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SEPARATION OF HUMAN Glu-PLASMINOGEN, Lys-PLASMINOGEN AND PLASMIN BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY ON ASAHIPAK GS GEL COUPLED WITH *p*-AMINOBENZAMIDINE

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SUMMARY

Human Glu-plasminogen, Lys-plasminogen and plasmin were effectively separated by high-performance affinity chromatography. The affinity adsorbent was prepared by using a micro-particulate polyvinyl alcohol gel (Asahipak GS-gel) as the supporting material and *p*-aminobenzamidine as the specific ligand. All of the active enzyme and proenzymes were adsorbed. Glu-plasminogen was eluted by changing the pH of the eluent and Lys-plasminogen by using an eluent containing 6-aminohexanoic acid. This affinity adsorbent recognized the difference between these proenzyme species. For the elution of plasmin, addition of urea was necessary. Plasmin may have been adsorbed through a two-site interaction with the adsorbent. All proteins were eluted as sharp peaks and the time required for one cycle was about 1 h. Fluorimetric detection of eluted protein and on-line assay of enzyme activity using a fluorigenic substrate made it possible to analyse microgram amounts of proteins specifically.

INTRODUCTION

Affinity chromatography is very effective for the separation of biological molecules. High-performance affinity chromatography (HPAC), the combination of affinity chromatography and high-performance liquid chromatography (HPLC), should extend the range of application of affinity chromatography[1–5].

Plasminogen is a precursor of plasmin, which is a major fibrinolytic enzyme in the blood[6,7]. It has lysine-binding sites that play a key role in its adsorption by a fibrin clot and in its interaction with antiplasmin[8]. As these sites are not removed

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after activation, plasmin has two types of specific binding site, namely the catalytic site in the light chain and the lysine-binding sites in the heavy chain, both of which specifically bind benzamidine. Native human plasminogen (MW 92 000) contains glutamic acid as the amino-terminal residue and is referred to as Glu-plasminogen (Glu-Plg). An amino-terminal peptide (MW 8000) is often lost from Glu-Plg during the activation or purification procedure in the absence of protease inhibitors. The resulting plasminogen having lysine as the amino-terminal residue is termed Lys-plasminogen (Lys-Plg, MW 84 000). Although Lys-Plg is known to be more susceptible to activation by plasminogen activators and has a higher affinity for fibrin than Glu-Plg[9–11], it is unclear whether or not Lys-Plg is always present in the normal, circulating blood. Both Glu-Plg and Lys-Plg have been further separated into Glu-Plg I and Glu-Plg II, and Lys-Plg I and Lys-Plg II, respectively, by lysine–Sepharose affinity chromatography[12].

As all these species have different binding strengths to specific ligands, it should be possible to separate them by using only one affinity adsorbent if an effective adsorbent can be prepared. Benzamidine derivatives have been used as a specific ligand of agarose-based ordinary affinity adsorbents for proteases[13,14]. We have reported the effective separation of plasminogen (Plg) and plasmin (Plm) by using an affinity adsorbent based on a hydrophilic vinyl polymer gel (Toyopearl HW65S) in which *p*-aminobenzamidine (ABA) was used as a specific ligand[5]. However, to obtain higher resolution and a shorter operating time, a much smaller and more rigid supporting material is preferable. We therefore tried Asahipak GS-gel and have already reported its usefulness for the HPAC of trypsins[15]. In this study, we prepared an adsorbent with a much higher ligand content than that prepared previously and applied it to the plasminogen–plasmin system. It was very effective, because even the subspecies of plasminogen, namely Glu-Plg and Lys-Plg, were separated. Hence it is now possible to analyse these important proteins of the fibrinolytic system with high sensitivity in a short time.

EXPERIMENTAL

Materials

Asahipak GS-520 gel (exclusion limit molecular weight 3×10^5, particle size 9 ± 0.5 μm) was a product of Asahi Chemical Industry (Tokyo, Japan). 6-Aminohexanoic acid (AHA) was obtained from Nakarai Chemicals (Kyoto, Japan). *p*-Aminobenzamidine monohydrochloride (ABA · HCl) was purchased from Sigma (St. Louis, MO, U.S.A.). 7-(*tert.*-Butyloxycarbonyl-L-glutamyl-L-lysyl-L-lysineamido)-4-methylcoumarin (Boc-Glu-Lys-Lys-AMC) was a product of the Protein Research Foundation (Osaka, Japan). 1,1′-Carbonyldiimidazole (CDI) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (EDC · HCl) was a product of Dojin Chemical (Kumamoto, Japan). Acetone was dehydrated by the use of molecular sieve 4A. Plasminogen from human serum (List No. GCC-1089, 8–10 casein units per mg of protein) and urokinase from human urine (List No. GCC-4015, over 60 000 international units per mg of protein) were products of Green Cross (Osaka, Japan). Purified Glu-plasminogen I and II were prepared from human citrated plasma by affinity chromatography on lysine–Sepharose in the presence of bovine pancreatic trypsin

inhibitor (Trasylol). Lys-plasminogen from human placenta (KB-623, Lot No. 80-074) was a product of Kanebo (Osaka, Japan).

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[15]. In brief, Asahipak-GS gel was suspended in acetone and activation with CDI (17.5 mmol of reagent for 2.5 g of dry gel) was carried out for 15 min at room temperature. The activated gel was treated with AHA overnight at 4°C and pH 10. The product contained 369 μmol of AHA per gram of dry gel. ABA was immobilized on the carboxyl group as a ligand. The ABA content was 134 μmol per gram of dry gel.

Affinity chromatography on Asahipak GS-520–AHA–ABA

Affinity chromatography was carried out by using a system similar to that reported previously[15]. Asahipak GS-520–AHA–ABA was suspended in 0.2 *M* sodium sulphate solution and packed in a stainless-steel column (100 × 6 mm I.D.) for 1 h with the same solution. The final flow-rate was 1.8 ml/min. The substrate solution consisted of 20 μ*M* Boc-Glu-Lys-Lys-AMC in 0.5 *M* sodium phosphate buffer (pH 7.4). The following eluents were used. Eluent 0: 0.05 *M* sodium phosphate (pH 6.5). 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, 0.02 *M* AHA; eluent 2′, 0.04 *M* AHA; eluent 3, 0.02 *M* AHA–3 *M* urea; eluent 3′, 0.04 *M* AHA–1 *M* urea.

RESULTS

Asahipak GS-520–AHA–ABA proved to be very effective for the specific separation of plasminogen subspecies. The ligand, aminobenzamidine, was expected to bind to the lysine-binding sites of plasminogen, as was observed in the case of Toyopearl HW65S–AGG–ABA[5].

When the column was equilibrated with eluent 1, Lys-Plg was trapped on it and could be detached by eluent 2 (containing AHA) (Fig. 1A). Under the same conditions, Glu-Plg I and II were not adsorbed, but were retarded compared with the pass-through fraction (retention time, 2.2 min). The retardation of Glu-Plg II (5.4 min) was slightly greater than that of Glu-Plg I (4.4 min) (Fig. 1B). This difference was reproducible. Hence the order of binding strength was Lys-Plg $\gg$ Glu-Plg II > Glu-Plg I. As shown in Fig. 1A, Lys-Plg seemed to show similar behaviour to Glu-Plg: the main peak eluted with eluent 2 in Fig. 1A may be Lys-Plg I and the shoulder peak Lys-Plg II. Human plasminogen produced by Green Cross was examined to determine whether it consists of Glu-Plg or Lys-Plg. Fig. 1C shows that it consists mainly of Lys-Plg, because the main component was adsorbed on the column equilibrated with eluent 1 and eluted with eluent 2. The first peak (pass-through peak) may be due to impurities, because its retention time differed from those of Glu-Plg.

When Glu-Plg I and II were mixed and applied to the column, they did not give separate peaks under the same conditions as used in Fig. 1A (Fig. 2A). The binding of Glu-Plg was enhanced by lowering the pH. At pH 6.5 (eluent 0), Glu-Plg

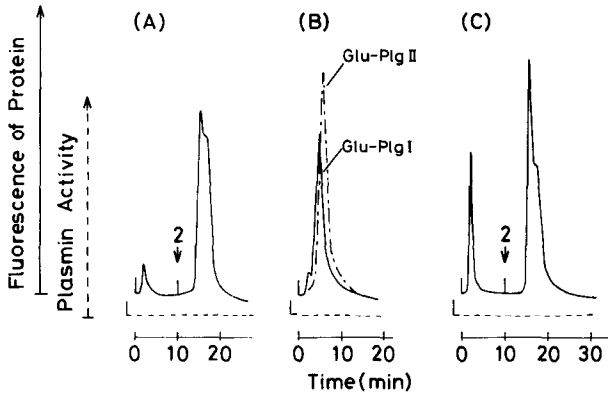

Fig. 1. Affinity chromatography of plasminogens on Asahipak GS-520–AHA–ABA. (A) The column was equilibrated with eluent 1. Lys-plasminogen (10 μg) was applied to the column at time 0. (B) Glu-plasminogen I (4.8 μg) and Glu-plasminogen II (4.4 μg) were applied at time 0. (C) Plasminogen from Green Cross (11 μg) was applied at time 0. The eluent was changed as indicated by the numbered arrows. The compositions of the eluents are described under Experimental. Proteins were dissolved in eluent 1.

was trapped and could be eluted with eluent 1 (pH 7.4) (Fig. 2B). These findings show that the affinity of Glu-Plg is extremely pH dependent. Ionic strength also has a significant effect. When NaCl was omitted from eluent 1, the peak was considerably retarded and broadened (Fig. 2C).

An adsorbent with a lower ligand content (40 μmol of AHA and 13 μmol of ABA per gram of gel), which had been shown to be a good adsorbent for trypsin[14], could not separate Glu-Plg and Lys-Plg (data not shown). Apparently, a high ligand content is required to provide a suitable adsorbent for plasminogen.

Conditions under which both Glu-Plg and Lys-Plg could be adsorbed and then eluted separately were found, but an effective separation of Glu-Plg I and II or of

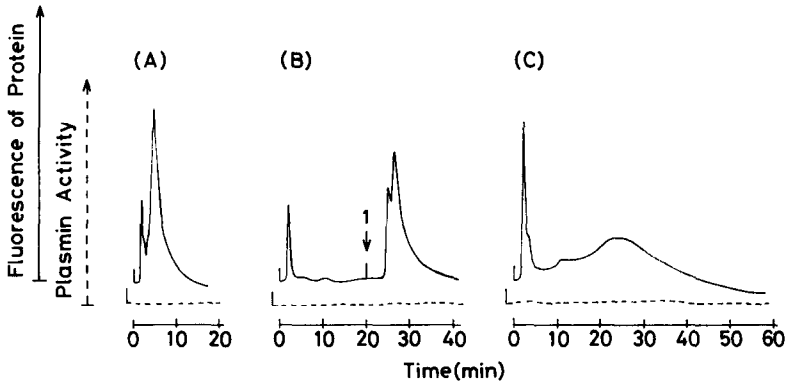

Fig. 2. Affinity chromatography of Glu-plasminogen on Asahipak GS-520–AHA–ABA. (A) The column was equilibrated with eluent 1. A mixture of Glu-plasminogen I (7.5 μg) and Glu-plasminogen II (5.5 μg) dissolved in 17.5 μl of eluent 1 was applied to the column at time 0. (B) The column was equilibrated with eluent 0. A mixture of Glu-plasminogen I (15 μg) and Glu-plasminogen II (11 μg) dissolved in 35 μl of eluent 1 was applied at time 0. The eluent change is indicated by the numbered arrow as in Fig. 1. (C) Same conditions as in (A), except that NaCl was omitted from eluent 1.

Lys-Plg I and II has not yet been achieved. A mixture of Glu-Plg and Lys-Plg was activated by urokinase and the activation mixture was applied to the column equilibrated with eluent 0. Adsorbed Glu-Plg was eluted with eluent 1 and Lys-Plg with eluent 2′ (0.04 *M* AHA) (Fig. 3A). The fraction eluted with eluent 3′ (0.04 *M* AHA + 1 *M* urea) had enzyme activity. Addition of urea was required to elute plasmin, as has been observed in our previous study using Toyopearl HW65S–AGG–ABA[5]. Separation of plasminogen subspecies and plasmin in one cycle of chromatography was achieved for the first time. The procedure takes less than 1 h and the amount of protein required is only about 1–5 μg. The chromatograms were highly reproducible. Hence the method might be useful in clinical applications. Under these conditions, human serum albumin, the major protein in human plasma, passed through the column (Fig. 3B). This would be advantageous for analysing fibrinolytic enzymes in blood. However, when the plasma was directly injected into the column, significant amounts of fluorescent substances were eluted and overlapped with the plasminogen peaks. UV detectors are not sensitive enough to detect the plasminogen peaks. Therefore, it will be necessary to develop methods to detect plasminogens specifically, such as measurement of enzyme activity after activation of immunochemical reactions, in order to apply this method to the direct analysis of plasma.

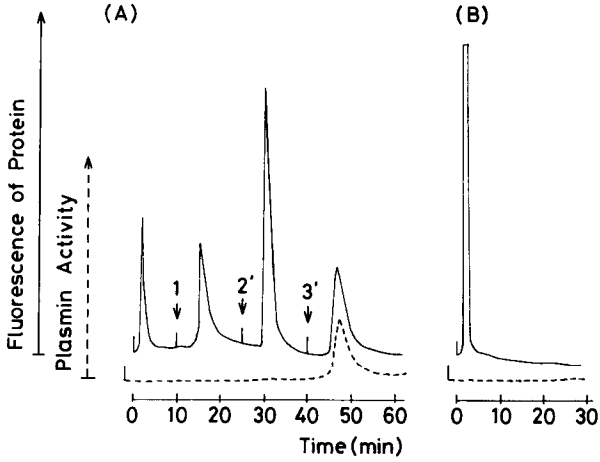

Fig. 3. (A) Affinity chromatography of activation mixture of plasminogen on Asahipak GS-520–AHA–ABA. The column was equilibrated with eluent 0. Glu-plasminogen I (4 μg), Glu-plasminogen II (3 μg) and Lys-plasminogen (4 μg) were activated for 3 min at 37°C and the activation mixture was immediately applied to the column (time 0). Eluent changes are indicated by the numbered arrows. (B) Chromatography of human serum albumin on Asahipak GS-520–AHA–ABA. The column was equilibrated with eluent 0. Human serum albumin (5 μg) was applied at time 0.

DISCUSSION

Synthetic polymer gels for HPLC have not yet been widely used for HPAC, in spite of their superior properties. In this and the previous study[15], the usefulness of Asahipak GS-gel was demonstrated. In our system, enzyme activity was nearly simultaneously measured with fluorescence of protein by using a fluorigenic sub-

strate. Therefore, a very small amount of plasmin could be specifically detected. The reproducibility and accuracy of the system were satisfactory, as had been observed for trypsins[15].

It is important to know the state of the fibrinolytic system in the blood to achieve accurate diagnosis of diseases such as thrombosis, myocardial infarction and disseminated intravascular coagulation syndrome. As Lys-Plg is more susceptible to activation by plasminogen activators than Glu-Plg, the amounts of Glu-Plg and Lys-Plg in the blood should be an important index of the potential of the fibrinolytic system. Although SDS polyacrylamide gel electrophoresis may be applicable to the analysis of Glu-Plg and Lys-Plg, a long time is required because, prior to the electrophoresis, plasminogen must be purified by some appropriate method such as lysine–Sepharose affinity chromatography. Moreover, the amount of Lys-Plg should be much smaller than that of Glu-Plg, and accurate determination of Lys-Plg will be seriously interfered with by large amounts of Glu-Plg. It is also difficult to analyse extremely small amounts of Lys-Plg by conventional affinity chromatography using agarose gel. Moreover, modification of plasminogen, *e.g.*, conversion from Glu-Plg to Lys-Plg or activation from Plg to Plm, might occur during a prolonged analysis. Our affinity adsorbent and chromatographic system proved to have the potential for the direct and rapid measurement of these materials.

In conclusion, the efficiency (*e.g.*, the peak sharpness and specific separation of Glu-Plg and Lys-Plg and the possibility of separating plasminogen I and II) was much improved by using Asahipak GS-gel instead of Toyopearl HW65S, and the range of application should be much greater. If a sensitive and specific method for the detection of the proenzymes can be developed, our HPAC system should be widely applicable, especially in fields where fast and specific separations with high sensitivity is required, *e.g.*, clinical diagnosis, monitoring of medical treatment and analysis of the causes of disease.

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