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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF PLASMIN AND PLASMINOGEN ON A HYDROPHILIC VINYL-POLYMER GEL COUPLED WITH *p*-AMINOBENZAMIDINE

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SUMMARY

p-Aminobenzamidine was covalently attached via a spacer moiety to a microparticulate hydrophilic vinyl-polymer gel (Toyopearl HW65S) and this affinity adsorbent was used for the separation of plasmin and plasminogen by high-performance affinity chromatography. Toyopearl HW65S was alkylated with chloroacetylglycylglycine in dimethyl sulphoxide using methylsulphinyl carbanion as a catalyst, then *p*-aminobenzamidine was coupled to the carboxyl group of glycylglycine to form an acid amide bond. A column packed with the adsorbent retained both plasmin and plasminogen. Plasminogen was eluted with 6-aminohexanoic acid, a haptenic compound for the lysine-binding sites of plasminogen. For the elution of plasmin, the coexistence of 6-aminohexanoic acid and leupeptin (a competitive inhibitor for plasmin) was necessary. The results indicate a two-site interaction of plasmin with the immobilized ligand, *i.e.*, at the lysine-binding sites and the catalytic site. Fluorometric detection of eluted protein and on-line assay of plasmin activity using a fluorogenic substrate, peptidylmethylcoumarylamide, revealed that effective chromatographic separation of the enzyme could be achieved with high sensitivity (10 μg) within 1 h.

INTRODUCTION

Affinity chromatography is a very effective method for the separation of biological molecules, and has inherently high resolving power. By utilizing the techniques and equipment developed for high-performance liquid chromatography, it should be possible to extend considerably the range of applicability of affinity chromatography procedures.

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A rigid and micro-particulate matrix is preferable for operation at high flow-rates and from the viewpoint of the stability of the column bed[1–3]. Toyopearl™[4], which consists of hydrophilic vinyl-polymer gel particles developed as a new packing material for molecular sieve chromatography, seems to be a promising candidate. Its mechanical and chemical stability is superior to that of agarose gel, it is very stable over a wide range of pH (1–14) and it scarcely adsorbs proteins. The pore surface of Toyopearl is enriched with hydroxyl groups which can be used for immobilization of affinity ligands. In this work, we have investigated the usefulness of Toyopearl as a new matrix for affinity chromatography and constructed an integrated chromatographic system for the separation of human plasmin and plasminogen.

Plasmin, a major fibrinolytic enzyme in blood, consists of two polypeptide chains (mol. wt. 56,000–58,000 and 25,000) held together by two disulphide bridges. It is formed from plasminogen by selective cleavage of polypeptide bonds[5,6]. Plasminogen has lysine-binding sites which plays a key rôle in the interactions of plasmin with fibrin and antiplasmin[7]. It can readily be purified from blood plasma by affinity chromatography on lysine-substituted agarose[8]. Since the lysine-binding sites are not removed after activation, plasmin has two types of specific binding site, the catalytic site in the light chain and the lysine-binding sites in the heavy chain. Thus, for the separation of plasmin, affinity adsorbents which distinguish the catalytic site and the lysine-binding sites are preferable. Benzamidine derivatives, which have been used as specific ligands for trypsin[9,10], are also expected to be effective for plasmin, because plasmin is one of the trypsin family of enzymes.

Derivatizations of Toyopearl to yield affinity adsorbents have been accomplished by the use of several activation reagents which have been used for particulated agarose gel, *e.g.*, CNBr[11] and epichlorohydrin[12]. In the present work, Hakomori's permethylation method for saccharides was applied to derivatize the gel. Chloroacetylglycylglycine was coupled to Toyopearl by O-alkylation to produce an ω-carboxyl spacer, and *p*-aminobenzamidine (ABA) was immobilized on the carboxyl group. The new affinity adsorbent was very effective for the separation of plasmin and plasminogen.

EXPERIMENTAL

Materials

Toyopearl HW65 superfine (exclusion limit molecular weight $5 \cdot 10^6$, particle size 20–40 μm) was a product of Toyo Soda Co. (Tokyo, Japan). Toyopearl is supplied also by E. Merck (Darmstadt, F.R.G.) under the trade-mark, Fractogel TSK. *p*-Aminobenzamidine monohydrochloride (ABA · HCl) was purchased from Sigma (St. Louis, MO, U.S.A.). Benzamidine hydrochloride was obtained from Tokyo Chemical Industry (Tokyo, Japan). 7-(*tert.*-Butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysineamido)-4-methylcoumarin (Boc-Glu-Lys-Lys-AMC) and 7-amino-4-methylcoumarin (AMC) were products of the Protein Research Foundation (Osaka, Japan). Leupeptin hemisulphate was a generous gift from Professor Shin-ichi Ishii (Faculty of Pharmaceutical Science, Hokkaido University, Sapporo, Japan). N-Chloroacetylglycylglycine (CAGG) was synthesized according to Fischer[13]. Dimethyl sulphoxide (DMSO) was dehydrated by the use of molecular sieve 4A. Methylsulphinyl carbanion was prepared basically according to Greenwald *et al.*[14]. Plasminogen from human

serum (List No. GCC-1089, 8–10 casein units per mg protein) and urokinase from human urine (List No. GCC-4015, over 60,000 international units per mg protein) were products of Green Cross Corporation (Osaka, Japan). Amino-terminal analysis of the plasminogen preparation by the dansyl chloride method showed that the preparation consisted mainly of Lys-plasminogen and other partially degraded forms[5].

Synthesis of N-chloroacetyl-6-aminohexanoic acid (CAAHA)

6-Aminohexanoic acid (20 g) was acylated by dropwise addition of chloroacetyl chloride (25 g) in ice-cold 2 *M* sodium hydroxide solution (80 ml) for over 30 min at pH 10–10.5, maintained by the addition of 6 *M* sodium hydroxide solution (54 ml). Addition of 6 *M* hydrochloric acid (26 ml) produced a creamy precipitate, which was filtered off and dried. CAAHA was extracted from the solid (32.5 g) with chloroform (325 ml) at 25–30°C and precipitated from the extract by the addition of petroleum ether (325 ml). The product was recrystallized from water (yield 16.4 g; 52%). M.p.: 85–86.5°C. Elemental analysis: calculated for $C_8H_{14}NO_3Cl$, C 46.27, H 6.80, N 6.75, Cl 17.07; found, C 46.36; H 6.78; N 6.74; Cl 17.28.

Preparation of affinity adsorbent for plasmin (Toyopearl HW65S-AGG-ABA) (Fig. 1)

Toyopearl and its derivatives were weighed as wet cakes after filtration by suction on a sintered glass funnel (pore size G3). They were washed on the same funnel.

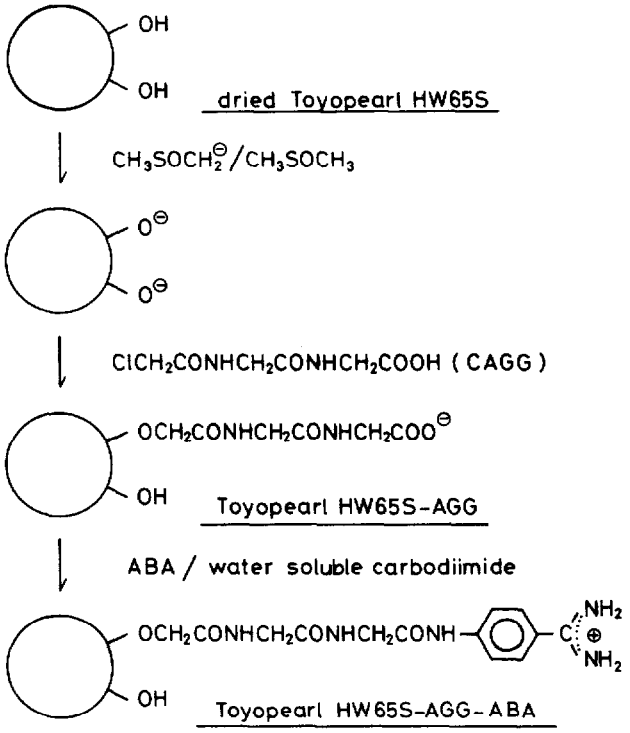

Fig. 1. Derivatization of Toyopearl.

Drying of Toyopearl. Toyopearl HW65S was washed with water until the filtrate became clear. A 100-g amount of the wet gel was suspended in 50 ml of 0.03 *M* sodium hydroxide solution containing 0.3 *M* $NaBH_4$ and allowed to stand overnight at room temperature. The gel was washed with 1 l of water, then with 500 ml of dioxane and freeze-dried. To prevent loss of gel particles, the container was capped with a filter-paper. Eighteen grams of dry powder were obtained.

Preparation of the Toyopearl derivative bearing an ω-carboxyl spacer (Toyopearl HW65S-AGG). The dried gel (18 g) in a 500-ml pear-shaped flask was swollen with 140 ml of dry dimethyl sulphoxide containing 1.8 g of $NaBH_4$. The flask was plugged to exclude moisture throughout the following alkylation procedure. Reagents were introduced under a stream of dry nitrogen. The flask was immersed in a sonication bath for 10 min with occasional gentle shaking. The resulting homogeneous suspension was treated with 36 mmol of methylsulphinyl carbanion (2.25 *M* in DMSO), and the mixture was left for 5 min. A solution of CAGG (7.2 mmol) in 14 ml of dry DMSO was then added, and the whole was left for 1 h at room temperature with occasional gentle shaking. The reaction mixture was poured into 1.8 l of ice-cold water. The gel (Toyopearl HW65S-AGG) was washed successively with 2 l each of water, 0.05 *M* hydrochloric acid, 0.05 *M* sodium hydroxide containing 1 *M* sodium chloride and again with water until the washings were neutral. The AGG content was 55 μmol per g wet gel.

The results of a study of the coupling conditions with CAAHA as alkylating reagent are shown in Figs. 3 and 4.

Coupling of ABA to Toyopearl HW65S-AGG. Toyopearl HW65S-AGG (90 g, wet) was suspended in 90 ml of 0.2 *M* 2-(morpholino)ethanesulphonic acid–NaOH buffer, pH 4.75, and 3.45 g (18 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 1.56 g (9 mmol) of ABA · HCl were added. The pH was readjusted three times to 4.75 at 30-min intervals with 1 *M* hydrochloric acid or 1 *M* sodium hydroxide solution. The suspension was gently shaken at room temperature overnight. The gel (Toyopearl HW65S-AGG-ABA) was washed successively with 1 l each of water, 0.05 *M* sodium hydroxide solution containing 1 *M* sodium chloride, 0.05 *M* hydrochloric acid containing 1 *M* NaCl and water again until the washings were neutral. The content of ABA was 27 μmol per g wet gel.

Determination of the contents of spacer and ABA

The spacer contents were determined by amino acid analysis of the supernatant of the acid hydrolyzates (6 *M* hydrochloric acid, 110°C, *in vacuo*, 16 h) of the derivatives. The content of ABA was determined by spectrophotometry ($\varepsilon_{292} = 1.53 \cdot 10^4$ $l\,mol^{-1}\,cm^{-1}$ in 0.05 *M* sodium dihydrogenphosphate, pH adjusted to 7 with sodium hydroxide) of the acid hydrolyzate, with a correction for the recovery of the hydrolysis procedure (91%).

Activation of plasminogen

Plasminogen was dissolved in 0.05 *M* sodium dihydrogenphosphate, 0.1 *M* sodium chloride, pH 7.4 to a concentration of 2 mg/ml (determined on the basis of $E^{1\%}_{280} = 18.4$). Urokinase was dissolved in the same buffer to give 3000 units per ml. Activation was carried out with urokinase at 37°C for 1.5 or 3 min (see Figs. 6–10) at a ratio of 1.5 units of urokinase per μg of plasminogen.

Chromatographic system (Fig. 2)

The Pyrex glass column (100 × 6 mm I.D., GCH6W) and a packer (PA-H-10) were from Umetani Precision Co. (Osaka, Japan). The reciprocating single-plunger pump (flow-rate 0.1–1.4 ml/min, KHU26-1/2), pulse damper (KU-AIR$_2$), pressure gauge (0–20 kg/cm^2, KPG-20N) and line-filter (KLF-D) were from Kyowa Seimitsu Co. (Tokyo, Japan).

Toyopearl HW65S-AGG-ABA was suspended in 0.05 *M* sodium phosphate, 0.1 *M* sodium chloride, pH 7.4, and packed in the column for 1 h at a flow-rate of 1.4 ml/min with the same buffer. Reservoirs of the eluents were connected to the pump through an eight-way valve connector (Omnifit, U.K.). Samples (5–100 μl) were introduced through a line-sample injector equipped with a rubber septum (LSI-1S; Gasukuro Industry Co., Tokyo, Japan) by using a micro syringe (1710RN; Hamilton, Reno, NV, U.S.A.). All tubing (PTFE) was 1.5–1.6 mm O.D. The inner diameter was 0.5 mm before and 0.25 mm after the column.

The proteins eluted were detected by measuring tryptophan fluorescence with a Hitachi 650-10S fluorescence spectrophotometer (xenon lamp; excitation at 285 nm, emission at 340 nm) (Hitachi, Tokyo, Japan) equipped with an LC micro flow cell (volume 18 μl). The effluent was collected with a fraction collector at low temperature (4°C).

On-line detection of plasmin activity (Fig. 2)

Plasmin activity in the effluent was determined by the use of an on-line assay system. The effluent was delivered at a point just after the protein detector by a peristaltic pump (Minipuls II, HP4; Gilson, France), which simultaneously pumped a substrate solution. The substrate solution comprised 20 μM Boc-Glu-Lys-Lys-AMC[15] in 0.5 *M* sodium phosphate buffer, pH 7.4. The pumping rates were 0.05

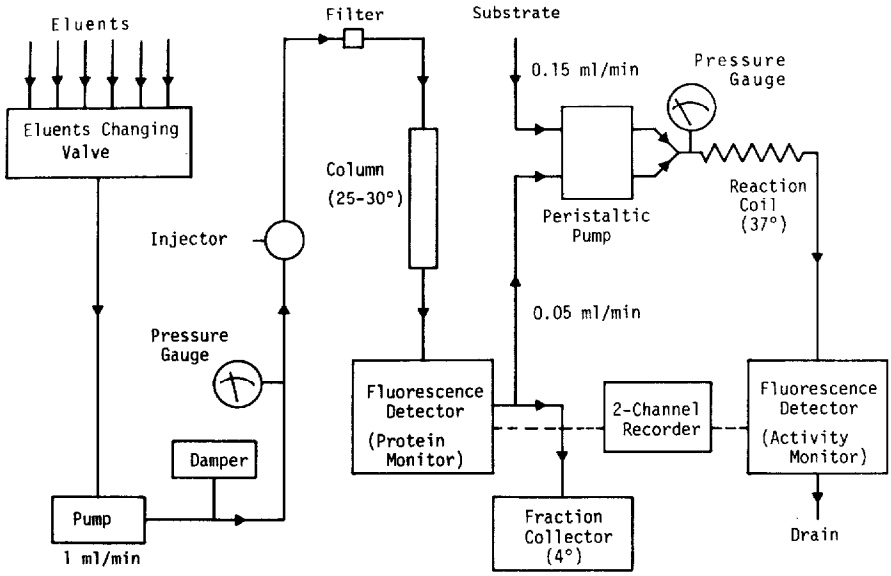

Fig. 2. Diagram of the chromatographic system.

ml/min (0.25 mm I.D., Tygon tube) for the effluent and 0.15 ml/min (0.5 mm I.D., Tygon tube) for the substrate solution. The reagents were mixed just after the pump with a three-way connector and reacted in a coiled tube (PTFE, 4 m × 0.25 mm I.D.) equilibrated at 37°C in a water-bath. AMC produced by the reaction was detected with a JASCO FP110-C fluorescence spectrophotometer (medium-pressure mercury lamp; excitation at 365 nm, emission at 460 nm (Tokyo, Japan).

Affinity chromatography on Toyopearl HW65S-AGG-ABA

The following eluents were used. Eluent 1: 0.05 *M* sodium phosphate–0.1 *M* sodium chloride (pH 7.4). Other eluents contained the components described below in addition to those of eluent 1. Eluent 2, 20 m*M* 6-aminohexanoic acid (AHA); eluent 3, 20 m*M* AHA–3 *M* urea; eluent 4, 20 m*M* AHA–1 *M* urea; eluent 5, 20 m*M* AHA–1 m*M* leupeptin; eluent 6, 20 m*M* AHA–1 m*M* reduced leupeptin; eluent 7, 1 m*M* leupeptin; eluent 8, 20 m*M* AHA–100 m*M* benzamidine. All eluents (pH 7.4) were passed through a 0.45-μm Millipore filter before use and were pumped at a flow-rate of 1 ml/min. The pressure drop of the column at the above flow-rate was <2 kg/cm^2. Prior to a series of chromatographic runs, the column was irrigated alternately with eluent 1 and eluent 3 until a constant baseline was obtained. For sample sizes of 5–100 μl (5–100 μg protein), the sample solution was introduced from the injector onto the column equilibrated with eluent 1. Samples of over 100 μl were introduced through the eluent changing valve. All operations were performed at room temperature (23–28°C).

Determination of plasmin activity

A 2-ml volume of substrate solution (20 μM Boc-Glu-Lys-Lys-AMC in 0.05 *M* sodium phosphate, 0.1 *M* sodium chloride, pH 7.4) in a fluorescence cell (1 × 1 × 4 cm) were equilibrated at 37°C in a constant-temperature cell holder. A 100-μl volume of enzyme solution (0.1–0.5 μg of plasmin) was added and the increase in fluorescence was recorded (Hitachi 650-10S; excitation at 380 nm, emission at 460 nm). The liberation of 1 nmol of AMC per min was defined as one unit of activity.

Electrophoresis

The proteins (reduced) were separated on a 10% polyacrylamide slab gel (95 × 45 × 1 mm) in the presence of sodium dodecyl sulphate (SDS) according to Laemmli[16]. The protein bands were stained with silver according to Merril *et al.*[17].

Reduction of leupeptin

Leupeptin was reduced to dihydroleupeptin with $NaBH_4$ according to Kondo *et al.*[18] and used without purification. While 4.8 μM leupeptin inhibited about 70% of plasmin activity in the assay system used, 480 μM reduced leupeptin inhibited only 5% of the activity.

RESULTS

Coupling of the spacer to Toyopearl HW65S

ω-Carboxyl spacers were introduced onto Toyopearl by O-alkylation (in DMSO). Hydroxyl groups of Toyopearl were first converted into alkoxide groups by

treatment with methylsulphinyl carbanion and then alkylated with N-chloroacetyl-6-aminohexanoic acid (CAAHA) or chloroacetylglycylglycine (CAGG). The procedure is an application of Hakomori's permethylation method for carbohydrates[19]. The alkoxide formation seemed to be very fast, because the highest coupling yield was observed when the alkylating reagents were added only 2 min after the addition of the anion (data not shown). On the other hand, the alkylation proceeded more slowly. As shown in Fig. 3, the maximum yield was reached at 30 min after the addition of the alkylating reagent.

Fig. 4 shows the coupling yield of the spacer as a function of the amount of methylsulphinyl carbanion and CAAHA. In the presence of a sufficient amount of the anion (3.6 mmol per g dry gel), an almost linear relationship was observed between the coupling yield, up to 140 μmol per g wet gel, and the added CAAHA. About 70% of the added CAAHA bound to the gel. When a smaller amount of the anion was used (1.2 mmol per g dry gel), the maximum coupling of the spacer was only 30 μmol per g wet gel. CAGG reacted similarly.

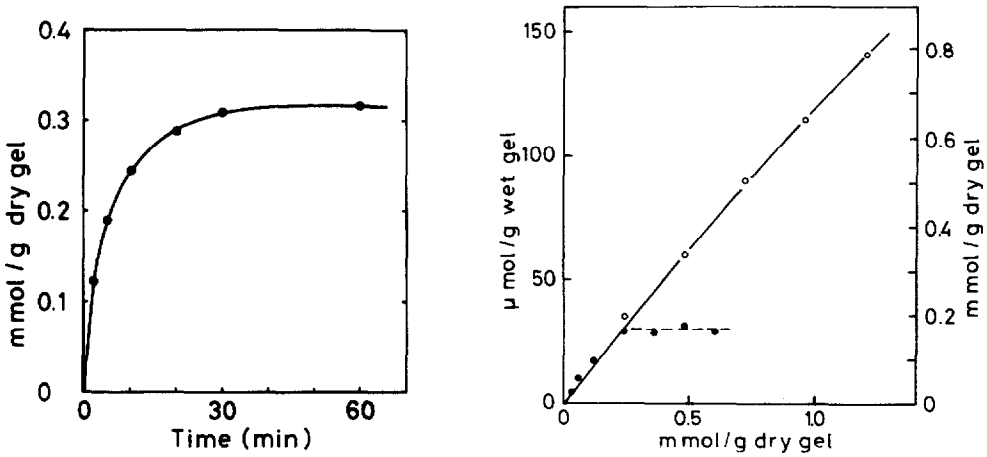

Fig. 3. The time course of the coupling reaction of CAAHA to the alkoxide of Toyopearl. Dried Toyopearl HW65S (1 g) was suspended in 7.5 ml of dry DMSO for 10 min under dry nitrogen. Two mmol (1.2 *M*, 1.7 ml) of methylsulphinyl carbanion in DMSO were added and the mixture was stirred for 5 min, followed by the addition of 83 mg (0.4 mmol) of CAAHA dissolved in 1 ml of DMSO. At each time point, a 1-ml portion of the suspension was removed and the reaction was stopped by dilution in water. After washing of the gel as described in the Experimental section, the content of the spacer was determined by amino acid analysis of 25 mg of dried gel (corresponding to 0.14 g of wet gel). Ordinate, content of the spacer; abscissa, time after the addition of CAAHA.

Fig. 4. Coupling of CAAHA to Toyopearl HW65S as a function of the amounts of CAAHA and methylsulphinyl carbanion added to the reaction mixture. Portions of 0.5 g each of dried Toyopearl HW65S were suspended in 4 ml of DMSO in vials and allowed to stand for 10 min. The anion solution (2.2 *M* in DMSO) was added to two series of suspensions which differed in the amount of anion added. After 10 min, 1 *M* CAAHA in DMSO was added in various amounts and the mixture was allowed to react for 1 h with occasional shaking. Vials were tightly capped throughout the procedure unless opening was necessary. The suspensions were diluted in water, the gels were washed and the spacer contents were determined as described in Fig. 3. Ordinate, content of the spacer; abscissa, amount of CAAHA added. Amount of anion: ○, 3.6 mmol per g dry gel; ●, 1.2 mmol per g dry gel.

Affinity chromatography of plasminogen

Toyopearl HW65S-AGG-ABA proved to be very efficient for the affinity chromatography of plasminogen, providing good separation with high sensitivity in a short time. The ligand, benzamidine, was expected to bind to the catalytic site (S_1) of plasmin. However, plasminogen was trapped on the column packed with Toyopearl HW65S-AGG-ABA and detached by 20 m*M* AHA (see Fig. 5A). Since AHA is known to bind to the lysine-binding sites of plasminogen[5], these sites seem to be responsible for the interaction with the benzamidine moieties on the adsorbent. When 100 μg of plasminogen were applied, the recovered protein in the 20 m*M* AHA eluate (10.4 ml) was about 80 μg (determined by measuring the fluorescence of Trp in the protein).

Bovine serum albumin, a control protein, passed through the column without tailing (Fig. 5B); the recovery was 97%. When eluent 1 diluted two-fold (0.025 *M* sodium phosphate, 0.05 *M* sodium chloride, pH 7.4) was used, bovine serum albumin was eluted as a broad, tailing peak. A certain ionic strength was necessary to minimize ionic adsorption of proteins.

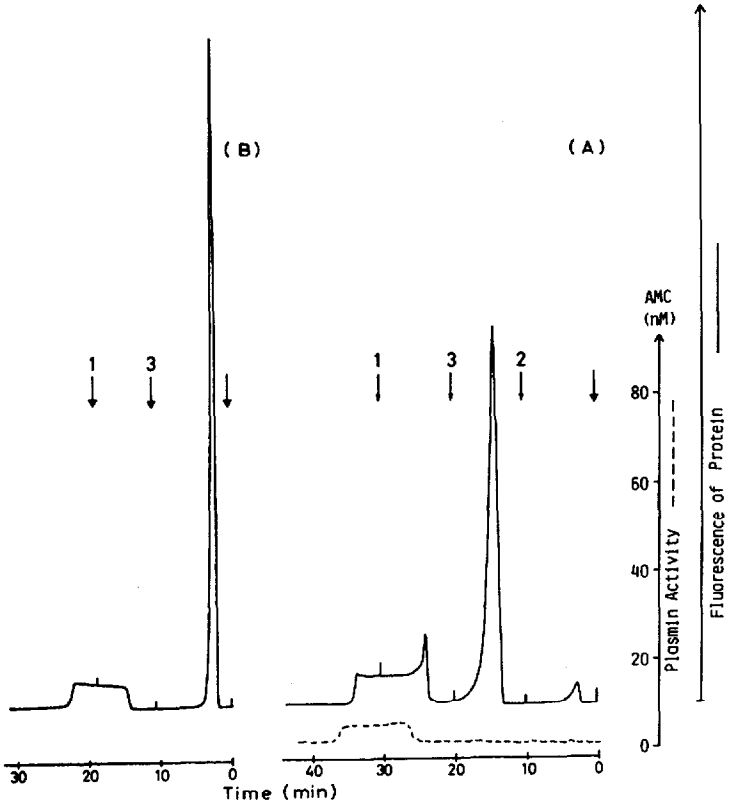

Fig. 5. Affinity chromatography of plasminogen on Toyopearl HW65S-AGG-ABA. (A), The column was equilibrated with eluent 1. Plasminogen (10 μg) dissolved in 5 μl of eluent 1 was applied to the column at time 0. The eluent was changed to those indicated by the numbered arrows. The compositions of the eluents are as described in the Experimental section. (B), Bovine serum albumin (10 μg) dissolved in 10 μl of eluent 1 was applied at time 0.

Affinity chromatography of plasmin

Plasminogen (100 μg) was activated by urokinase and applied to the system. Adsorbed proteins were eluted successively with eluents 2 and 3 (Fig. 6A). Eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). The 20 m*M* AHA (eluent 2) eluate contained plasminogen as the major component (appearing as a doublet on the electropherogram; Fig. 7, lane 3). The fraction eluted with 20 m*M* AHA and 3 *M* urea (eluent 3) showed enzymatic activity. The activity profile was recorded with an about 3-min delay because of the passage through the reaction coil. The recovery of activity was nearly complete. When 18 units of plasmin were applied the apparent recovery in this fraction (9.8 ml) was 19 units. SDS-electrophoresis showed the presence of plasmin heavy chain and light chain (Fig. 7, lane 4). When AHA was omitted from the eluent, plasmin was eluted more slowly (data not shown).

The fact that plasmin could not be eluted with AHA alone indicates that plasmin binds to the immobilized ligand through not only the lysine-binding sites on the heavy chain but also the catalytic site on the light chain.

Competitive elution of plasmin

Plasmin was eluted with 20 m*M* AHA and 1 m*M* leupeptin (Fig. 6B and Fig.

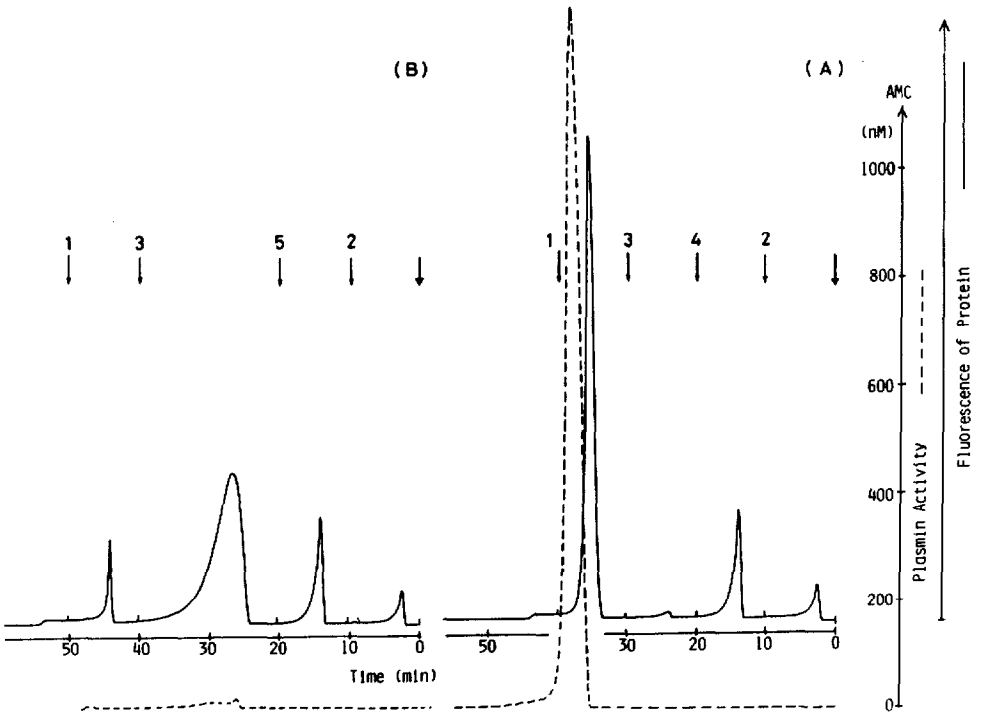

Fig. 6. Affinity chromatography of the activation mixture of plasminogen on Toyopearl HW65S-AGG-ABA. Plasminogen (100 μg) was activated for 1.5 min and the activation mixture was immediately applied to the column (time 0). Eluent changes are indicated as described as in Fig. 5. The effluent was collected at the rate of one fraction per minute. (A), Effect of AHA and urea; (B) effect of AHA and leupeptin.

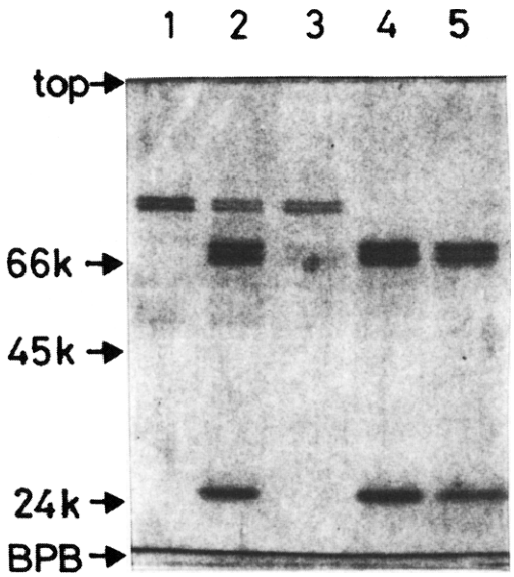

Fig. 7. SDS-polyacrylamide gel electrophoresis of fractions obtained in the experiments described in Fig. 6. Lanes: 1, plasminogen (0.1 μg); 2, activation mixture of plasminogen (0.2 μg, activated at 37°C for 1.5 min); 3 and 4, the materials eluted at 14–15 min and 35–36 min respectively in the experiment shown in Fig. 6A; 5, the material eluted at 26–27 min in the experiment shown in Fig. 6B. Bovine serum albumin, chicken ovalbumin and bovine trypsinogen were used as molecular weight markers. BPB indicates the position to which bromophenol blue migrated.

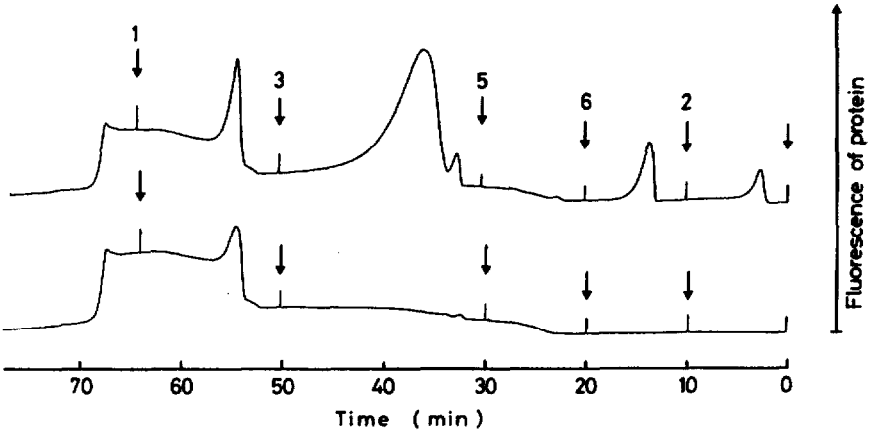

Fig. 8. Affinity chromatography of the activation mixture of plasminogen on Toyopearl HW65S-AGG-ABA; effect of reduced leupeptin. Upper line: plasminogen (10 μg) was activated for 3 min and the activation mixture was immediately applied to the column (time 0); eluent changes are indicated as described in Fig. 5. Lower line: the baseline obtained with the same eluent changes as in the case of the upper line, but without sample application.

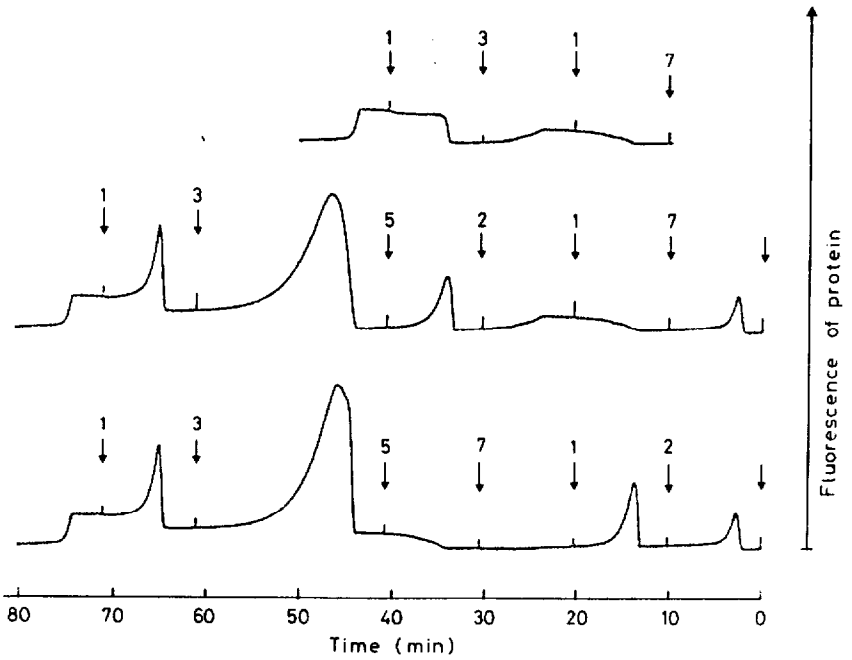

Fig. 9. Affinity chromatography of the activation mixture of plasminogen on Toyopearl HW65S-AGG-ABA; effect of leupeptin alone. Middle and lower line: plasminogen (10 μg each) was activated for 3 min and immediately applied to the column (time 0); eluent changes are indicated as in Fig. 5. Upper line: baseline changes with same eluents.

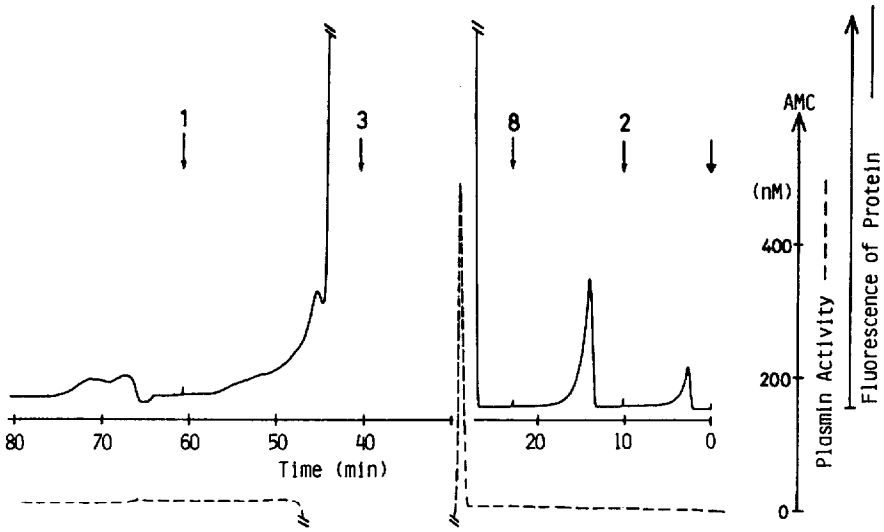

Fig. 10. Affinity chromatography of the activation mixture of plasminogen on Toyopearl HW65S-AG-G-ABA; effect of benzamidine. Plasminogen (100 μg) was activated for 1.5 min and immediately applied to the column (time 0). Eluent changes are indicated as in Fig. 5. The effluent was collected at the rate of one fraction per minute.

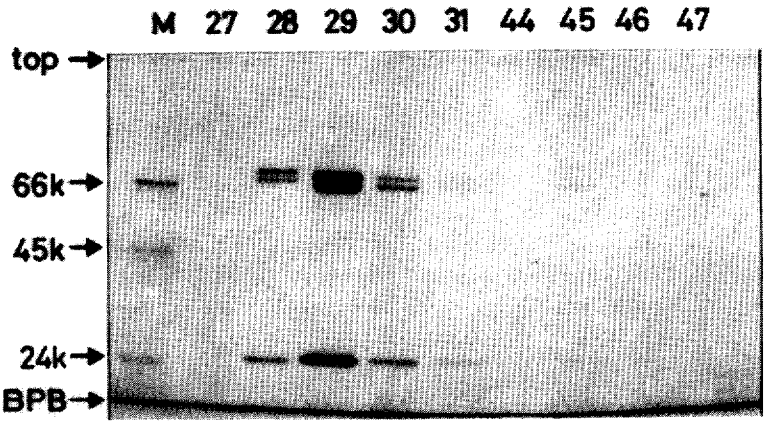

Fig. 11. SDS-polyacrylamide gel electrophoresis of the fractions obtained in the experiment described in Fig. 10. Ten μl of each fraction were applied to the gel. The number above each lane correspond to the time in minutes in Fig. 10. Markers (lane M) were as in Fig. 7.

7, lane 5). Leupeptin is a potent competitive inhibitor of trypsin-type proteases, including plasmin[20]. The aldehyde group of leupeptin is thought to form a hemiacetal with the hydroxyl group of the active site serine residue. Dihydroleupeptin, which is produced by reduction of the aldehyde group of leupeptin and scarcely inhibits plasmin[18], was not effective in eluting plasmin from the adsorbent (Fig. 8). Evidently, plasmin was adsorbed on the column through its catalytic site.

Leupeptin (1 m*M*) alone could elute neither plasminogen nor plasmin from the column (Fig. 9). The coexistence of AHA (20 m*M*) and leupeptin (1 m*M*) was necessary for the elution of plasmin. This indicates that plasmin is bound via two different types of binding sites.

Competitive elution of plasmin with benzamidine was also effective, but the UV absorption of benzamidine interfered with the detection of both protein and activity (Fig. 10). SDS-polyacrylamide gel electrophoresis of eluted fractions, however, confirmed the successful competitive elution of plasmin (Fig. 11).

Similar chromatographic patterns were obtained with any of these elution methods for up to 1 mg of the activation mixture on a column of the same size (100 × 6 mm I.D.).

DISCUSSION

Investigations to improve the efficiency of affinity chromatography have been carried out using silica gel[1,2] or hydroxyalkyl methacrylate gel[3]. The results reported here showed that a hydrophilic vinyl-polymer gel, Toyopearl, is also useful for this purpose.

The Hakomori reaction[19] was applied to the derivatization of cross-linked agarose gel by Rosengren and Glad[21], but details of the procedure and practical use of the product in affinity chromatography were not supplied. We applied this reaction to Toyopearl by using CAAHA and CAGG. The coupling yield was very high: about 70% of the added chloroacetyl derivative was bound to the gel. As shown in Fig. 4,

we could prepare Toyopearl derivatives having a desired amount of spacer without undesirable over-derivatization of the gel. The affinity ligand, ABA, was coupled to the carboxyl group of the spacer with an acid amide linkage by the carbodiimide method[22]. The advantage of the present procedure is that no undesirable electric charge is introduced either between the matrix and the spacer or the spacer and the ligand. The treatment of Toyopearl with the anion in DMSO introduced an unknown ionic group or groups titratable in the weakly acidic region (pH 4.5–7). Pretreatment of the gel with $NaBH_4$, and the addition of $NaBH_4$ to the reaction mixture, however, lowered the ionic group content to 10–30 μequiv. per g wet gel. Toyopearl derivatives having such ω-carboxyl spacers should be useful intermediates for preparing various adsorbents.

The chromatographic system with the new affinity adsorbent was found to be very efficient, and the fluorometric detection of both protein and activity was effective. The recovery of both protein and activity was nearly quantitative. A clear chromatogram was obtained with as little as 10 μg of protein. One cycle of chromatography, including the initial treatment of the column, could be completed within 1 h. Thus, the system seems to be very suitable for analytical purposes.

Ultraviolet absorption detection often produces pseudo-peaks on changing the eluents. Especially in the case of elution with a high concentration of urea, a large baseline change which overlapped an eluted protein peak was noted with an UV absorption detector (JASCO, Uvidec 100-III, 280 nm) (data not shown). The detection of protein by fluorescence measurement is preferable to UV detection, since the former offers high sensitivity and a smaller shift in baseline when the eluent is changed.

The use of a fluorogenic substrate, Boc-Glu-Lys-Lys-AMC[15], enabled us to detect the plasmin activity nearly simultaneously with the protein, with high sensitivity. Since a series of peptidyl-AMC substrates for various proteases is now available[23,24], the on-line assay system described here should be applicable to many other proteases.

Both plasminogen and plasmin bound to the adsorbent. Only plasminogen was eluted from the adsorbent with 20 m*M* AHA, and plasmin remained bound to the immobilized ligand. Thus, 20 m*M* AHA is enough to displace the benzamidine moiety of the adsorbent (27 μmol per g wet gel) from the lysine-binding sites of plasminogen and plasmin, but not enough to displace the moiety occupying the catalytic site of plasmin. This is in agreement with the observation of Christensen[25] that AHA binds very weakly to the catalytic site of plasmin (K_i = 58 m*M*) in comparison with its binding to the allosteric sites (K_a = 0.3 m*M*), which we suppose to be the same as the lysine-binding sites. Plasmin was eluted when both AHA (20 m*M*) and leupeptin (1 m*M*) were contained in the eluent. The observation that leupeptin alone could not detach plasmin suggests that leupeptin has only weak affinity for the lysine-binding sites, in contrast to AHA.

In conclusion, the effectiveness of derivatized Toyopearl in our integrated chromatographic system for the separation of plasminogen and plasmin by affinity chromatography was demonstrated. This system has many advantages, and should be widely applicable, *e.g.*, in clinical diagnosis, analytical biochemistry and the production of enzyme drugs.

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