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Immobilized urokinase column as part of a specific detection system for plasminogen species separated by high-performance affinity chromatography

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ABSTRACT

Immobilized urokinase was used as part of a post-column reactor for the specific detection of human plasminogen species which were fractionated using a high-performance affinity column. After on-line activation of each peak, plasmin activity was measured by mixing the eluate with a specific fluorogenic substrate and the product was detected by a fluorescence monitor. This detection system gave linear calibration graphs for both purified plasminogens (0.1–50 μg) and plasminogens contained in plasma (25–100 μl). Relative standard deviations for the determination of plasminogens in plasma were 6.1–6.6% (n = 12), showing good reproducibility. The detection limit was as low as 0.1 μg of plasminogen. Immobilized urokinase was very stable and no appreciable decrease in activity was found after 100 cycles of operation. In combination with an immobilized benzamidine column, this system made it possible to separate and detect Glu-plasminogen and Lys-plasminogen contained in human plasma samples as small as 100 μl without any pretreatment.

INTRODUCTION

We have previously developed an analytical system for enzymes involved in the fibrinolytic system by using high-performance affinity chromatography [1–5]. Monitoring of the fibrinolytic system is important for diagnostic purposes, be-

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cause it is also related to various disorders occurring in blood vessels. The principal fibrinolytic enzyme, plasmin (EC 3.4.21.7), is produced from its inactive precursor plasminogen [6]. Most plasminogen molecules have N-terminal glutamic acid (Glu-plg), which is the parental form, although another molecular species having N-terminal lysine (Lys-plg) is often obtained [7]. Lys-plg is readily produced by *in vitro* digestion of Glu-plg with plasmin [7,8], and thus has been considered as an artifact produced during the purification process. However, we were able to demonstrate its presence in normal plasma using our analytical system [4], although the amount found was minute.

In vitro experiments showed that Lys-plg is activated by urokinase and plasminogen activator much faster than Glu-plg [9]. Hence it could be important to determine Lys-plg in circulating blood of patients for a more accurate diagnosis, because it may play a crucial role at a very early stage of fibrinolysis. High-performance affinity chromatography using Asahipak GS-520 bearing *p*-aminobenzamidine (ABA) was found to be effective in separating Glu-plg and Lys-plg [2]. Asahipak GS-520 gel, a newly developed packing material for high-performance liquid chromatography (HPLC) which is composed of poly(vinyl alcohol), is mechanically and chemically very stable. The pore surface is enriched with hydroxyl groups, which can be used for immobilization of affinity ligands. The immobilized ligand ABA interacts with both the lysine-binding site of plasminogen [10] and the active site of plasmin. Thus, in addition to Glu-plg and Lys-plg, active plasmin is also adsorbed. These three proteins can be eluted separately by an appropriate elution programme. However, the detection of the minor component Lys-plg was extremely difficult. Although active plasmin can be readily detected by using a specific fluorogenic substrate, this procedure is not directly applicable to the inactive precursors.

We therefore devised an on-line activitation system by introducing urokinase into the eluate from the affinity column [3–5]. Activated enzyme was then mixed with the fluorogenic substrate and the fluorescence produced was measured. This system proved to be effective to detect and determine minute amounts of Lys-plg in plasma. It had some drawbacks, however; for example, the use of urokinase as a solution often resulted in poor reproducibility because of its instability. To overcome these difficulties, the use of immobilized urokinase was tried. Urokinase was immobilized on AF-tresyl Toyopearl 650M, which is a preactivated matrix for medium-pressure chromatography composed of a hydrophilic vinyl polymer. It is mechanically and chemically stable and can be used to immobilize amino and thiol compounds. A column packed with this immobilized urokinase connected to the affinity column activated Glu-plg and Lys-plg in the eluate (Fig. 1). This improvement resulted in both increased stability and reduced consumption of urokinase. The reproducibility and reliability of the measurement were also greatly improved.

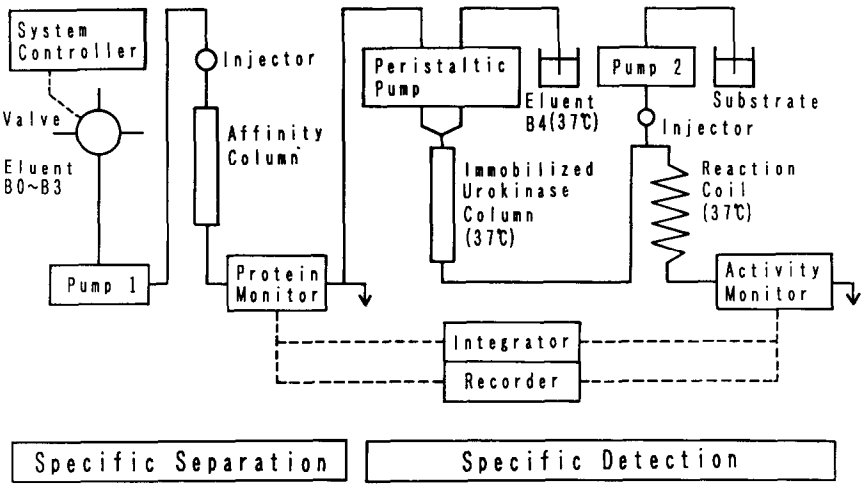

Fig. 1. Schematic diagram of the system.

EXPERIMENTAL

Materials

The sources of the materials used were as follows: Asahipak GS-520 gel (exclusion limit molecular mass $3 \cdot 10^5$; particle size 9 ± 0.5 μm) from Asahi Chemical Industry (Tokyo, Japan), AF-tresyl Toyopearl 650M gel (exclusion limit molecular mass $5 \cdot 10^6$; particle size 44–88 μm; tresyl group content 100 μmol per gram of dry gel) from Tosoh (Tokyo Japan), 6-aminohexanoic acid (AHA) from Nacalai Tesque (Kyoto, Japan), *p*-aminobenzamidine monohydrochloride (ABA) from Sigma (St. Louis, MO, U.S.A.), 7-(*tert.*-butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysineamido)-4-methylcoumarin (Boc-Glu-Lys-Lys-AMC), 7-(glutaryl-L-glycyl-L-arginineamido)-4-methylcoumarin (Glt-Gly-Arg-AMC) and aminomethylcoumarin (AMC) from the Protein Research Foundation (Osaka, Japan), 1,1′-carbonyldiimidazole (CDI) from Tokyo Chemical Industry (Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (EDC) from Dojin Chemical (Kumamoto, Japan) and urokinase for research use [high-molecular-mass type, from human urine, more than 60 000 international units (I.U.) per mg of protein], Urokinase Inj.-Green Cross for therapeutic use (from human urine, 60 000 I.U. per vial) and plasminogen from human serum (8–9 casein units per mg of protein, mixture of Lys-plg I and II) from Green Cross (Osaka, Japan). Purified Glu-plg I and II were prepared from human citrated plasma by affinity chromatography on lysine-Sepharose in the presence of bovine pancreatic trypsin inhibitor (Trasylol).

Preparation of affinity adsorbent (Asahipak GS-520–AHA–ABA)

The activation of Asahipak GS gel with CDI and the coupling of spacer and ligand were carried out by methods similar to those reported previously [2,11]. In brief, Asahipak GS gel was suspended in dry acetone and activation with CDI was carried out for 15 min at room temperature. The activated gel was treated with an excess of AHA overnight at 4°C and pH 10. The amount of the imidazoyl group introduced seemed to be more than 440 μmol/g. Then ABA was immobilized as a ligand on the introduced carboxyl groups by using EDC (water-soluble carbodiimide). The product contained 437 μmol of AHA and 48 μmol of ABA per gram of dry gel.

Preparation of immobilized urokinase gel

AF-tresyl Toyopearl was suspended in 10 ml of coupling buffer containing urokinase (for research use or for therapeutic use, 15 000–120 000 I.U.) and the suspension was gently shaken overnight at 4°C. The coupling buffer was 0.2 *M* sodium hydrogencarbonate containing 0.5 *M* sodium chloride (pH 8.0). Then, to block unreacted tresyl groups, 100 ml of 0.1 *M* Tris–HCl buffer (pH 8.0) were added and the suspension was gently shaken overnight at 4°C. The gel was washed sequentially with 200 ml of water, 100 ml of 0.05 *M* sodium phosphate–0.1 *M* sodium chloride solution (eluent B1) (pH 7.4) and 200 ml of water.

A matrix coupled with Tris base was prepared as an experimental control. To prepare this gel, 0.1 *M* Tris–HCl containing 0.1 *M* sodium chloride (pH 8.0) was mixed with AF-tresyl Toyopearl. The other conditions were similar to those for the immobilized urokinase.

Affinity chromatography on Asahipak GS-520–AHA–ABA

Affinity chromatography was carried out by using a system similar to that reported previously [2,11]. Asahipak GS-520 coupled with ABA was suspended in 0.2 *M* sodium sulphate solution and packed into a stainless-steel column (100 mm × 6 mm I.D.). The final flow-rate was 1.7 ml/min. The following eluents were used: B0, 0.05 *M* sodium phosphate (pH 5.5); B1, 0.05 *M* sodium phosphate–0.1 *M* sodium chloride (pH 7.4); B2, as B1 + 0.02 *M* AHA; and B3, as B1 + 0.02 *M* AHA + 3 *M* urea. Glu-plg, Lys-plg and plasmin were trapped by the column, which was equilibrated with eluent B0. Glu-plg was eluted with eluent B1. Lys-plg was eluted with eluent B2 and plasmin with eluent B3. The eluents were changed according to an elution programme by the use of an eight-way connector (FCV-3AL; Shimadzu, Kyoto, Japan) and pumped at a flow-rate of 1.0 ml/min.

On-line detection of plasminogen species by the immobilized urokinase system

Immobilized urokinase gel was suspended in eluent B1 and packed into a stainless-steel column (100 mm × 6 mm I.D.) (Umetani Seiki, Osaka, Japan) by changing the flow-rate in a stepwise manner from 1.0 to 2.6 ml/min during 30

min. The column contained an amount of the gel corresponding to a dry weight of 0.6 g. After passage of the effluent through a fluorescence monitor (Jasco FP-110, excitation at 254 nm, emission at 340 nm) to detect protein, a portion was taken at a flow-rate of 0.32 ml/min by a peristaltic pump (Gilson Minipulse 2). This was mixed with solvent B4 (eluent B1 + 0.08 *M* AHA), which was kept in a water-bath at 37°C, and led to the immobilized urokinase column, which was also immersed in a water-bath at 37°C. After the immobilized urokinase column, a solution of a specific substrate (40 μM Boc-Glu-Lys-Lys-AMC in 0.05 *M* phosphate buffer, pH 7.4) was introduced into the eluates by using a pump for HPLC (Shimadzu LC-6A) at a flow-rate of 0.3 ml/min. This mixture was passed through a reaction coil (10 m × 0.5 mm I.D. PTFE tubing) immersed in a water-bath at 37°C. Fluorescent AMC produced by plasmin was detected by a fluorescence monitor (Shimadzu RF-530; excitation at 380 nm, emission at 460 nm; flow-rate, 0.94 ml/min) and recorded. To calibrate the fluorescence intensity, a known amount of AMC was injected into the reaction coil. After a series of experiments, the immobilized urokinase column was flushed with eluent B1 containing 0.02% sodium azide and stored at 4°C.

Evaluation of linearity and reproducibility of the detection system

Authentic plasminogens. As authentic plasminogens, stock solutions of Glu-plg II (0.98 mg/ml) and Lys-plg (mixture of Lsy-plg I and II, 1.27 mg/ml) were serially diluted with eluent B1 containing 0.02% bovine serum albumin, which was necessary to prevent loss of plasminogen at very low concentrations, and injected into the system. With Glu-plg, prior to sample application, the affinity column was equilibrated with eluent B0. After application of Glu-plg, the column was washed with the same eluent for 15 min and the eluent was switched to eluent B1. With Lys-plg, the affinity column was equilibrated and run for 5 min after sample application with eluent B1, and adsorbed Lys-plg was eluted with eluent B2. To evaluate the reproducibility, a fixed volume of Lys-plg diluted as above was injected repeatedly into the system.

Evaluation of linearity and reproducibility of the determination of plasminogens in plasma. Human plasma was injected directly into the system without pretreatment. The conditions of affinity chromatography were as follows. A plasma sample was injected into the affinity column which had been equilibrated with eluent B0 and the column was washed with the same eluent. Then, eluents B1 (for Glu-plg), B2 (for Lys-plg) and B3 (for plasmin) were applied for 30, 10 and 5 min, respectively. For the evaluation of linearity, 25, 50, 75 and 100 μl of human plasma were applied. For the evaluation of reproducibility, 50 μl of human plasma were analysed repeatedly (two or three times per day during a five-day period).

RESULTS

Preparation of immobilized urokinase

A urokinase preparation for research use and also a preparation for therapeutic purposes were each effectively immobilized on AF-tresyl Toyopearl 650M. Additives contained in the therapeutic preparation (mainly human serum albumin) did not seem to have interfered with the immobilization of urokinase. After blocking of unreacted tresyl groups, the derivatized gel was packed in a stainless-steel column. Six preparations differing in the amount of immobilized urokinase were obtained (2500–49 000 I.U. of urokinase per gram of dry gel). The yield in the immobilization reaction in terms of activity was 25–40%. The preparation having the largest amount of urokinase, obtained by using therapeutic urokinase, was mainly used in the experiments unless stated otherwise. The amount of immobilized urokinase on 1 g of the dry gel corresponded to about 1.6 mg of a preparation having a specific activity of 30 000 I.U./mg. In the column (100 mm × 6 mm I.D.), about 0.6 g of immobilized urokinase gel was packed (about 29 000 I.U. per column).

Detection of plasminogens separated by the affinity column

According to the established chromatographic protocol [2,4], Glu-plg, Lys-plg and plasmin were adsorbed on the column which had been equilibrated with eluent B0 (0.05 *M* phosphate buffer, pH 5.5), and sequentially eluted with eluent B1 (containing 0.1 *M* NaCl), eluent B2 (containing 0.02 *M* AHA) and eluent B3 (containing 0.02 *M* AHA and 3 *M* urea), respectively. Although the immobilized urokinase column adsorbed plasminogens to some extent, this was prevented by the addition of AHA. Thus, 0.08 *M* AHA solution (B4) was added to the effluent from the affinity column by using a peristaltic pump to make the final concentration of 0.04 *M* AHA. Because of the large particle size of AF-tresyl Toyopearl (44–88 μm), the back-pressure of the flow system did not increase. Fig. 2 shows a chromatogram obtained after application of a mixture of authentic Glu-plg, Lys-plg and plasmin. The proenzymes and active enzyme separated by the affinity column were clearly detected on the basis of their enzymatic activity.

The reproducibility and the linearity of the relationship between integrated area for peaks of activity and amount injected were examined. Up to 50 μg of Glu-plg (corresponding to the amount of Glu-plg contained in 300 μl of human plasma) or Lys-plg were injected to the system. As the flow-rate for the affinity column was 1.0 ml/min, and a fraction of the effluent was continuously withdrawn at a flow-rate of 0.32 ml/min for introduction into the immobilized urokinase column, up to 16 μg of the proenzymes were provided for activation. Fig. 3 shows the relationship between the area of the peak (expressed as AMC produced) and the amount of the proenzyme applied. Linearity was apparent for both Glu-plg and Lys-plg. The amount of AMC produced by Lys-plg was about 1.4 times that produced by Glu-plg. It is well known that the rate of activation of

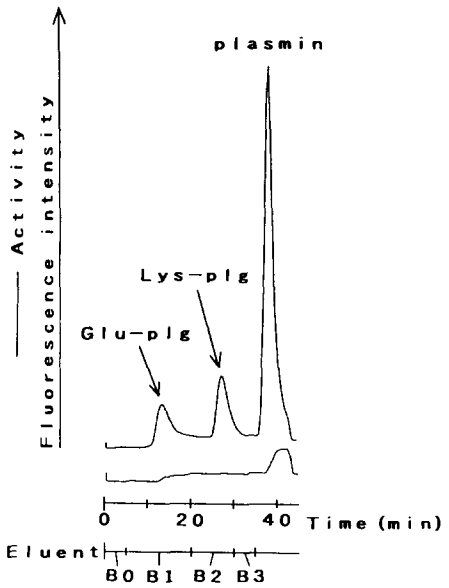

Fig. 2. Chromatogram obtained for a mixture of authentic Glu-plasminogen (Glu-plg), Lys-plasminogen (Lys-plg) and plasmin (top curve). The affinity column was equilibrated with eluent B0. Under these conditions, Glu-plg, Lys-plg and plasmin are adsorbed. A mixture of Glu-plg (5.5 μg) and Lys-plg (4.2 μg) was activated with 10 I.U. of urokinase for 30 s at 37°C, and the activation mixture was immediately applied to the system. The bottom curve was obtained in a blank run. The eluents were changed as indicated.

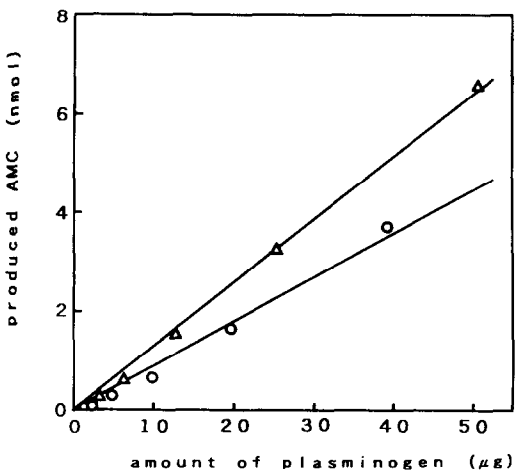

Fig. 3. Relationship between peak area [aminomethylcoumarin (AMC) produced] and amount of plasminogen applied. Glu-plg (0.98 mg/ml) and Lys-plg (1.27 mg/ml) were serially diluted with eluent B1 containing 0.02% bovine serum albumin, and 80 μl of the diluted solution were applied to the system. The peak area was expressed as the amount of AMC produced. For chromatographic conditions, see Experimental. (○) Glu-plg; (△) Lys-plg.

Glu-plg by urokinase is about one tenth of that of Lys-plg, but addition of AHA or lysine markedly enhances the activation of the former to almost the same rate as that of the latter [9]. Although, in the present system, Glu-plg was activated by the immobilized urokinase column in the presence of AHA, this process was still less efficient than the activation of Lys-plg. If as little as 6 pmol of AMC (corresponding to 0.1 μg of plasminogen) was produced, it could be detected as a peak on the chromatograms. Table I shows the reproducibility of the measurement when 3.2 μg of Lys-plg were repeatedly analysed during five days.

Application to plasma samples

As good linearity and reproducibility were confirmed when authentic plasminogens were analysed, a similar examination was made for plasma. Fig. 4 shows a chromatogram obtained when 50 μl of human plasma were injected without any pretreatment into this system. A large peak of Glu-plg and a small peak of Lys-plg appeared, but no plasmin was detected (a slight elevation of the baseline due to urea was observed at about 60 min). When we used a control column packed with AF-tresyl Toyopearl coupled with Tris base instead of urokinase, no peak of activity was detected.

Fig. 5 shows the dependence of the area of activity peaks on the volume of plasma. Linearity was satisfactory up to 100 μl of plasma. In this series of experi-

TABLE I

REPRODUCIBILITY OF THE PRESENT ASSAY SYSTEM

With authentic plasminogen, 40 μl (3.2 μg) of Lys-plg were repeatedly injected into the system. With plasminogens in plasma, 50 μl of human plasma were analysed repeatedly (2 or 3 times per day during a 5-day period).

	n	Amount of AMC produced (mean ± S.D.) (pmol)	Relative standard deviation (%)
Authentic plasminogen			
Between-day	14	300 ± 20	6.7
Within-day	3	310 ± 12	3.9
	3	295 ± 11	3.7
Plasminogens in plasma			
Between-day			
Glu-plg	12	608 ± 37	6.1
Lys-plg	12	21.1 ± 1.4	6.6
Within-day			
Glu-plg	3	667 ± 5.1	0.8
	3	582 ± 2.1	0.4
Lys-plg	3	21.0 ± 0.71	3.4
	3	22.8 ± 0.53	2.3

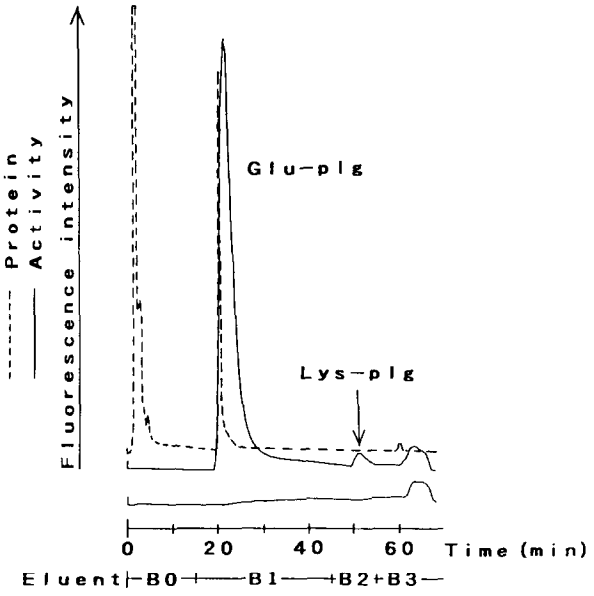

Fig. 4. Chromatogram obtained for human plasma (top solid curve). The affinity column was equilibrated with eluent B0 and 50 μl of normal plasma without any pretreatment were applied to the system. The bottom solid curve was obtained in a blank run. The eluents were changed as indicated.

ments, Glu-plg was adsorbed on the affinity column, eluted after washing the column for 15 min and determined (Fig. 5A). When we used elution conditions where Glu-plg passes through the affinity column, however, the linearity for Glu-plg was lost, especially when larger amounts of plasma were applied (Fig. 5B). This seems to be due to the presence of plasmin inhibitor in the fraction that passes through. Hence it proved to be necessary to remove plasmin inhibitor prior to activation in the immobilized urokinase column.

Reproducibility was examined by analysing 50 μl of plasma repeatedly during five days. Relative standard deviations of about 7% were obtained even for Lys-plg, which gave only small peaks (Table I). The contents of Glu-plg and Lys-plg in normal plasma were calculated as 13–15 mg/dl, and 0.3–0.4 mg/dl by using Fig. 3 as the calibration graph. The plasminogen concentration in human plasma has been reported to be 15–20 mg/dl, although no distinction between Lys-plg and Glu-plg was made. The present study showed that several percent of total plasmin activity produced by urokinase is attributable to Lys-plg. In the previous report, we roughly estimated the content of Lys-plg as 0.1–1% of that of Glu-plg by using a primitive analysis system. The more precise value obtained by the present system is slightly larger than expected. The reliability of the present procedure was verified by comparison with a currently used diagnosis kit (Testzyme; Kabi Diagnostica, Sweden), which is composed of streptokinase and a chromo-

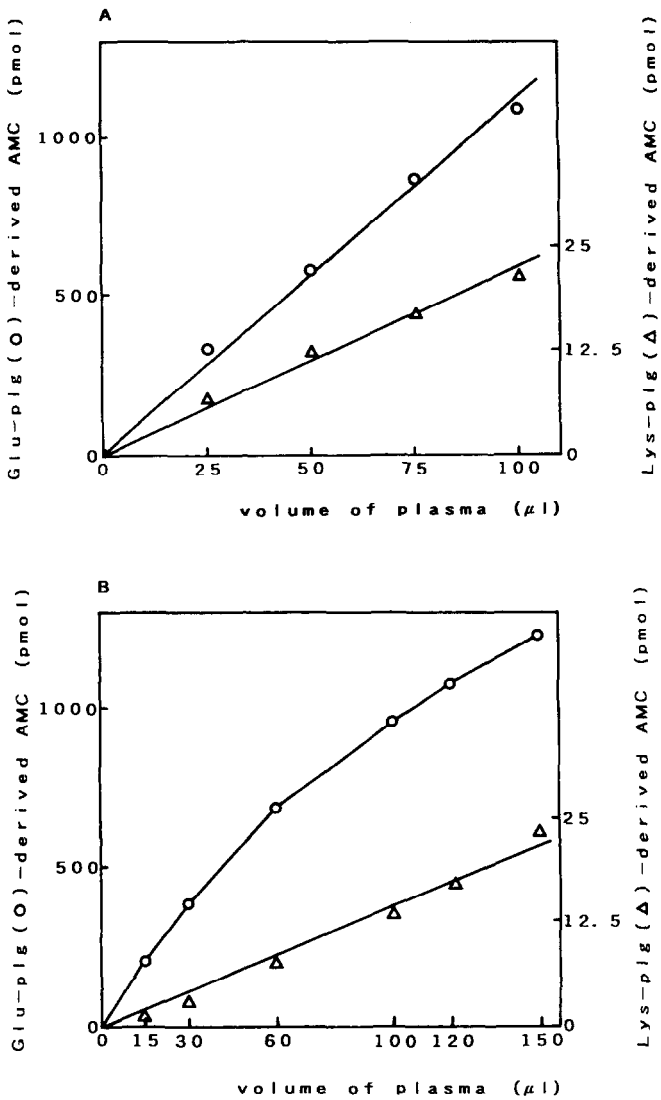

Fig. 5. Relationship between peak area and volume of plasma applied. (A) Chromatography was carried out under conditions where Glu-plg, Lys-plg and plasmin are adsorbed. The affinity column was equilibrated with eluent B0, and 25–100 μl of plasma without any pretreatment were applied to the system. (B) Chromatography was carried out under conditions where Glu-plg passes through the column. The affinity column was equilibrated with eluent B1, and 15–150 μl of plasma were applied to the system. Peak area was expressed as the amount of AMC produced. Values on the left-hand ordinate are for Glu-plg and those on the right-hand ordinate are for Lys-plg.

genic substrate. Plasma samples obtained from a normal individual and a patient with a hereditary abnormality in plasminogen were analysed, and plasminogen contents relative to a plasma for calibration (provided by Kabi) were obtained.

Testzyme gave values of 80% for the normal plasma and 54% for the abnormal plasma, whereas the present system gave values of 81 and 49% (sum of Glu-plg and Lys-plg), respectively. Hence the accuracy of the present system seems to be satisfactory.

Analysis of plasma containing abnormal plasminogen

Plasma samples from patients with hereditary abnormal plasminogen [12,13] were analysed using the present system. These patients have plasminogen that is reactive to antibodies but which cannot be activated to plasmin. This abnormality was attributed to substitution of one amino acid residue at the active site (from Ala to Thr) [14]. Fig. 6 shows results obtained for abnormal plasmas from a heterozygote (Fig. 6B) and a homozygote (Fig. 6C). The peak at about 60 min is due to the elevation of the baseline. The contents of both Glu-plg and Lys-plg in the plasma from the heterozygote were about half of those in the normal plasma. Hence the proportion of Lys-plg being produced from Glu-plg, that is, the process of the limited hydrolysis of the N-terminal part of Glu-plg, is unchanged in the abnormal plasminogen. In the plasma from the homozygote, a peak corresponding to only 5% of normal Glu-plg was detected. Lys-plg was below the limit of detection. These results are consistent with previous observations from other laboratories [12,13,15] except that the information concerning Lys-plg was previously unavailable.

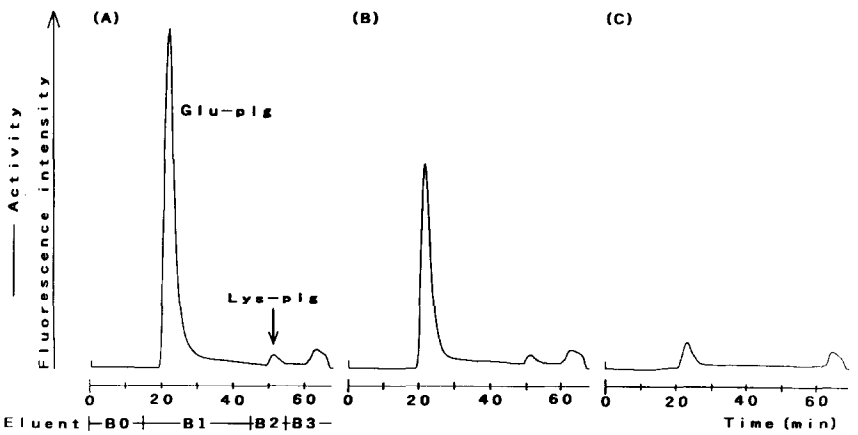

Fig. 6. Chromatograms of plasmas containing abnormal plaminogens. Chromatographic conditions as in Fig. 4. A 50-μl aliquot of plasma without any pretreatment was applied to the system. (A) Normal plasma; (B) plasma from a patient with abnormal plasminogen (heterozygote); (C) plasma from a patient with abnormal plasminogen (homozygote).

DISCUSSION

In previous studies, we showed the usefulness of a high-performance affinity chromatographic system for the analysis of the blood fibrinolytic system [1–5]. The analytical system consisted of specific separation on an affinity adsorbent and specific detection of proenzyme (plasminogen). The latter procedure involved the combination of specific activation of plasminogen species by adding urokinase solution and reaction of the resulting active plasmin with a specific fluorogenic substrate. However, that system had some weak points, mainly due to the use of urokinase solution. As urokinase is unstable in solution, a concentrated stock solution was divided into small portions, each of which was expected to be consumed within a day, and stored in a freezer. Each portion of stock solution was thawed, diluted and used as required. In spite of such precautions, it was difficult to keep the activity constant, thus decreasing the reproducibility of determination. The consumption of large amounts of urokinase was also a problem, because constant mixing with the effluent from the affinity column was essential to obtain a flat baseline even during the washing process.

Use of the immobilized urokinase column finally solved these problems. After immobilization, urokinase maintained its activity towards both the synthetic substrate (glutaryl-Gly-Arg-AMC) and plasminogen. It proved to be very stable and could be used repeatedly. One hundred cycles of use did not result in any appreciable decrease in the ability to activate plasminogen. Hence it was unaffected not only by inhibitors but also by proteases contained in the blood. The reproducibility of quantitative data was satisfactory.

One unexpected disadvantage of the present procedure was that if Glu-plg in plasma was measured without the adsorption process on the affinity column, linearity was lost, probably because of the presence of plasmin inhibitor in the fraction that passes through. Plasmin produced from Glu-plg seemed to have been trapped by plasmin inhibitor during passage through the reaction coil for the hydrolysis of the substrate. In the previous system using urokinase solution, such a loss of linearity was not observed [3–5]. The reason for the difference seems to be as follows. In the previous system, active plasmin was continuously produced even during the period of hydrolysis of the substrate, because urokinase was provided as a solution. This probably diminished the effect of the inhibitor on the determination. However, in the present system, production of active plasmin occurs exclusively in the urokinase column, and some inhibition of the active enzyme by the inhibitor presumably occurs during the passage through the reaction coil.

In this work, pre-tresyl-activated Toyopearl was used as the supporting matrix to immobilized urokinase. We tried various chromatographic media to find the most suitable matrix. However, every matrix tested showed more or less nonspecific adsorption of protein. As plasminogen is inherently very sticky towards a variety of kinds of surface, it was a serious problem to determine Lys-plg accu-

rately because the loss was proportionally great. For example, Asahipak coupled with urokinase proved to adsorb plasminogen strongly. It also raised the back-pressure considerably because the particle size was very small (9 μm). Even Superose, a derivatized agarose gel designed for high-flow-rate chromatography, showed some extent of non-specific adsorption. Although the derivatized Toyopearl also showed adsorption of plasminogen, such unfavourable adsorption became negligible in the presence of 0.04 *M* AHA. Addition of AHA was also favourable for the activation of Glu-plg. Owing to the large particle size, the back-pressure of the total system did not increase too much. Therefore, we finally adopted AF-tresyl Toyopearl as the support.

In conclusion, the use of immobilized activating enzyme proved to be useful as part of the post-column reactor for the specific detection of enzyme precursors. This principle should be applicable to many other proenzyme systems such as blood clotting, renin–angiotensin and complements.

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