ELSEVIER

Journal of Chromatography B, 742 (2000) 71–78

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Affinity chromatography for purification of two urokinases from human urine

Ryoki Takahashi*, Kiyoshi Akiba, Motofumi Koike, Takayasu Noguchi, Yohji Ezure

Sagami Research Laboratories, Wakamoto Pharmaceutical Co., Ltd., 378 Kanate, Ohi-machi, Ashigarakami-gun, Kanagawa, 258-0018 Japan

Received 19 October 1999; received in revised form 1 February 2000; accepted 4 February 2000

Abstract

A new affinity chromatography (hydrophobic-mediated affinity chromatography), which was characterized by the matrix having both affinity site to urokinase and hydrophobic site, was established for the purification of urokinase from human urine. The hydrophobic affinity matrix (tentatively named PAS in the text) was prepared by immobilizing 6-aminocaproic acid on Sepharose CL-6B, followed by a coupling *p*-aminobenzamidine to a part of the hydrophobic site on the matrix. The PAS matrix was applied to the purification of urokinase from human urine, and high- and low-molecular weight pure urokinases were efficiently obtained in high yield by the present method.

Keywords: Hydrophobicity; Enzymes; Urokinase; 6-Aminocaproic acid; *p*-Aminobenzamidine

1. Introduction

Urokinase (UK; EC 3.4.99.26) has been widely used as an effective thrombolytic agent for medication of acute myocardial infarction, peripheral arterial occlusion, and acute cerebral infarction [1–3]. Tissue-type plasminogen activator (t-PA) has been also used as one of the most representative thrombolytic agents; however, the side effects of t-PA such as bleeding and reocclusion [1,4,5] were significant. Recently, several groups have independently reported that a combination therapy of t-PA and UK for the patients decreased those side effects [1,6–8]. On the other hand, it is well known that there are two kinds of UKs, high- and low-molecular weight urokinases (HUK and LUK, respectively) in human urine, and HUK is the major component of urinary UK [9]. LUK is supposed to be derived from HUK by proteolytic degradation [10] and/or autolytic cleavage [11]. Although the enzymatic activities of HUK and LUK are nearly equivalent at the presence of plasma in vitro [12], the activity of HUK is considered to be more significant than that of LUK based on a higher affinity of HUK to their native substrate, Glu-plasminogen in vivo [11,13]. Recently, Abaza et al. [14], Koopman et al. [15], Shetty et al. [16] and Sillaber et al. [17] have reported evidence of the biological importance of HUK on proliferation [14], metastasis [15] and cell migration [16], relating to the expression of HUK receptor on various tumor cells such as human brain malignant AA cell lines, GB cell lines, human melanoma cell M14 and IF6, mesothelioma cells MS-1, and human

*Corresponding author. Tel.: +81-465-83-8047; fax: +81-465-85-1153.

E-mail address: wakamoto_sagami@biglobe.ne.jp (R. Takahashi)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00127-4

mast cells [17], respectively. They also suggested that the biological role of LUK in cancer metastasis might be less than that of HUK because of the absence of LUK receptor on various tumor cells, and the utilization of LUK has recently been reported [18,19]. Based on these observations, the biological roles of LUK are attracting a great deal of attention.

There are many reports on the purification of UK using affinity chromatography [20–26]; however, there are few reports on the purification of HUK and LUK from human urine. On the other hand, we have already reported a new affinity matrix named PAS prepared by immobilizing 6-aminocaproic acid (6-ACA) on Sepharose CL-6B, followed by coupling a part of carboxyl group of 6-ACA with *p*-aminobenzamidine (*p*-AB) [26]. Furthermore, we applied this matrix to the purification of UK containing both HUK and LUK from human urine. In this paper, we have developed a new affinity matrix, tentatively named hydrophobic-mediated affinity chromatography, having both affinity site and hydrophobic site. Furthermore, we showed a large-scale preparation of purified HUK and LUK from crude urine samples by the hydrophobic-mediated affinity chromatography.

2. Material and methods

2.1. Reagents

Sepharose CL-6B was purchased from Pharmacia-Biotech (Tokyo, Japan). 1,1′-Carbonyldiimidazole (CDI), *p*-aminobenzamidine dihydrochloride (*p*-AB·2HCl), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were from Aldrich (Milwaukee, WI, USA). Anhydrous dioxane (less than 0.5% of water) was purchased from Aldrich and used. The partially purified UK was purchased from Polyamine (Taipei, Taiwan; 15 000 IU/mg protein). All other chemicals were of analytical reagent grade and used without further purification.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and gel permeation chromatography (GPC)

SDS–PAGE and GPC were performed according to the modified methods of Laemmli [27] and Someno et al. [28], respectively. Briefly, SDS–PAGE was performed at 4–20% gradient gel (SDS–PAGE mini-gel, TEFCO, Tokyo, Japan) and protein was stained with 0.25% Coomassie brilliant blue in 10% acetic acid with 50% methanol.

The molecular weight of samples were determined by SDS–PAGE using the following proteins as references: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), β-lactalbumin (14.4 kDa); and then estimated by a Digitizer densitometer equipped with an image analyzer (Model TIAS-2300S, ACI Japan, Tokyo, Japan).

GPC was carried out with TSK-GEL3000SW column (60 cm×7.5 mm I.D., Toyo Soda, Tokyo, Japan). The analyses of HUK and LUK were carried out with Jasco-UP980 liquid chromatograph (Jasco, Tokyo, Japan) equipped with a syringe loading injector with a 50-μl sample loop (Rheodyne, USA). The eluent used 0.2 *M* KH_2PO_4 solution (pH 3.0) with 0.7 ml/min flow-rate, detector wavelength of 280 nm and sensitivity of 20 mV. A 20-μg protein sample was injected into the liquid chromatograph.

2.3. Measurement of UK activity

UK activity was determined by the modified fibrin plate method of Walton [29] using the WHO standard. The urokinase standards (120 000 units/mg protein) obtained from the National Institute of Medicinal and Food Sciences were used for the determination of the enzyme activity. The amount of protein was determined by the Folin method of Lowry et al. [30], using bovine serum albumin as a standard protein.

2.4. Preparation of standard HUK and LUK

Standard HUK (115 000 IU/mg protein, M_r 54 000) and LUK (135 000 IU/mg protein, M_r 33 000) were prepared from partially purified UK according to the modified method of Holmberg et al. [20]. Briefly, the HUK/LUK mixture was purified by affinity chromatography of *p*-AB-Sepharose 4B and gel filtration on Sephadex G100. The purity of each HUK and LUK was confirmed by both SDS–PAGE and GPC. The purified enzymes are stable for 2

months under the chromatographic condition (0.1–1.0 *M* NaCl and pH 4–8 at room temperature).

2.5. Preparation of 6-ACA-Sepharose CL-6B, PAS and p-AB-Sepharose CL-6B

6-ACA-Sepharose CL-6B, PAS and *p*-AB-Sepharose CL-6B were prepared according to the previously described method [26]. Preparation of 6-ACA-Sepharose CL-6B: Sepharose CL-6B (20 ml) was washed sequentially with 250 ml of water, 30% aqueous dioxane, 70% aqueous dioxane and dioxane on a glass filter-funnel and suspended in 20 ml of dioxane. CDI (1.2 g) was added, and the suspension was stirred at room temperature for 30 min and then washed sequentially with 250 ml of dioxane, 70% aqueous dioxane, 30% aqueous dioxane and water on a glass filter-funnel. The activated Sepharose CL-6B (20 ml) was reacted at room temperature with 6-ACA (9.4 g) in 20 ml of water at pH 10 for 10 h and washed sequentially with 250 ml of water, 0.1 *M* NaOH, water, 0.1 *M* HCl, and water. Fifty-eight μmol of 6-ACA was immobilized to 1 ml of Sepharose CL-6B.

Preparation of PAS: to a solution of 6-ACA-Sepharose CL-6B (20 ml) suspended in 20 ml of water, *p*-AB·2HCl (220 mg), and EDC (1.4 g) were added, and the suspension was stirred at room temperature for 10 h. The pH was adjusted to 4.5 and maintained at the pH for 10 h with 3 *M* HCl and 3 *M* NaOH. The modified matrix was washed in the same way described for the coupling step of 6-ACA. Twenty-nine μmol of *p*-AB as a ligand and 29.0 μmol of 6-ACA as a residual spacer were immobilized on 1 ml of Sepharose CL-6B.

Preparation of *p*-AB-Sepharose CL-6B: 6-ACA-Sepharose CL-6B, in which 30.5 μmol of 6-ACA were introduced to 1 ml of Sepharose CL-6B, was prepared by procedures similar to those described for 6-ACA-Sepharose CL-6B, except that 0.6 g of CDI was used. Then, *p*-AB-Sepharose CL-6B was prepared by procedures similar to those described for PAS by using EDC (1.4 g) and *p*-AB-2HCl (220 mg). Thirty μmol of *p*-AB were immobilized on 1 ml of the 6-ACA derivative.

For the determination of the content of 6-ACA and *p*-AB on matrix, first it was dried, and the total nitrogen content was determined by total nitrogen analyzer, MT-700 (Yanaco, Tokyo, Japan). The content of 6-ACA and *p*-AB was estimated to be mole equivalent to the total nitrogen content.

These procedures should be performed under conditions controlled precisely for the reproducibility of the affinity matrix. When the resolution and affinity of the resin to the enzymes are lost, the resin should be washed with 0.05 *M* NaOH and 0.05 *M* HCl containing 0.1–0.5% SDS, repeatedly.

2.6. Larger-scale purification of HUK and LUK from human urine

Human urine (1240 l; 9.9×10^6 IU) was collected from healthy male volunteers. Urine was cooled at 10°C, and the pH was adjusted to 9 with 12 *M* NaOH, and then the sample was kept at 10°C for 1 h. The precipitate was removed by a cotton membrane filter and the pH was adjusted to 6.5 with 12 *M* HCl, and then the sample was loaded into a column (30 cm I.D.×1.5 m) which was used for nitrogen gas bubbling to produce foam from the urine sample. The produced urinary foam was transformed from the bubbling column to the bottle cooled at 4°C. The foam was turned to liquid form by addition of deforming agent (silicone mist, TSA-732, Toshiba silicone, Tokyo). Consequently, we collected 100 l of concentrated urine fraction from 1240 l of neat urine, and the fraction was processed further purification procedures. One hundred liters of the fraction (10.1×10^6 IU; 48 IU/mg protein, total protein 210.4 g) were collected by bubbling, and the pH was adjusted to 7.5 by 12 *M* NaOH. To this solution, silica gel B40 beads (30 kg; Fuji Davison, Tokyo, Japan) were added, and the mixture was stirred at 300 rpm at 10°C for 10 h. The silica gel B40 beads, on which UK was adsorbed, were packed into a column (30 cm I.D.×51 cm). The column was washed by 3% ammonium borate (pH was adjusted to 9 by conc. NH_4OH), and UK activity was eluted by 1% ammonium hydroxide containing 1 *M* NH_4Cl. The pH of a UK fraction was adjusted to 7.3 by 12 *M* H_2SO_4, and $(NH_4)_2SO_4$ was added to the fraction to 60% of saturation. The precipitate (9.0×10^6 IU; 2300 IU/mg protein, total protein 3.913 g) was recovered by centrifugation at 7000 *g*, and was dissolved in 0.1 *M* phosphate buffer (pH 7.0, 300 ml) followed by centrifugation at 7000 *g*. The obtained supernatant was loaded onto a column (2.4 cm I.D.×3.5 cm) containing 15 ml of PAS, which

was equilibrated with 0.1 *M* phosphate buffer (pH 7.0) solution containing 0.1 *M* NaCl. The column was washed sequentially by 0.1 *M* phosphate buffer (pH 7.0) solution containing 0.1 *M* NaCl (Buffer A) and containing 1 *M* NaCl (Buffer B) until the optical absorbance of effluent at 280 nm became less than 0.01. LUK was eluted by 0.1 *M* NaH_2PO_4 solution containing 1 *M* NaCl (pH 4.0) (Buffer C), and then HUK was eluted by decreasing NaCl concentration from 1 to 0.1 *M* in Buffer C. The fractions were monitored by measuring both the optical absorbance at 280 nm and UK activity. The molecular weight of each UK was confirmed by SDS–PAGE. Both SDS–PAGE and GPC confirmed the purity of each UK sample.

3. Results and discussion

3.1. Preparation of matrix for purification of HUK and LUK

Although there may be many methods for preparing matrix that is applicable to hydrophobic-mediated chromatography, the shortest synthetic route should be designed. The strategy was as follows. First, a functional group that can be used for hydrophobic site and spacer for affinity site was introduced. Second, parts of functional group were reacted with a ligand to form the affinity site so that the appropriate amount of unreacted functional group should remain. For this purpose, ω-amino alkyl carboxylic acid or diaminoalkane could be selected as a functional group. In the case of ω-amino alkyl carboxylic acid, a coupling reaction between carboxylic acid of ω-amino alkyl carboxylic acid and the amino group of the ligands was controlled so that the appropriate amount of carboxylic acid should remain. By changing the ligand to carboxylic acid ratio, various types of matrices have been obtained. It is also well established that UK is one of the typical serine proteases and *p*-AB is a strong inhibitor against serine proteases [31,32]. Based on these observations, 6-ACA and *p*-AB had been chosen as a functional group and a ligand, respectively.

To clarify the nature of the hydrophobic-mediated affinity chromatography, two kinds of matrices were prepared; one is a matrix composed of the hydrophobic part of PAS alone (6-ACA-Sepharose CL-6B), and the other is a matrix composed of the affinity part of PAS alone (*p*-AB-Sepharose CL-6B). Their structures are shown in Fig. 1.

3.2. Effect of matrix ligand on adsorption of HUK and LUK under various pH and ionic strength conditions

Adsorption behaviors of HUK and LUK on the three matrices, 6-ACA, PAS, and *p*-AB, were examined under various pH and ionic strength conditions. Briefly, four columns (1.0×4.0 cm) packed with 3 ml of each matrix resin were equilibrated by Buffer A, Buffer B, Buffer C, or 0.1 *M* NaCl (pH 4.0) (Buffer D). A standard HUK (1.3×10^6 IU) or LUK (1.3×10^6 IU) solution was loaded onto the column. The column was eluted until the optical absorbance of each effluent at 280 nm became less than 0.01. The amount of each UK adsorbed was calculated by measuring the unadsorbed enzyme activity.

Adsorption of HUK and LUK on three kinds of matrices, 6-ACA-Sepharose CL-6B, PAS and *p*-AB-Sepharose CL-6B, is summarized in Table 1. As shown in Table 1, both HUK and LUK were adsorbed to a PAS column in the ranged of 83–100% under the conditions of 0.1 and 1 *M* NaCl, pH 7.0; however, only LUK was not adsorbed at pH 4.0. On the other hand, HUK was not adsorbed at 0.1 *M* NaCl and pH 4.0, but was adsorbed at 1 *M* NaCl and pH 4.0.

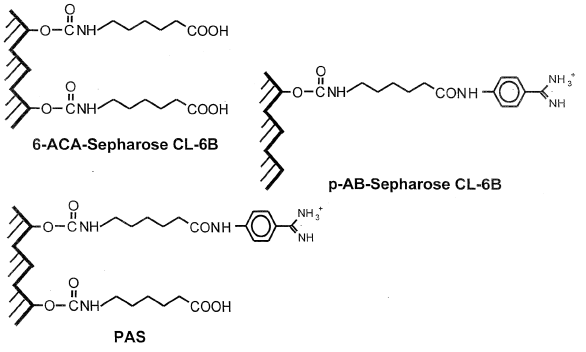

Fig. 1. Structures of matrices used for this study.

Table 1
Effect of pH and ionic strength on adsorption of HUK and LUK to matrix[a]

Matrix	Exp. no.	NaCl (*M*)	pH	HUK (%)	LUK (%)
p-AB-Sepharose CL-6B	1	0.1	7.0	99.6	98.0
	2	1.0	7.0	85.0	83.0
	3	0.1	4.0	0.8	0.5
	4	1.0	4.0	19.1	5.0
6-ACA-Sepharose CL-6B	5	0.1	7.0	5.0	3.5
	6	1.0	7.0	3.0	2.0
	7	0.1	4.0	15.0	32.0
	8	1.0	4.0	70.0	15.0
PAS	9	0.1	7.0	100	100
	10	1.0	7.0	100	95
	11	0.1	4.0	0.2	0
	12	1.0	4.0	97.0	0

[a] *p*-AB-Sepharose CL-6B: 30 μmol of *p*-AB was immobilized on 1 ml of Sepharose CL-6B. 6-ACA-Sepharose CL-6B: 30.5 μmol of 6-ACA was immobilized on 1 ml of Sepharose CL-6B. PAS: 30 μmol of *p*-AB and 30 μmol of 6-ACA were immobilized on 1 ml of Sepharose CL-6B.

The adsorption–elution mechanism of enzymes with PAS may be explained as follows: at pH 4.0, enzymes are generally supposed to be adsorbed by hydrophobicity because the carboxyl group of 6-ACA residue does not dissociate at pH 4.0, and should affect the hydrophobicity of the molecule. When the pH of the solution was increased, the dissociation of the carboxyl group might be increased, resulting in both decrease of hydrophobicity and increase of the affinity composed by *p*-AB residues. On the other hand, when the salt concentration was increased, the hydrophobicity could be enhanced. Interestingly, the hydrophobic interaction between LUK and PAS disappeared at pH 4.0. This might be explained as follows: PAS is composed of *p*-AB residues and 6-ACA residues, and both residues are distributed at random on the surface of a resin. The chain length of the *p*-AB residue is longer than that of 6-ACA residue. The lack of kringle module in LUK might not contribute to its hydrophobicity at pH 4.0. Furthermore, non-specific hydrophobic interaction between LUK and 6-ACA residues could be greatly influenced by the steric hindrance due to *p*-AB residues. Although HUK has a hydrophobic kringle module, its hydrophobicity potential was weakened at 0.1 *M* NaCl and pH 4.0, resulting in the lower contribution of the kringle module to the adsorption. Consequently, it was considered that the decrease of adsorption was caused by the steric hindrance due to *p*-AB residues, as in the case of LUK. We speculated that the above

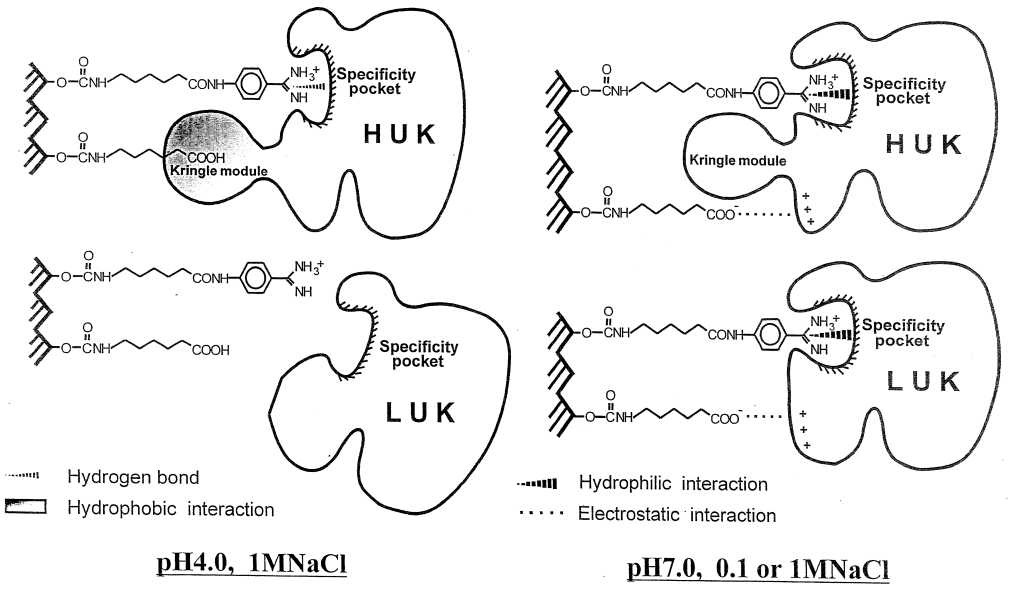

Fig. 2. Speculation of interaction between PAS and HUK under various conditions.

adsorption mechanism might be depicted as shown in Fig. 2.

3.3. Large-scale purification of HUK and LUK from human urine

Table 2 summarizes the purity of UK mixture from human urine by the present method. Highly purified UK was efficiently obtained from human urine by the combination of bubbling, silica gel chromatography and hydrophobic-mediated affinity chromatography, with a final yield of 80–90%. Fig. 3 shows each UK elution profile of hydrophobic-mediated affinity chromatography on PAS, which was performed as a final step.

The analysis of the purified UK by SDS–PAGE and GPC revealed that the hydrophobic-mediated affinity chromatography was very effective in separating HUK and LUK without contamination of any impurity (Figs. 4 and 5). As shown in Table 2 and SDS–PAGE (Fig. 4), the resulting HUK showed a specific activity of 120 000 IU/mg and a molecular weight of 54 kDa, and those of LUK were 135 000 IU/mg and 33 kDa, respectively. To determine the maximal binding capacity of a PAS column, various concentrations of crude UK dissolved in 0.1 *M* phosphate buffer (pH 7.0) were loaded to 1 ml of the column, and then the column was washed with Buffer A. The adsorption capacity of the column was estimated by measuring the specific activity and UK activity of the eluted fraction by Buffer D. As shown in Fig. 5, the maximal binding capacity of PAS was evaluated to be 9×10^5 IU per 1 ml of the gel without a loss of specific activity.

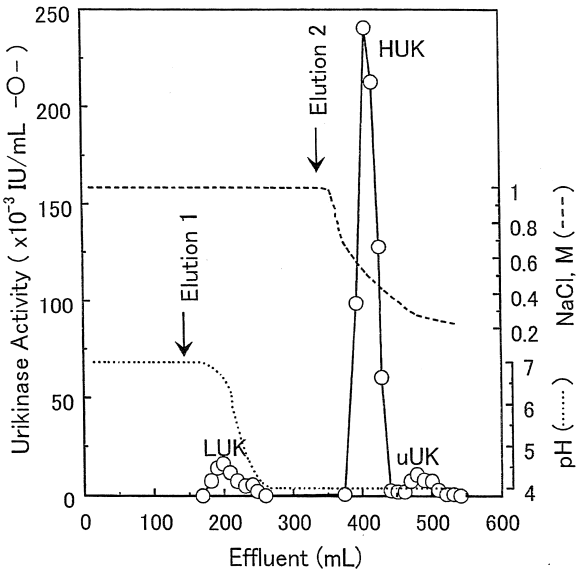

Fig. 3. Hydrophobic-mediated affinity chromatography of UK on the PAS column. After application of the crude UK (2300 IU/mg protein) eluted from the silica gel column, the PAS column was washed with Buffer A and B. At the indicated arrow, elution 1 was carried out with Buffer C and elution 2 with reducing NaCl concentration in Buffer C. LUK, HUK and uUK (unidentified UK-like protease) fractions were collected, respectively.

There have been barely successful reports on the purification of HUK and LUK, and many kinds of ligands have been tried for affinity chromatography

Table 2
Purification of HUK and LUK from human urine

Step	Volume (ml)	Urokinase		Protein (mg)	Specific activity (IU/mg protein)	Purification fold
		Activity ($\times10^{-4}$ IU)	Recovery (%)			
Urine	1.24×10^6	990	100	7.4×10^5	13	1
Foam fraction	1.0×10^5	1010	102	2.1×10^5	48	3.9
Silica gel eluate (ppt)[a]	8.2×10^3	900	91	3.9×10^3	2300	177
PAS affinity						
LUK	190	72	7.3[b]	5.33	135 000	10 380
HUK	55	747	75.4[b]	62.25	120 000	9230
uUK	100	36	3.6[b]	4.73	76 000	5840

[a] Precipitation (ppt) was obtained by 65% ammonium fractination of the eluate from silica gel chromatography.
[b] Estimated values based on UK activity. uUK, unidentified UK-like protese.

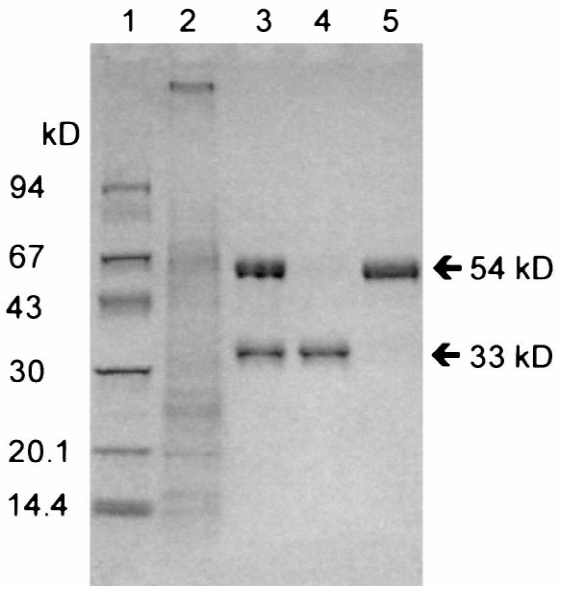

Fig. 4. SDS–PAGE of HUK and LUK. The electrophoresis was carried out at 18 mA for 2 h. The sample was dipped into a boiling water bath for 5 min before electrophoresis. Lanes: (1) molecular weight standard proteins (94 kDa, phosphorylase b; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20.1 kDa, soybean trypsin inhibitor; 14.4 kDa, lactalbumin); (2) crude urokinase (10 μg, ppt. of silica gel eluate in Table 2); (3) a mixture of HUK and LUK standards (3 μg each); (4) purified LUK (3 μg); and (5) purified HUK (3 μg).

of UK. Among these reports, the affinity ligands to HUK and LUK seem to be very similar [20,23,31], and there is no report on complete purification of HUK and LUK. The fact suggests that it might be impossible to separate completely HUK and LUK

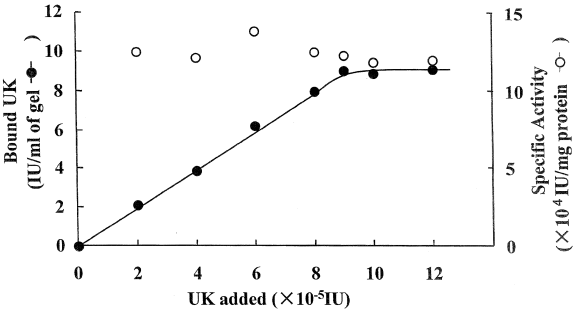

Fig. 5. Adsorption capacity of PAS. Increasing amounts of crude UK were loaded on a constant PAS. After washing PAS, UK was eluted with Buffer C. The eluted UK activity was estimated as amount of bound UK per 1 ml of PAS.

each other by general affinity chromatography. Consequently, the utilization of new types of ligand(s) should be added to the affinity chromatography used before. On the other hand, there is a big difference in reported molecular weight between HUK (M_r 47 000–54 000) and LUK (M_r 31 000–33 000) [9,11,20,25,33]. It is well known that 6-ACA can bind to plasminogen kringle [34], and also it is a homologue of UK kringle, and this UK kringle module is found only in the HUK molecule [35,36]. As the kringle module is hydrophobic [37], this hydrophobicity should be noted as one of the key points for building up a matrix for the purification of HUK and LUK. Based on this consideration, we intended to study chromatography by a matrix having a general affinity site and a new hydrophobic site, which was named hydrophobic-mediated chromatography, for the purification of HUK and LUK. The concept shown in this paper on hydrophobic-mediated affinity chromatography could be generally applicable to the purification of other enzymes and proteins.

References

[1] J. Lopez-Sendon, S.E. de Lopez, J.F. Bobadilla, R. Rubio, J. Bermejo, J.L. Delcan, Rev. Esp. Cardiol. 48 (1995) 407–439.
[2] A.J. Comerota, G.S. Cohen, Can. J. Surg. 36 (1993) 342–348.
[3] M. Fujishima, T. Omae, K. Tanaka, K. Iino, O. Matsuo, H. Mihara, Angiology 37 (1986) 487–498.
[4] E.J. Topol, R.M. Califf, B.S. George, D.J. Kereiakes, D. Rothbum, R.J. Candela, C.W. Abbotsmith, C.A. Pinkerton, D.C. Stump, D. Collen, Circulation 77 (1988) 1100–1107.
[5] GUSTO Investigator, New Engl. J. Med. 329 (1993) 673–682.
[6] R.M. Califf, E.J. Topol, R.S. Stack, S.G. Ellis, B.S. George, D.J. Kereiakes, J.K. Samaha, S.J. Worley, J.L. Anderson, L. Harrelson-Woodlief, Circulation 83 (1991) 1543–1556.
[7] W. Rutsch, Z. Schmutzler, Herz 19 (1994) 336–352.
[8] C. Bode, H. Baumann, E. von Hodenberg, M. Freitag, T. Nordt, Z. Kardiol. 82 (1993) 125–128.
[9] W.F. White, G.H. Barlow, M.M. Mozen, Biochemistry 5 (1966) 2160–2169.
[10] W.A. Günzler, G.J. Steffens, F. Ötting, G. Buse, L. Flohé, Hoppe-Seyler Z. Physiol. Chem. 363 (1982) 133–141.
[11] M. Nobuhara, M. Sakamaki, H. Ohnishi, Y. Suzuki, J. Biochem. 47 (1981) 41–45.
[12] M. Samama, M. Castel, O. Matsuo, M. Hoylaerts, H.R. Lijnen, Thromb. Haemost. 47 (1982) 36–40.

[13] N. Toki, S. Takasugi, H. Sumi, Enzyme 26 (1981) 221–224.
[14] M.S. Abaza, F.A. Shaba, R.K. Naraya, M.Z. Atassi, Br. J. Cancer 78 (1998) 1578–1585.
[15] J.L. Koopman, J. Slomp, A.C. Bart, P.H. Quax, J.H. Verheijen, J. Biol. Chem. 273 (1998) 33267–33272.
[16] S. Shetty, A. Kumar, A. Johnson, S. Pueblitz, S. Idell, Am. J. Physiol. 268 (1995) 972–982.
[17] C. Sillaber, M. Baghestanian, R. Hofbauer, I. Virgolini, H.C. Bankl, W. Fureder, H. Agis, M. Willheim, M. Leimer, O. Scheiner, B.R. Binder, H.P. Kiener, P. Valent, J. Biol. Chem. 272 (1997) 7824–7832.
[18] X.C. Chen, Z.C. Hua, D.X. Zhu, Biochem. Mol. Biol. Int. 39 (1996) 455–460.
[19] X.C. Chen, Z.C. Hua, D.X. Zhu, Biochem. Mol. Biol. Int. 39 (1996) 797–803.
[20] L. Holmberg, B. Bladh, B. Astedt, Biochim. Biophys. Acta 445 (1976) 215–222.
[21] V. Yomotova, B. Tchorbanov, S. Novkova, B. Blagoev, J. Appl. Biochem. 2 (1980) 1–6.
[22] X.J. Cao, J.W. Zhu, D.W. Wang, X.Y. Wu, G.C. Dai, Ann. NY Acad. Sci. 799 (1996) 454–459.
[23] T. Someno, T. Saino, K. Katoh, H. Miyazaki, S. Ishii, J. Biochem. 97 (1985) 1493–1500.
[24] J. Wojta, J.C. Kirchheimer, L. Turcu, G. Christ, B.R. Binder, Thromb. Haemost. 55 (1986) 347–351.
[25] M.E. Soberano, E.B. Ong, A.J. Johnson, M. Levy, G. Schoellmann, Biochim. Biophys. Acta 445 (1976) 763–773.
[26] R. Takahashi, Yakugaku Zasshi 108 (1988) 331–338.
[27] U.K. Laemmli, Nature 227 (1970) 680–685.
[28] T. Someno, K. Katoh, K. Niijima, H. Miyazaki, J. Chromatogr. 188 (1980) 185–192.
[29] P.L. Walton, Clin. Chim. Acta 13 (1966) 680–684.
[30] O.H. Lowry, J.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[31] J.D. Geratz, S.R. Shaver, R.R. Tidwell, Thromb. Res. 24 (1981) 73–83.
[32] J.D. Geratz, M.C.F. Cheng, Thromb. Diathes. Haemorrh. 33 (1975) 230–243.
[33] A. Lesuk, L. Terminiello, J.H. Traver, Science 147 (1965) 880–882.
[34] G. Markus, J.L. DePasquale, F.C. Wissler, J. Biol. Chem. 253 (1978) 727–732.
[35] W.A. Günzier, G.J. Steffens, F. Ötting, Sung-Man, A. Kim, E. Frankus, L. Flohé, Hoppe-Seyler Z. Physiol. Chem. 363 (1982) 1155–1165.
[36] G.J. Steffens, W.A. Günzier, F. Ötting, E. Frankus, L. Flohé, Hoppe-Seyler Z. Physiol. Chem. 363 (1982) 1043–1058.
[37] A.M. Bokman, J. Jimenez-Barbero, M. Llinas, J. Biol. Chem. 268 (1993) 13858–13868.