). The less alkaline a subform of H- or L-UK was, the less activity it showed. As far as H-UK is concerned, this is consistent with the results from isotachoelectrophoresis[1] that the subform with pI 9.4 was predominant. However, with respect to L-UK, the results of isoelectric focusing were not consistent with those from isotachoelectrophoresis that the subforms with pI 8.3 and 8.8 were dominant.[1]

Isoelectric focusing does not allow the activities of the two most alkaline subforms of L-UK to be evaluated strictly. This is mainly because highly purified L-UK is moderately (relative activities of 19—26%) activated at alkaline pH's (pH 8—10) and low temperatures (5—25°C),[2] and the two more alkaline subforms of L-UK were exposed to these conditions (pH 9—10; 5°C) during isoelectric focusing.

The finding of apparently maximal activity for the most alkaline subform of L-UK after isoelectric focusing is assumed to arise as follows. (1) The subforms of L-UK with high pI's might be subject to synergistic alkaline and cold activations[2] during the electrophoresis. (2) Fibrinolytic activity might be modified in the assay system[5] by the carrier ampholyte and/or sucrose diluted 20-fold before the assay. (3) A rather large amount of protein was detected

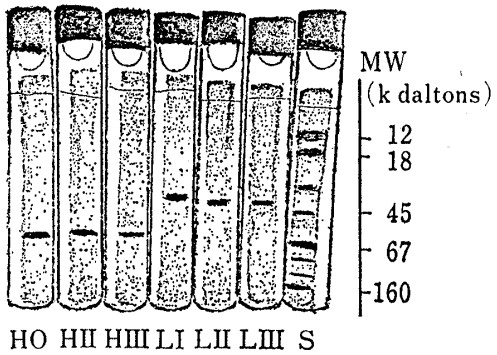

Fig. 2. SDS-Polyacrylamide Gel Electrophoregrams of the Main Subforms of H- and L-UK

H, H-UK; L, L-UK; O, preparation before isoelectric focusing; I—III, subform numbers illustrated in Fig. 1; S, the standard markers for molecular weight determination (human fraction II γ-globulin, bovine albumin, ovalbumin, sperm whale myoglobin and horse heart cytochrome c).

for the most alkaline subform of L-UK as compared with the other subforms of L-UK (Fig. 2).

Homogeneity Analyses of UK Subforms fractionated by Isoelectric Focusing by SDS-Polyacrylamide Gel Electrophoresis

Each subform fractionated by isoelectric focusing was investigated in terms of homogeneity of molecular weight by SDS-polyacrylamide gel electrophoresis (Fig. 2). Two and three main subforms of H- and L-UK, respectively, were homogeneous.

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References and Notes

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