[Chem. Pharm. Bull. 29(2) 463—471 (1981)]

Comparative Studies on Two Active Enzyme Forms of Human Urinary Urokinase. I. Purification by Serial Column Chromatography and Homogeneity Analyses of Molecular Weight and Isoelectric Point[1)]

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(Received May 29, 1980)

A method for the efficient further purification of two active forms of urokinase (UK) [EC. 3.4.21.31] from partially purified human urinary UK was established by serial column chromatography on Sephadex G-100, Sephadex G-75, and SP-Sephadex C-50. According to Ferguson plots two active forms showed molecular weights of 5.5×10^4 (H-UK) and 3.6×10^4 (L-UK).

Both UK forms were shown to be homogeneous by SDS-polyacrylamide gel electrophoresis, gel gradient electrophoresis, and Ouchterlony's double immunodiffusion method. Amino acid analysis showed that there was no marked difference in composition between H- and L-UK. The specific activity of H-UK was 1.2×10^5 IU/mg protein, which exceeds the highest specific activity that has ever been obtained by other investigators, and that of L-UK was 1.52×10^5 IU/mg protein.

These results suggest that this serial column chromatography procedure is effective for the purification of H-UK, which is known to be a pharmacologically advantageous form.

As judged by isotachoelectrophoresis, H-UK contained five subforms with pI values of 8.7, 8.9, 9.1, 9.2, and 9.4, and L-UK contained the same number of subforms with pI values of 7.5, 8.3, 8.8, 9.4 and 9.7. The pI values of the subforms with the highest specific activity were 9.4 for H-UK and 8.3 and 8.8 for L-UK. Judging from the dissimilarity in the pI values of UK preparations obtained so far by other investigators and ourselves, we consider that the subforms of UK obtained after purification do not correspond to the forms present *in vivo*, but are artifacts of the purification procedures used.

Keywords——urokinase; purification of protein; column chromatography; SDS-polyacrylamide gel electrophoresis; isotachoelectrophoresis; amino acid analysis; Ouchterlony's double immunodiffusion method

Various methods, mainly involving column chromatography, have been attempted for the purification of urokinase (UK) [EC3.4.21.31] from human urine.[2–8)] However, previous purification methods have proved to be unsuitable for raising the specific activity of a large quantity of UK to a high level. In addition, little work has been done to compare the two active forms of UK, namely, the MW $5.4—5.5 \times 10^4$ form (H-UK) and the MW $3.3—3.6 \times 10^4$ form (L-UK), as regards the properties and the protein secondary structure of purified preparations. Most studies have been based on a clinical or a pharmacological standpoint.[9–12)]

We report in this paper an efficient purification method for both H- and L-UK, and the results of homogeneity assessment by analyses of molecular weight, isoelectric point, immunogenicity. and amino acid composition.

Materials and Method

Materials——A partially purified UK preparation was obtained by column chromatography on Sepharose 4B with the affinity ligand, α-benzylsulfonyl-*p*-amino-phenylalanine essentially as described by Maciag *et al.*.[8)] except that the crude UK preparation, which had been obtained from human urine by celite adsorption, precipitation with ammonium sulfate, and desalting with a hollow fiber-type artificial kidney (Asahi Kasei Co.), was applied to the column without any added detergents or fatty acids.

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 and Sephandex G-100, Sephadex G-75, and SP-Sephadex C-50 were from Pharmacia Fine Chemicals Co.

Determination of Plasminogen Activator Activity——Using the International Standard preparation for calibration, we determined the plasminogen activator activity of UK in terms of fibrinolytic activity by means of the fibrin tube method of Ploug and Kjeldgaard.[13]

Protein Determination——The amount of protein was determined colorimetrically by the method of Lowry *et al.*[14] with bovine serum albumin as a standard.

SDS-Polyacrylamide Gel Electrophoresis and Densitometry——Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed essentially according to the method of Weber and Osborn.[15] Molecular weights were determined by comparison with a simultaneous run of several reference proteins which included phosphorylase A (MW 94000), fibrinogen (alpha-, 70900; beta-, 60400; gamma-chain, 50700), and chymotrypsinogen A (25700). After being stained with Amide Black 10B, the cylindrical gels were scanned with a Schoeffel Co. densitometer.

Isotachoelectrophoresis——The isoelectric points (pI's) of UK were determined with an LKB Co. isotachoelectrophoresis apparatus 2127 TACHOPHOR.

Ten μl of protein sample (1 mg/ml) was applied to the apparatus with Ampholine (pH gradient 4—6 and 9—11) as a spacer. Various proteins were used as pI markers, *e.g.* horse heart acetylated cytochrome (cyt.) c (pI 3.9), *Rhodospirillum rubrum* cyt. c, (5.6). *Rhodospirillum rubrum* cyt.c_2 (6.2), horse myoglobin (7.6), whale sperm myoglobin (8.7), and horse heart cyt.c (10.6), which were purchased from Oriental Yeast Inc., Osaka. Leading electrolytes and terminating electrolytes were 10 mM potassium acetate adjusted with 1 M acetic acid to pH 5.0 –0.4% methyl cellulose and 10 mM β-alanine, respectively. The capillary tube was 23 cm long. The migration current was set at 100 μA. The oven was thermostatically controlled to 15°.

Gradient Gel Electrophoresis——PAA 4/30, gradient gel plates purchased from Pharmacia Fine Chemicals Co. were equilibrated with 350 mM β-alanine-140 mM acetic acid (Soln. I) inside an electrophoresis apparatus filled with Soln. I. Ten μg of a protein sample mixed with an equal volume of 40% sucrose-Soln. I was applied to a PAA 4/30 plate. The electrophoresis was conducted for eight hr. at 10° under a constant potential of 70 volts.

After the electrophoresis, the gel plate was stained with Coomassie Brilliant Blue R 250 and destained electrically in 7% acetic acid for 30 min. at 36 volts. Several kinds of proteins with various values of molecular weight were used as standards: bovine serum albumin, ovalbumin, α-chymotrypsinogen, horse myoglobin, and horse heart cytochrome c.

Ouchterlony's Double Immunodiffusion Method——Antiserum was obtained from a rabbit to which partially purified UK preparations emulsified in Freund's adjuvant had been administrated as an antigen either by the footpad technique or by the intraperitoneal route. Double diffusion was done on an agar dish by Ouchterlony's method.[16]

Amino Acid Analyses——The highly purified H- and L-UK preparation were analyzed in a JEOL JLC-5AH automatic amino acid analyzer after hydrolysis for 24 hr in 6 N HCl at 110° under a vacuum. L-Cysteic acid or DL-norleucine was used as an internal standard, whose recovery was used for the calculation of minimun molecular weight. Corrections were made for the decomposition of serine (10%) and threonine (5%).[17]

Results and Discussion

Gel Filtration Column Chromatography on Sephadex G-100

The lyophilized sample (1.4 g; 2.8×10^4 IU/mg protein) of partially purified UK, prepared by the method of Maciag *et al.*,[8] was dissolved in 25 ml of 20 mM Na-glycine buffer-300 mM NaCl pH 8.6 (buffer II) and passed through a 5×120 cm column packed with Sephadex G-100 equilibrated with buffer II. The elution patterns of absorbancy at 280 nm and plasminogen activator activity obtained by the gel filtration chromatography on Sephadex G-100 are shown in Fig. 1.

The absorbancy pattern was composed of five peaks, of which the second (Fr. G100-II) and third (Fr. G100-III) peak were enzymatically active.

The purity of the two active fractions was examined by SDS-polyacrylamide gel electrophoresis; the densitometric tracings are shown in Fig. 2. Fr. G100-II and Fr. G100-III each contained a few minor components and a single main component, whose molecular weight was determined by means of a Ferguson plot[18] to be 5.5×10^4 daltons for Fr. G100-II (H-UK) and 3.6×10^4 daltons for Fr. G100-III (L-UK).

Gel Filtration Column Chromatography on Sephadex G-75

The two active fractions (Fr. G100-II and Fr. G100-III) obtained by gel filtration of Sephadex G-100 were each concentrated, to 15 ml by ultrafiltration with an Amicon membrane Diaflo PM-10 and fractionated by gel filtration on a column (5×120 cm) of Sephadex G-75, whose upper limit as a molecular sieve (MW 7×10^4 as a globular protein) is slightly above the molecular weight of H-UK.

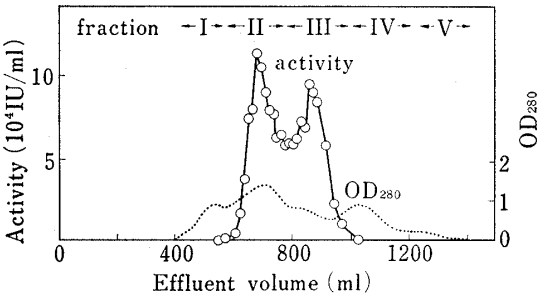

Fig. 1. Gel Filtration of Partially Purified Urokinase on Sephadex G-100

The lyophilized sample of UK (1.4 g; 2.8×10^4 IU/mg protein) was applied to a column (5×120 cm) of Sephadex G-100 and eluted with 20 mM Na-glycine buffer-300 mM NaCl pH 8.6.

The main fraction (Fr. G75-II-H) obtained by the gel filtration of Fr. G100-II on Sephadex G-75 showed a slight shoulder in the chromatogram (Fig. 3a) at a position of lower molecular weight corresponding to L-UK, whereas a relatively symmetrical peak was seen on gel filtration of Fr. G100-III with the same column (Fig. 3b). The main fractions of H- and L-UK

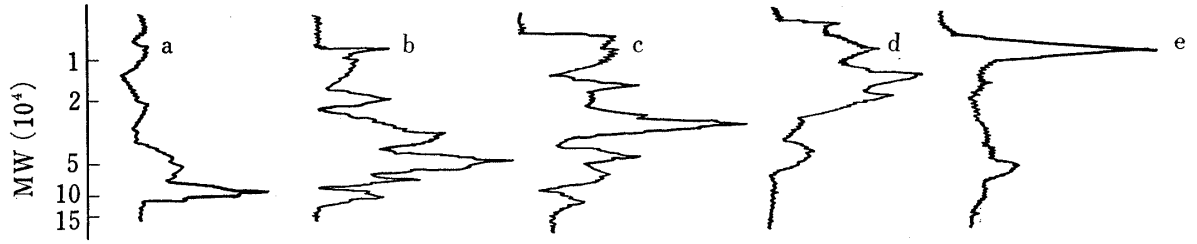

Fig. 2. Densitometry of SDS-Polyacrylamide Gel Electrophoregrams of Fractions obtained by Chromatography of Partially Purified UK on Sephadex G-100

a) Fr. G100-I, b) Fr. G100-II, c) Fr. G100-III, d) Fr. G100-IV, e) Fr. G100-V.

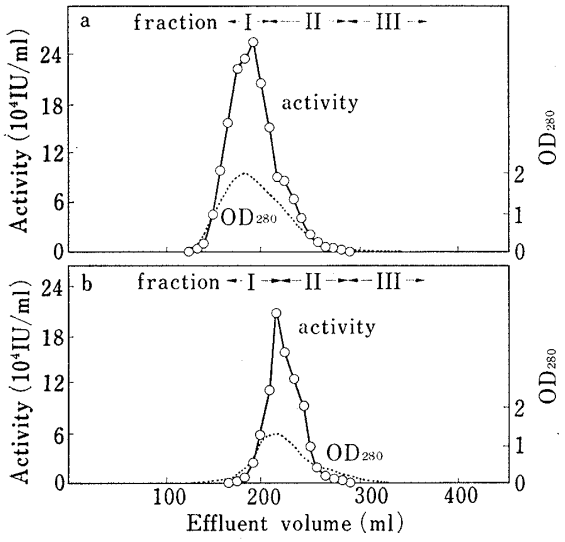

Fig. 3. Gel Filtration of H- and L-UK on Sephadex G-75

Fr. G100-II (445 mg; 4.61×10^4 IU/mg protein) and Fr. G100-III (259 mg; 5.22×10^4 IU/mg protein) were each applied to a column (5×120 cm) of Sephadex G-75 and eluted with 20 mM Na-glycine buffer-300 mM NaCl pH 8.6.

a) Fr. G100-II (H-UK). b) Fr. G100-III (L-UK).

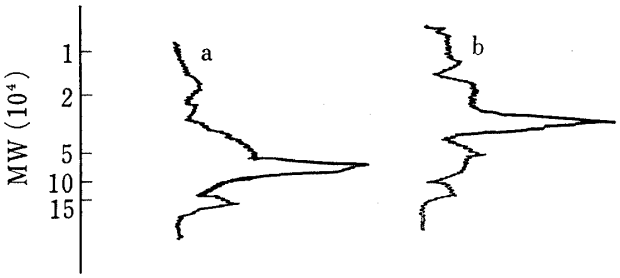

Fig. 4. Densitometry of SDS-Polyacrylamide Gel Electrophoregrams of Fractions obtained by Chromatography in Sephadex G-75

a) Fr. G75-II-H, b) Fr. G75-II-L.

(Fr. 75-II-H and Fr. G75-II-L) obtained by the gel filtration on Sephadex G-75 were shown by SDS-polyacrylamide gel electrophoresis (Fig. 4) to contain much smaller amounts of minor components than the Sephadex G-100 fractions (Fig. 2).

Ion Exchange Column Chromatography on SP-Sephadex C-50

Fr. G75-II-H and Fr. G75-II-L were each dialyzed against 20 mM Na-glycfne buffer, pH 8.6, without NaCl (buffer III) at 5° for about 4 hr until the electroconductivity of the dialysate had decreased nearly to that of buffer III and then applied to a column (5×120 cm) of SP-Sephadex C-50 equilibrated with buffer III. The column was washed with buffer III until the absorbancy of the effluent at 280 nm fell below 0.01. The protein adsorbed on the top of the column packed with the cation exchanger was eluted with a linear gradient from 0 to 0.4 M NaCl dissolved in buffer III.

By means of the ion exchange chromatography, H- and L-UK were each fractionated into several kinds of subforms (Figs. 5 and 6). On a cation exchanger such as SP-Sephadex, subforms with low pI values mostly tend to appear at lower effluent volume than subforms with high pI values, especially in the case of subforms possessing the same molecular weight. However, it was observed by Ogawa *et al.*[6] that the L-UK preparation was not chromatographically separated into more than two fractions on CM-Sephadex, although this preparation was shown by electrofocusing to contain five kinds of subforms.

Thus, although the separation of UK subforms cannot be generally achieved on all types of cation exchangers, SP-Sephadex C-50 combined with salt gradient elution, is effective.

Apart from various minor subforms, most fractions obtained by the chromatography of H-UK on SP-Sephadex possessed one or two major components in the isotachoelectrophoretic patterns (Fig. 6).

Since both H- and L-UK possessed five kinds of subforms, H-UK was purified relatively highly from the standpoint of an isoelectric point, as compared with L-UK, each fraction of which contained two or three subforms as major components.

Specific Activity and Activity Recovery

The fractions with the highest specific activity (Fr. SP-V-H and Fr. SP-V-L) obtained by chromatography on SP-Sephadex, were lyophilized and examined for plasminogen activator activity and the protein content; the latter was determined by the micro-Kjeldahl method and by ultraviolet absorption measurement, using an extinction coefficient of 12.6 for an optical path of 1 cm at 1% UK concentration, in addition to the method of Lowry *et al.*[14] The specific activity and the activity recovery at each purification stage of UK are shown

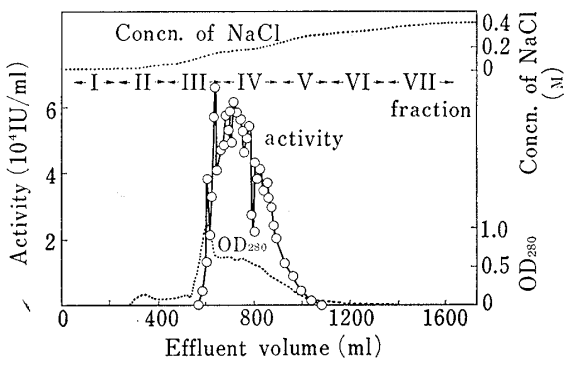

Fig. 5a. Ion Exchange Chromatography of H-UK on SP-Sephadex C-50

Fr. G75-II-H (335 mg; 4.63×10^4 IU/mg protein) was adsorbed on a column (5×120 cm) of SP-Sephadex C-50 and eluted with a linear gradient of NaCl concentration from 0 to 0.4 M.

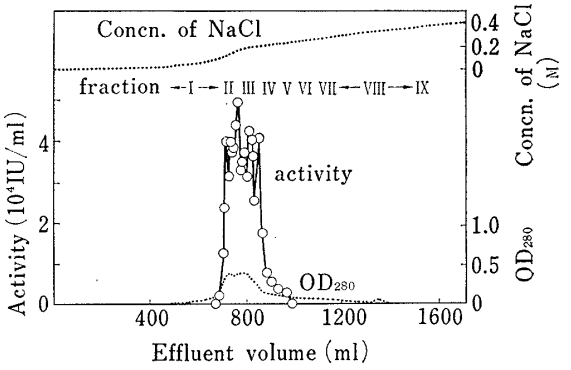

Fig. 5b. Ion Exchange Chromatography of L-UK on SP-Sephadex C-50

Fr. G75-II-L (198 mg; 7.56×10^4 IU/mg protein) was adsorbed on a column (5×120 cm) of SP-Sephadex C-50 and eluted with a linear gradient of NaCl concentration from 0 to 0.4 M.

in Table I. The specific activity of H-UK (1.20×10^5 IU/mg protein), which is generally recognized to be a native form of UK[19] and is known to possess a higher thrombolytic capacity *in vivo* than L-UK,[20] exceeded the highest level (1.04×10^5 IU/mg protein)[3] that has ever been obtained by other investigators. This was accomplished by the use of serial column chromatography including SP-Sephadex C-50. Although the purification stage with SP-Sephadex seems to result in large losses of activity from both Fr. G75-II-H and Fr. G75-II-L (Table I), fractions other than Fr. SP-V-H and Fr. SP-V-L obtained by the chromatography also showed high activity.

The activity recovery in the chromatography on SP-Sephadex amounted to 66.4 and 42.1% for H- and L-UK, respectively (sums of all active fractions). The purification factors of the main fractions at the SP-Sephadex stage were 2.48 and 1.81 for H- and L-UK, respectively. SP-Sephadex, accordingly, was better for the purification of H-UK, which was shown to be a pharmacologically advantageous form,[20] than for that of L-UK.

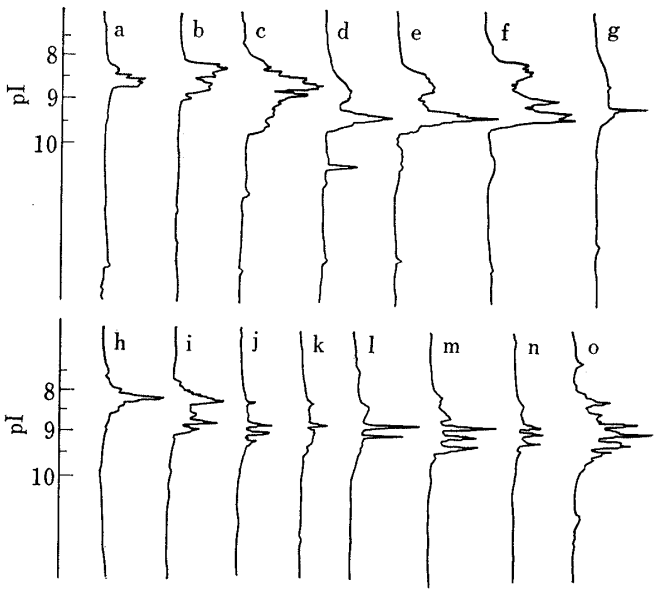

Fig. 6. Isotachoelectrophoresis of Fractions Obtained by Chromatography of H- and L-UK on SP-Sephadex C-50

H-UK: a) Fr. I, b) Fr. II, c) Fr. III, d) Fr. IV, e) Fr. V, f) Fr. VI, g) Fr. VII.
L-UK: h) Fr. I, i) Fr, II, j) Fr, III, k) Fr. IV, l) Fr. V, m) Fr. VI, n) Fr. VII, o) Fr. VIII.

Purity of Highly Purified UK in terms of Molecular Weight and Immunological Homogeneity

The cylindrical gels obtained by SDS-polyacrylamide gel electrophoresis of highly purified UK (Fr. SP-V-H and Fr. SP-V-L) were measured densitometrically (Fig. 7). The densitometric tracings showed an almost ultrasharp main peak for both UK forms but a minor peak at a position of higher molecular weight which can be assumed to correspond to the dimerized form of H- or L-UK.

The UK preparation at each purification stage was analyzed by gradient gel electrophoresis (Fig. 8), which could detect sensitively more components in the UK preparation than SDS-polyacrylamide gel electrophoresis. The crude UK preparation before application on Sephadex G-100 was shown to contain at least nfne components.

Table I. Specific Activity and Activity Recovery at Each Purification Stage of UK

Purification stage		Fraction	Total protein (mg)	Total activity (10^5IU)	Specific activity (IU/mg protein)	Activity recovery (%)
Partially purified prepn.			1400	392	28000	100.0
H-UK	Sephadex G-100	II	445	205	46100	52.4
	Sephadex G-75	II	335	155	46300	39.6
	SP-Sephadex C-50	V	33	38	114000	9.7
	Lyophilization		33	40	120000	10.2
L-UK	Sephadex G-100	III	259	135	52200	34.5
	Sephadex G-75	II	198	150	75600	38.2
	SP-Sephadex C-50	V	14	19	134000	4.9
	Lyophilization		14	21	152000	5.4

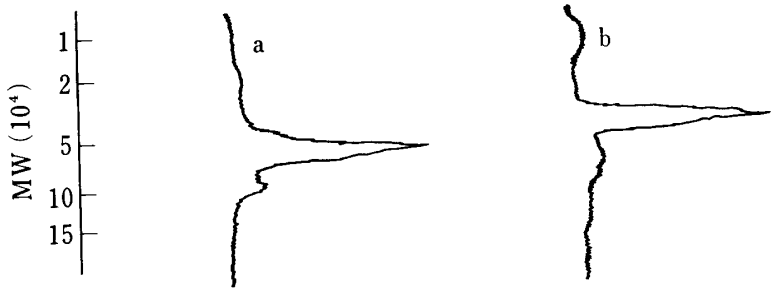

Fig. 7. Densitometry of SDS-Polyacrylamide Gel Electrophoregrams of Highly Purified UK

a) Fr. SP-V-H (H-UK), b) Fr. SP-V-L (L-UK).

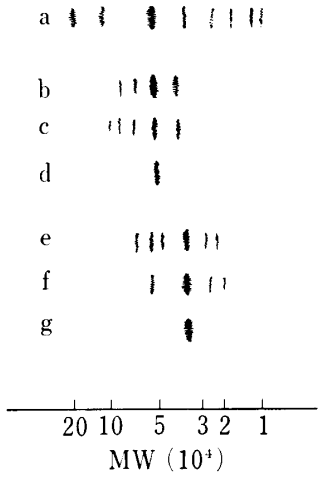

Fig. 8. Comparison of Gradient Gel Electrophoregrams of UK Preparations at Each Purification Stage

a) partially purified prepn. before application to Sephadex G-100, b) Fr. G100-II, c) Fr. G75-II-H, d) Fr. SP-V-H, e) Fr. G100-III, f) Fr. G75-II-L, g) Fr. SP-V-L.

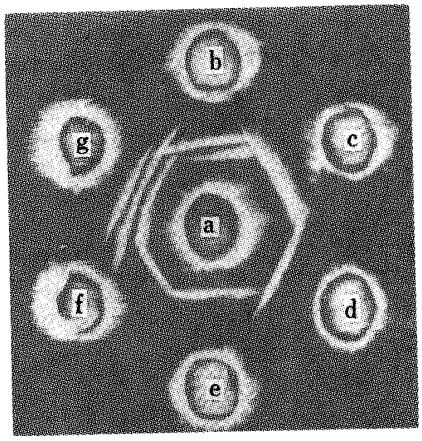

Fig. 9. Ouchterlony's Double Diffusion Test for Various UK Preparations

a) antiserum, b) Fr. G100-I, c) Fr. SP-V-H, d) Fr. SP-V-L, e) Fr. G100-IV, f) Fr. G100-V, g) partially purified UK before application to Sephadex G-100.

The amounts of components decreased as the purification proceeded in the case of both UK forms. The most highly purified UK (Fr. SP-V-H and Fr. SP-V-L) which was obtained by the chromatography on SP-Sephadex, gave essentially a single spot.

The immunological homogeneity of highly purified H- (Fr. SP-V-E) and L-UK (Fr. SP-V-L) was examined by Ouchterlony's method (Fig. 9).[16] Both H- and L-UK were immunologically homogeneous, showing a single precipitation line between the antiserum and each UK form used as an antigen, while crude UK preparation (before Sephadex G-100) gave at least three precipitation lines.

As regards antigenic structure, H-UK is assumed to be similar to L-UK because neither a spur nor a cross was seen; the precipitation lines of the two UK forms fused completely. The antigen-determining group presumably exists on the heavy chain, where the active site was indicated to be situated,[3,18] not on the light chain.

Amino Acid Composition

The amino acid compositions of H- and L-UK showed no striking difference (Table II). It is assumed, accordingly, that the polypeptide segment which is present in H-UK but not in L-UK and presumably corresponds to the light chain (MW 2.0×10^4) of H-UK, did not differ markedly from L-UK or the remainder of H-UK as regards amino acid composition.

The contents of basic amino acids (lysine, histidine, and arginine) of H-, L-UK and the

Table II. Amino Acid Compositions of H-UK and L-UK

	g%		Residues per molecule	
	H-UK	L-UK	H-UK	L-UK
Lysine	7.21	7.23	27	18
Histidine	4.76	3.88	17	9
Arginine	7.89	7.60	25	16
Aspartic acid	9.98	8.40	41	23
Threonine[a)]	5.35	5.95	25	18
Serine[a)]	6.36	6.69	33	23
Glutamic acid	10.91	12.15	41	30
Proline	5.23	5.23	25	16
Glycine	5.46	5.47	40	26
Alanine	3.52	3.35	22	14
Half-cystine	4.00	2.87	18	9
Valine	4.79	4.12	23	13
Methionine	2.07	2.36	8	6
Isoleucine	4.05	4.79	17	13
Leucine	8.32	8.83	35	24
Tyrosine	6.28	6.22	19	12
Phenylalanine	3.81	3.97	13	9
Tryptophan	nd	nd	nd	nd
Total number of residues			429	279

a) Corrected for decomposition of threonine (5%) and serine (10%).

light chain were all in the range from 15 to 17% as a molar fraction of total amino acid residues; those of acidic amino acids (aspartic acid and glutamic acid) were 18—19%; those of hydrophobic amino acids (valine, leucine, isoleucine, tyrosine, and phenylalanine) were 23—25%.

In terms of the amino acid composition, L-UK is slightly more similar to the B chains with active site peptide segments from the serine proteases, thrombin and plasmin,[21,22)] than H-UK and the light chain of H-UK are. In comparison with these two serine enzymes, however, L-UK possesses a larger amount of serine and glutamic acid and a smaller amount of valine.

The three molecular types of UK known so far, *i.e.* MW 54000—55000 form, MW 47,000 form, and MW 33000—36000 form,[20)] all possess similar amino acid composition; the MW 47000 form and MW 33000 form of UK, obtained by Soberano *et al.*,[3)] did not differ strikingly in composition from our H- and L-UK. This suggests that the three molecular types of UK originate from the same precursor protein or that the two smaller ones are derived from H-UK itself, which contains a heavy chain with an active site which is similar to the B chains of thrombin and plasmin from the standpoint of amino acid composition.

Determination of Molecular Weight

The molecular weight of UK was determined by SDS-polyacrylamide gel electrophoresis; Ferguson's plot[18)] was applied to correlate the gel concentrations and migration distances. In addition to gradient gel electrophoresis and gel filtration on Sephadex G-100, the molecular weights were calculated from the results of amino acid analyses. The values obtained were in close agreement with one another: H-UK gave a molecular weight of 5.5×10^4 daltons and L-UK 3.6×10^4 daltons. This value for H-UK is in fair agreement with the results of Sumi *et al.*[23a)] (MW 54000) and Nishida[23b)] (MW 54000), whereas the value for L-UK is consistent with the result of Clemmensen and Christensen[23c)] (MW 36000), but significantly larger than the results of Andrassy and Ritz[23d)] (MW 32000) and Ogawa *et al.*[6)] (MW 33000).

The molecular weight of a protein composed of more than two chains is generally difficult to determine exactly and is likely to be underestimated in the unreduced form. However,

this was not the case for H-UK, which is composed of two polypeptide chains.[3] SDS-polyacrylamide gel electrophoresis, gave a molecular weight of 5.5×10^4 for unreduced but partially unfolded form of H-UK in 1.2% SDS without any reductants, and this is nearly equal to the sum of the molecular weights of the heavy chain (3.6×10^4) and the light chain (2.0×10^4) separated from H-UK by reduction in 0.2% SDS with β-mercaptoethanol. This suggests that the neighborhood of interchain SS bridges in H-UK is not folded markedly even in a native state, and/or that there are few intrachain SS loops and intercha in SS bridges, so that most regions of the polypeptide are sufficiently accessible to SDS.

Characterization of pI Pattern

The UK preparations purified by other investigators differed from one another in terms of pI values as well as molecular weight. This is not regarded as arising from the dissimilarity in their methods for determining pI values. Soberano *et al.*[3] reported the purification of two molecular forms of UK, each of which contained five subforms with pI values within the range from 8.05 to 8.70; Ogawa *et al.*[6] purified the MW 33000 form, which also contained five subforms with a pI range similar to that of our L-UK (from pI 7.5 to 9.7), *i.e.*, from 6.9 to 9.6; Walasek[23c] moreover, isolated multimolecular forms of UK with pI values which ranged from 7.5 to 9.8; Andrassy and Ritz[23b] purified MW 32000 form and detected two or three bands by disc gel electrophoresis.

The isotachoelectrophoretic profiles (Figs. 6h—o) show that Fr. SP-V-L (corresponding to L-UK) contained five subforms with pI values of 7.5, 8.3, 8.8, 9.4, and 9.7. By comparing the ratio of activity to absorbancy at 280 nm (Fig. 5b) with the isotachoelectrophoretic profiles (Figs. 6h—o), the subforms of L-UK with the lowest pI value (7.5) and with the highest pI value (9.7) were shown to be almost inactive, whereas the most active subforms showed pI values of 8.8 and 8.3.

These findings are similar to the results of Ogawa *et al.*,[6] who purified MW 33000 form alone (corresponding to our L-UK) and found that the most acidic subform (pI 6.9) was almost inactive while the most alkaline one (pI 9.6) showed less than one-half of the specific activity of the most active subform (pI 8.5) of the five subforms contained in MW 33000 form of UK.

It was found (Figs. 5a and 6a—g) that five kinds of subforms with pI values of 8.7, 8.9, 9.1, 9.2, and 9.4 were also present in Fr. SP-V-H (corresponding to H-UK) and that the most acidic subform (pI 8.7) was almost completely inactive, as in the case of L-UK. In contrast to L-UK, the most active subform was the most alkaline one (pI 9.4) of the five kinds of subforms contained in H-UK.

The MW 47000 form and MW 33000 form, both contained four subforms with the same pI values (8.05, 8.40, 8.60, and 8.70),[3] but there was no subform with the same pI value in the case of our H- and L-UK, although the MW 47000 form was shown not to be identical to H-UK (MW 54000—55000).[20] The difference in the molecular weight of these subforms may be so slight that SDS-polyacrylamide gel electrophoresis cannot separate them, if they were produced by elimination of a short peptide from the precursor protein. Alternatively, the difference in the pI values may depend on variation in the saccharide content of the glycoprotein, *e.g.* sialic acid, glucosamine, and galactosamine (present at 1.67, 0.74, and 0.29 wt%, respectively,[24]) and/or may be due to "nicking" within the intrachain SS loops at constant molecular weight.

Aside from the question of whether the subforms of UK are classified as "isozymes" or "multiple enzyme forms" it seems likely that the subforms do not exist *in vivo* (in contrast to the case of heart form and muscle form of lactate dehydrogenase,[25] which are typical isozymes), but arise in the purification procedure as an artifact produced by protease,[26] as in the cases of yeast hexokinase,[27] phytochrome,[28] and human serum choline esterase,[29] and/or by nonenzymatic hydrolysis of amide groups,[26] as in the case of cytochrome c,[30] and *Escherichia coli* sugar carrier protein.[31] This is because one would expect the same subform pattern or at least a main subform with the same pI value, in common among various lots

of UK prepared by various investigators[3,6,23] and the present authors if the subforms of UK which have been detected so far had existed *in vivo* in the same pI pattern as found after purification.

Homogeneity of Highly Purified UK Preparations

Fr. SP-V-H and Fr. SP-V-L, which were obtained by serial column chromatography, appeared to be essentially homogeneous as regards specific activity (Table I), molecular weight (Figs. 7 and 8), immunogenicity (Fig. 9), and isoelectric point (Fig. 6).

We regard the most active subforms, whose pI values were 9.4 for H-UK and 8.8 and 8.3 for L-UK, as the representatives of each UK form, and we intend to carry out further studies of the enzymological and physico-chemical properties of H- and L-UK with these highly purified preparations.

Acknowledgement We are grateful to Dr. I. Utsumi, general manager of the Pharmaceuticals Center, Kanebo, Ltd. for his encouragement and to Miss Y. Nakata for her technical assistance.

References and Notes

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