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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF TRYPSINS ON ASAHIPAK GS-GEL COUPLED WITH *p*-AMINOBENZAMIDINE

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SUMMARY

An adsorbent for high-performance affinity chromatography of trypsins was prepared, based on a micro-particulate polyvinyl alcohol gel for high-performance liquid chromatography, Asahipak GS-gel. After the hydroxyl groups had been activated with 1,1'-carbonyldiimidazole, 6-aminohexanoic acid was coupled as a spacer, then *p*-aminobenzamidine, a specific ligand for trypsin-family enzymes, was immobilized on the spacer. Fluorometric detection of eluted protein and on-line assay of enzyme activity using a fluorogenic substrate, peptidylmethylcoumarylamide, made it possible to attain very high sensitivity. Microgram amounts of bovine trypsin and *Streptomyces griseus* trypsin could easily be analyzed in a short time (< 1 h).

INTRODUCTION

Affinity chromatography will become a very effective analytical tool for biological molecules if improvements similar to those made in the field of high-performance liquid chromatography (HPLC) can be made. Agarose gel is no longer a suitable support material for affinity chromatography; supports used for HPLC seem to be greatly superior[1–4]. In the present work, we tested Asahipak GS-gel, a newly developed packing material for HPLC[5], which is composed of polyvinyl alcohol. It is mechanically and chemically stable, and can be used over a wide range of pH (3–12). The pore size is large (molecular weight exclusion limit $3 \cdot 10^5$) and the particle size is small (9 ± 0.5 μm), so high resolution is expected in the analysis of proteins. The pore surface is enriched with hydroxyl groups, which can be used for immobilization of affinity ligands. We have already reported the separation of plasminogen and plasmin with a hydrophilic vinyl-polymer gel (Toyopearl HW-65S, particle size 20–40 μm) coupled with *p*-aminobenzamidine (ABA)[6]. In the present work, we used a

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gel with much smaller particles (Asahipak GS-gel) as a new matrix in order to increase the scope of high-performance affinity chromatography.

The hydroxyl groups of Asahipak GS-gel were activated with 1,1'-carbonyldiimidazole, and 6-aminohexanoic acid was coupled as an ω-carboxyl spacer. The ligand, *p*-aminobenzamidine[7], was reacted with the carboxyl groups by using water-soluble carbodiimide. This new affinity adsorbent proved to be very effective for the high-performance affinity chromatography of trypsins.

EXPERIMENTAL

Materials

Asahipak GS-520 gel (molecular weight exclusion limit 3×10^5, particle size 9 ± 0.5 μm) was obtained from Asahi Chemical Industry Co. (Tokyo, Japan). 6-Aminohexanoic acid (AHA) was obtained from Nakarai Chemicals Co. (Kyoto, Japan), *p*-aminobenzamidine monohydrochloride (ABA · HCl) from Sigma (St. Louis, MO, U.S.A.), 7-(benzoyl-L-arginineamido)-4-methylcoumarin (Bz-Arg-AMC) from the Protein Research Foundation (Osaka, Japan), 1,1'-carbonyldiimidazole (CDI) from Tokyo Chemical Industry Co. (Tokyo, Japan) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (EDC · HCl) from Dojin Chemical Co. (Kumamoto, Japan). Dioxane was dehydrated by the use of molecular sieve 4A. Trypsin (bovine pancreas, Type III) and trypsinogen (bovine pancreas, Type I) were obtained from Sigma. Purified *Streptomyces erythreus* trypsin (*S. erythreus* trypsin) and *Streptomyces griseus* trypsin (*S. griseus* trypsin) were gifts from Professor F. Sakiyama and Dr. M. Nakano, respectively. Pronase P was obtained from Kaken Chemical Co. (Tokyo, Japan).

Preparation of affinity adsorbent for trypsins (Asahipak GS520-AHA-ABA) (Fig. 1)

Activation procedure. The dried gel (10 g) was swollen with water and sonicated for 5 min to ensure complete swelling. The swollen gel was washed with 200 ml of water, then with 200 ml of dioxane, and was suspended in 100 ml of dioxane. CDI (3.24 g) was added and the suspension was stirred gently at room temperature for 15 min. The activated gel was washed with 200 ml of dioxane and used immediately.

Asahipak GS-gel bearing an ω-carboxyl spacer (Asahipak GS520-AHA). The above material was suspended in 200 ml of 1 *M* sodium hydrogen carbonate solution (pH 10) containing 1 *M* AHA and gently shaken at 4°C for 25 h. The gel was washed sequentially with 200 ml of water, 100 ml of 1 *M* sodium chloride solution and 200 ml of water. The AHA content was 40 μmol per g dry gel.

Coupling of ABA. The above gel (2 g) was suspended in 15 ml of 0.2 *M* 2-(morpholino)ethanesulphonic acid–sodium hydroxide buffer (pH 4.75), and EDC · HCl (288 mg) was added. The suspension was gently stirred at room temperature for 30 min, then ABA · HCl (28.3 mg) was 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 for 24 h. The gel was washed sequentially with 150 ml of water, 100 ml of 0.05 *M* sodium hydroxide solution containing 1 *M* sodium chloride, 100 ml of 0.05 *M* hydrochloric acid containing 1 *M* sodium chloride and finally with water until the washings were neutral. The content of ABA was 13 μmol per g dry gel.

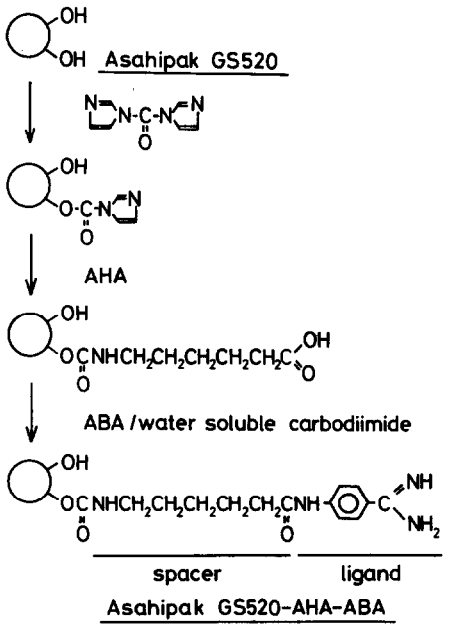

Fig. 1. Derivatization of Asahipak GS-gel.

Determination of the contents of AHA and ABA

The AHA content was determined by titration with sodium hydroxide solution. The content of ABA was determined by measurement of the absorbance (ε_{292} = 1.53 × 10^4 l mol^{-1} cm^{-1} in 0.05 *M* sodium dihydrogenphosphate, adjusted to pH 7 with sodium hydroxide) of the washings.

Chromatographic system (Fig. 2)

The stainless-steel column (100 × 6 mm I.D., SCE-06W) and packing device (PA-H-10) were from Umetani Precision Co. (Osaka, Japan), the reciprocating double plunger pump (flow-rate 0.1–9.9 ml/min, Twincle) was from Japan Spectroscopic Co. (Tokyo, Japan), pressure gauges (0–50 kg/cm^2, KPG-50N; 0–10 kg/cm^2, KPG-10N) and the line filter (KLF-D) from Kyowa Seimitsu Co. (Tokyo, Japan).

Asahipak GS520-AHA-ABA was suspended in 0.05 *M* sodium phosphate containing 0.1 *M* sodium chloride (pH 7.4) and packed in the column for 1 h with the same solution. The final flow-rate was 1.7 ml/min. Reservoirs containing the eluents were connected to the pump through an eight-way valve (Omnifit, U.K.). Samples were introduced through an injector valve (Model 7125; Rheodyne, CA, U.S.A.) by using a micro syringe (1702 RN; Hamilton, Reno, NV, U.S.A.). Stainless-steel tubing (1/16 in. O.D.) was used, except for the reaction coil (PTFE, 4 m × 0.25 mm I.D.). The inner diameter was 0.5 mm before and 0.25 mm after the column.

The eluted protein was detected by measuring tryptophan fluorescence with a Shimadzu RF-530 fluorescence spectrophotometer (xenon lamp; excitation at 285 nm, emission at 340 nm) (Shimadzu, Kyoto, Japan) equipped with an LC micro flow cell (volume 12 μl).

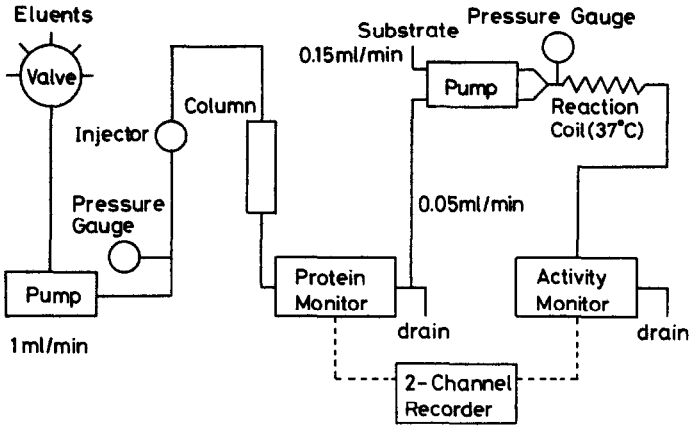

Fig. 2. Diagram of the chromatographic system.

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

Trypsin activity in the effluent was detected by the use of an on-line assay system. The effluent was delivered at a point just after the protein monitor by a peristaltic pump (minipuls II, HP4; Gilson, France), which simultaneously pumped a substrate solution. The substrate solution comprised 20 μM Bz-Arg-AMC[8] in 0.5 M sodium phosphate buffer, pH 7.4. The pumping rates were 0.05 ml/min (Tygon tube, 0.25 mm I.D.) for the effluent and 0.15 ml/min (Tygon tube, 0.5 mm I.D.), for the substrate solution. The reagents were mixed just after the pump with a three-way connector and reacted in a coil equilibrated at 37°C in a water-bath. AMC produced by the enzymatic reaction was detected with a JASCO FD110-C fluorescence spectrophotometer (medium-pressure mercury lamp; excitation at 365 nm, emission at 460 nm) (JASCO, Tokyo, Japan).

Affinity chromatography on Asahipak GS520-AHA-ABA

The following eluents were used: 1, 0.05 M sodium phosphate–0.1 M sodium chloride (pH 7.4); other eluents contained the components described in addition to those of eluent 1; eluent 2, 20 mM AHA; 3, 20 mM AHA–3 M urea. All eluents (pH 7.4) were passed through a 0.45-μm Millipore filter before use and were degassed. They were pumped at a flow-rate of 1 ml/min. The pressure drop in the column at this flow-rate was < 25 kg/cm^2. Prior to a series of chromatographic runs, the column was irrigated alternately with eluents 1 and 3 until a constant baseline was obtained. The sample solution was introduced from the injector onto the column equilibrated with eluent 1. All operations were performed at room temperature (20–25°C).

RESULTS

Affinity chromatography of bovine trypsin

Asahipak GS520-AHA-ABA proved to be very effective for the affinity chromatography of trypsins, providing good separation with high sensitivity in a short time. Bovine trypsin was applied to the column equilibrated with eluent 1 (Fig. 3A).

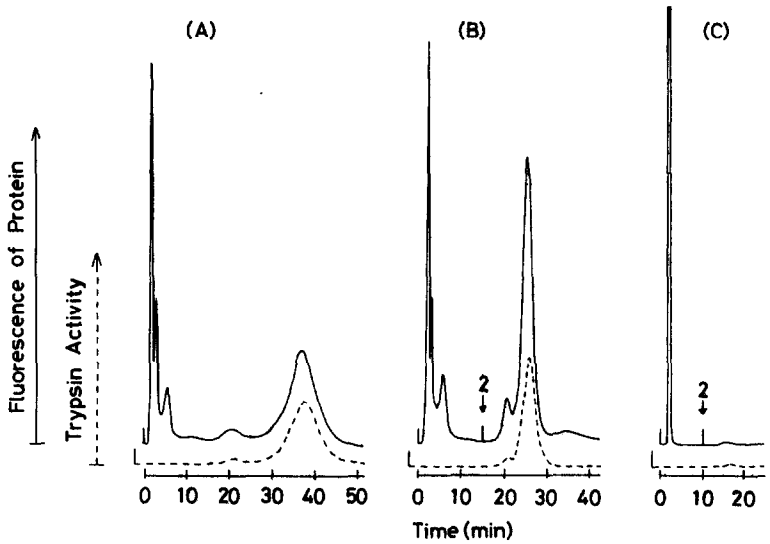

Fig. 3. Affinity chromatography of bovine pancreas trypsin and trypsinogen on Asahipak GS520-AHA-ABA. A, The column was equilibrated with eluent 1. Trypsin (10 μg) dissolved in 20 μl of 1 m*M* HCl was applied to the column at time 0. B, Trypsin (10 μg) dissolved in 20 μl of 1 m*M* HCl was applied at time 0. C, Trypsinogen (4 μg) dissolved in 10 μl of 1 m*M* HCl. The eluent was changed as indicated by the numbered arrow. The compositions of the eluents are given in the Experimental section.

The effluent which passed through the column had no enzyme activity, and two strongly retarded peaks of protein appeared later. Both peaks had trypsin activity. When the eluent was changed to eluent 2 (eluent 1 + 20 m*M* AHA) at 15 min, the elution of the active trypsins was accelerated and the peaks became sharper (Fig. 3B). Trypsinogen (bovine pancreas) passed through the column equilibrated with eluent 1 (Fig. 3C) without retardation. These findings indicate that Asahipak GS520-AHA-ABA is an affinity adsorbent for trypsins.

Jameson and Elmore[9] reported the separation of α- and β-trypsin on benzamidine-Sepharose, and on the basis of their results, the first peak in Fig. 3A, B may be α-trypsin and the main peak should be β-trypsin.

The chromatographic results proved to be reproducible. Essentially the same elution patterns (both for protein and activity) were obtained for replicate analyses. The difference in the retention times did not exceed 30 sec and that in the peak heights was less than 5%. The height of the main peak of trypsin was doubled when twice the amount of sample was applied, *e.g.*, 81 mm for 7.6 μg protein and 165 mm for 15.2 μg protein. The reproducibility and accuracy of the system seems to be satisfactory for analytical purposes.

Affinity chromatography of other trypsins

Fig. 4A shows that *S. griseus* trypsin[10] was retained on the column. It was not eluted with eluent 1 (after 1 h) (data not shown), and was eluted more slowly than bovine trypsin with eluent 2. Evidently, *S. griseus* trypsin has an extremely high affinity for the specific ligand, benzamidine. This is consistent with the properties of

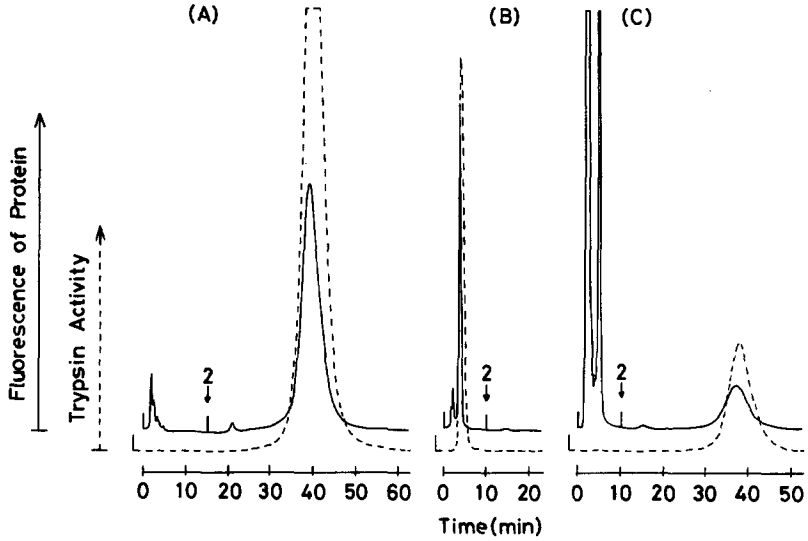

Fig. 4. Affinity chromatography of trypsins on Asahipak GS520-AHA-ABA. A, *Streptomyces griseus* trypsin (5 μg) dissolved in 5 μl of 0.05 *M* sodium acetate–0.02 *M* calcium chloride (pH 5.5) was applied to the column (time 0). B, *Streptomyces erythreus* trypsin (4.5 μg) dissolved in 5 μl of eluent 1 was applied to the column (time 0). C, Pronase P (20 μg) dissolved in 20 μl of eluent 1 was applied to the column (time 0). Eluent change is indicated by the numbered arrow as in Fig. 3.

this trypsin. Its specific activity was also larger than that of bovine trypsin. The reproducibility of the chromatography was also satisfactory.

Fig. 4B shows that *S. erythreus* trypsin[11], an anionic trypsin (p*I* 4.0), passed through the column equilibrated with eluent 1. Thus, it is not adsorbed by this adsorbent, presumably because of the ionic repulsion between the enzyme and the COOH groups of the spacer moieties which had not been coupled with the ligand.

Pronase P[12] is a mixture of proteinases, including *S. griseus* trypsin. Specific separation of *S. griseus* trypsin from Pronase P was achieved in a short time by using Asahipak GS520-AHA-ABA (Fig. 4C).

DISCUSSION

Various attempts[1–4,13] to improve the efficiency of affinity chromatography have been made by using silica gels or synthetic polymer gels. Though silica gel[1–3] is extremely stable at high pressure, it has a serious drawback in affinity chromatography, because the choice of eluents is limited due to its instability under alkaline conditions. Synthetic polymer supports are usually very stable over a wide pH range, but their mechanical stability is not as good. Turková *et al.*[4] reported the use of hydroxyalkyl methacrylate gel (Separon H1000, particle size 10 μm) for the separation of pepsin. They did not monitor the enzyme activity. We have reported an analytical system for human plasmin and plasminogen using medium-pressure liquid chromatography and a hydrophilic vinyl-polymer gel, Toyopearl HW-65S[6]. Since Toyopearl HW-65S is not suitable as a support for HPLC, we investigated an po-

lyvinyl alcohol gel, Asahipak GS-gel, in the present work. The small particle size and narrow size distribution (9 ± 0.5 μm) made it possible to increase the eluent flow-rate and thus decrease the analysis time. The results reported here show that affinity chromatography could be successfully performed under conditions similar to those of HPLC by using Asahipak GS-gel.

The OH group of the matrix was activated by use of CDI. This technique has many advantages[14–16]. It gives an adsorbent which has a non-basic urethane linkage, devoid of additional charged groups between the matrix and the compound coupled to it, in contrast to the cyanogen bromide method. The activation level can easily be controlled and is reproducible. The reagent is less poisonous, and so is much easier to handle, than CNBr. Thus CDI proved to be very convenient for the activation of Asahipak GS-gel.

The use of fluorometric detection, especially of enzyme activity, made it possible to analyze very small amounts of enzymes. The limit of detection would theoretically be about 5 ng for bovine trypsin and 100 pg for *S. griseus* trypsin based on the capacity of the detector. Even when only 0.5 μg of bovine trypsin were applied to this system, a chromatogram essentially similar to Fig. 3A was obtained (data not shown).

In conclusion, the use of derivatized Asahipak GS-gel in our chromatographic system proved to be very effective in the analysis of trypsins by affinity chromatography. This system should be applicable to many other trypsin-family proteases by using a series of specific peptidyl-AMC substrates for various proteases[17,18]. It should also be valuable in various fields, *e.g.*, in clinical diagnosis and analytical biochemistry. Further studies on such applications are in progress.

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