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# PREPARATION OF HIGH-CAPACITY AFFINITY ADSORBENTS USING FORMYL CARRIERS AND THEIR USE FOR LOW- AND HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF TRYPSIN-FAMILY PROTEASES

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## SUMMARY

To prepare stable and high-capacity affinity adsorbents for trypsin-family proteases, high concentrations of *m*-aminobenzamidine (ABA) were immobilized by reductive amination with sodium cyanoborohydride on two types of formyl carriers I and II having different spacer lengths. Formyl carriers I were prepared by periodate oxidation of glucose carriers obtained by coupling glucose to epoxy-activated carriers. Formyl carriers II were prepared by periodate oxidation of glyceryl carriers obtained by hydrolysis of epoxy-activated carriers. High concentrations of ABA (15–21 μmol per g wet gel) were efficiently immobilized on both types of carriers, and formyl groups remaining on the adsorbents were converted into hydroxymethyl groups by reduction with $NaBH_4$. Two types of adsorbents prepared with Sepharose gel were successfully used for affinity chromatography of bovine trypsin, *Streptomyces griseus* trypsin and serine proteases in urine, and exhibited high adsorption capacities, *e.g.*, about 30 mg bovine trypsin per ml gel. The adsorbents prepared with Toyopearl gel (Fractogel TSK) were successfully used for high-performance liquid chromatography of bovine trypsin, though they exhibited lower adsorption capacities than those prepared with Sepharose gels.

## INTRODUCTION

We have reported that derivatized carriers having formyl groups are useful for the immobilization of protein ligands[1]. In particular, higher concentrations of protein ligands were immobilized under mild conditions on formyl carrier I which has a long spacer than on formyl carrier II which has a short spacer. We have now applied this method to immobilize *m*-aminobenzamidine (ABA), a ligand for affinity chromatography of the trypsin-family proteases. New formyl carriers I, different from those reported previously were used, prepared by periodate oxidation of glucose carriers instead of reductive amination of glutaraldehyde and amino carriers. New formyl carriers I can be quickly prepared just before use and have a shorter but more hy-

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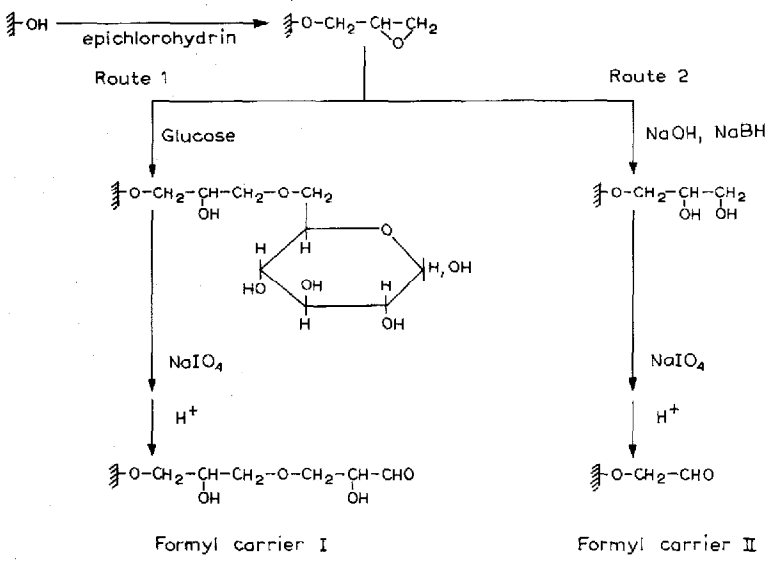
drophilic spacer arm than the formyl carrier I reported previously[1]. High concentrations of ABA were efficiently immobilized on both types of formyl carriers, and the adsorbents thus obtained were successfully used for low- and high-performance liquid affinity chromatography of trypsin-family proteases.

## MATERIALS AND METHODS

Sepharose 4B was obtained from Pharmacia Fine Chemicals, Toyopearl HW-65 (Fractogel TSK) from Toyo Soda Manufacturing Co. (Tokyo, Japan), sodium metaperiodate ($NaIO_4$) and trypsin (2000 units/g) from Wako (Osaka, Japan), sodium cyanoborohydride ($NaCNBH_3$) from Nakarai (Kyoto, Japan), *m*-aminobenzamidine (ABA) dihydrochloride monohydrate from Aldrich, benzoyl-L-arginine ethyl ester (BAEE) from Fluka, pronase E from Kaken (Tokyo, Japan) and Toyo membrane filters from Toyo Roshi (Tokyo, Japan).

### *Preparation of formyl carriers*

Two formyl derivatives of Sepharose 4B and of Toyopearl HW-65 were prepared by the routes shown in Scheme 1. Sepharose 4B and Toyopearl HW-65 were activated with epichlorohydrin as described previously[2,3].




Scheme 1. Preparation of formyl carriers.

*Route 1.* A 1-g amount of the epoxy-activated gel was suspended in 4 ml of 0.1 *M* sodium hydroxide containing 0.5 g of glucose and incubated at 40°C for 24 h with shaking. After washing extensively with water, the gel was suspended in 1.5 ml of 0.1 *M* sodium periodate and shaken for 1 h at 4°C. After washing with water, the gel was incubated in 0.1 *M* hydrochloric acid for 30 min at room temperature and again washed with water.

*Route 2.* A 1-g amount of the epoxy-activated gel was suspended in 10 ml of 0.1 *M* sodium hydroxide containing 2 mg $NaBH_4$ and incubated at 40°C for 24 h with shaking. Subsequent oxidation with periodate was performed under as in route 1.

*Coupling of ABA to formyl carriers*

A 2.0-g amount of the formyl carrier was suspended in 4 ml of 15 m*M* phosphate-buffer saline (PBS), pH 7.0, containing 9.0 mg of ABA, and the suspension was incubated with shaking at 4°C for 24 h. Then 10 mg of $NaCNBH_3$ were added, and the suspension was shaken for 6 h at 4°C. The gel was washed successively with PBS and water. The adsorbents were treated with 10 mg of $NaBH_4$ at 4°C for 4 h to convert the remaining formyl groups into hydroxymethyl groups.

*Determination of the contents of formyl spacer and ABA*

A 0.5-g amount of the suction-dried formyl carrier was suspended in 1 ml of 2 *M* ethylenediamine dihydrochloride adjusted to pH 7.0 with 2 *M* sodium hydroxide, and the suspension was incubated at 40°C for 1.5 h with shaking. Then 10 mg of $NaCNBH_3$ were added, and the suspension was incubated for another 1.5 h at 40°C. The gel was treated with a 4-g amount of succinic anhydride, and the carboxyl groups introduced into the gel were titrated with 0.01 *M* hydrochloric acid according to the method of Inman and Dintzis[4]. The concentration of formyl groups was estimated indirectly from the concentration of carboxyl groups. The amount of immobilized ABA was determined by difference analysis of the absorbance at 320 nm ($\varepsilon_{320\,nm} = 1.80 \cdot 10^3$ l mol$^{-1}$ cm$^{-1}$ in 15 m*M* PBS, pH 7.0) of the combined filtrate and washings.

*Affinity chromatography of trypsin on immobilized ABA gels*

A 1-g amount of crude trypsin was dissolved in 16 ml of 0.05 *M* Tris–HCl containing 0.5 *M* sodium chloride and 0.02 *M* calcium chloride, pH 7.8 (buffer 1) and the solution was centrifuged at 20250 *g* for 30 min. The supernatant was filtered successively through Toyo membrane filters with pore sizes of 3.0 and 1.0 $\mu$m. The filtrate was applied to a column of immobilized ABA gel equilibrated with buffer 1, the column was washed with buffer 1 and adsorbed trypsin was eluted with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride. Enzyme activity was determined from the change in absorbance at 253 nm using BAEE as a substrate, according to the method of Schwert and Takenaka[5].

To examine the adsorption capacity, the column was saturated with trypsin and adsorbed trypsin was eluted by the same procedure. The amount of bound trypsin was calculated from the elution curve assuming $A^{1\%}_{280\,nm} = 15.9$.

*Affinity chromatography of pronase E on ABA-Sepharose*

A 80-mg amount of pronase E dissolved in 1.6 ml of buffer 1 was applied to a column of ABA-Sepharose and affinity chromatography was performed as described for trypsin. The pronase activity was determined using casein as a substrate, according to the method of Hagihara[6].

*Affinity chromatography of serine proteases in urine on ABA-Sepharose*

Fresh urine was dialyzed against 0.05 *M* Tris–HCl, pH 7.6 (buffer 2), overnight at 4°C with stirring, then centrifuged at 20250 *g* for 15 min. The supernatant was applied to a column of ABA-Sepharose equilibrated with buffer 2, and the column was washed with buffer 2. Elution was then performed successively with 1.4 *M* sodium chloride and 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride. The enzyme activity was determined with BAEE as a substrate by the method of Hagihara[6].

*High-performance liquid affinity chromatography (HPLAC) of trypsin*

ABA-Toyopearl HW-65 (I) was packed into a 750 mm × 75 mm I.D. column by the slurry packing method at a flow-rate of 1 ml/min. An HPLC system with a high-pressure pump (Model HLC-803D, Toyo Soda) and a spectromonitor (UV-8 Model II, Toyo Soda) was used. A 100-μl aliquot of crude bovine trypsin was applied to the column at a flow-rate of 0.5 ml/min. The mobile phase was buffer 1; 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride was used to elute adsorbed trypsin from the column. The effluent was monitored at 280 nm.

## RESULTS

*Coupling of the spacers*

The concentrations of formyl groups introduced are shown in Table I. The difference in the concentrations seems to be due to the properties of the carrier, but not to the presence of the spacers derived from glucose. Each carrier had different concentrations of epoxy groups introduced with epichlorohydrin: 40 μmol per g wet Sepharose 4B and 103 μmol per g wet Toyopearl HW-65.

TABLE I

IMMOBILIZATION OF ABA

| *Adsorbent* | *Concentration of formyl groups (μmol/g wet gel*)* | *Concentration of immobilized ABA (μmol/g wet gel*)* | *Yield of immobilization** (%)* |
|---|---|---|---|
| ABA-Sepharose 4B (I) | 36 | 16 | 74 |
| ABA-Sepharose 4B (II) | 36 | 15 | 72 |
| ABA-Toyopearl HW-65 (I) | 106 | 21 | 98 |
| ABA-Toyopearl HW-65 (II) | 96 | 20 | 98 |

* The volume per gram of wet Sepharose gels was 1.5 ml and that of wet Toyopearl gels was 1.2 ml.

** The yield of immobilization indicates the proportion of bound ABA of the total ABA added.

*Immobilization of ABA*

The results of the immobilization of ABA are also shown in Table I. The Toyopearl gels had higher concentrations of immobilized ABA and higher yields of immobilization than the corresponding Sepharose gels, perhaps because the Toyopearl gels have higher contents of formyl groups than the Sepharose gels. The presence of the spacer derived from glucose did not affect the concentration of ABA immobilized on either carrier.

*Affinity chromatography of trypsin*

Affinity chromatography of trypsin on a column of ABA-Sepharose 4B (I) is shown in Fig. 1. All the trypsin activity was retained on the column and was eluted with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride as a sharp peak. The recovery of the activity was as high as 110%, suggesting that inhibitors or competitive protein substrates in the sample were also separated by the chromatography. A similar affinity chromatogram was obtained with a column of ABA-Sepharose 4B (II). The specific activities of trypsin eluted were increased by 70–80 fold (w/w) and by 3-fold per $A_{280nm}$ unit.

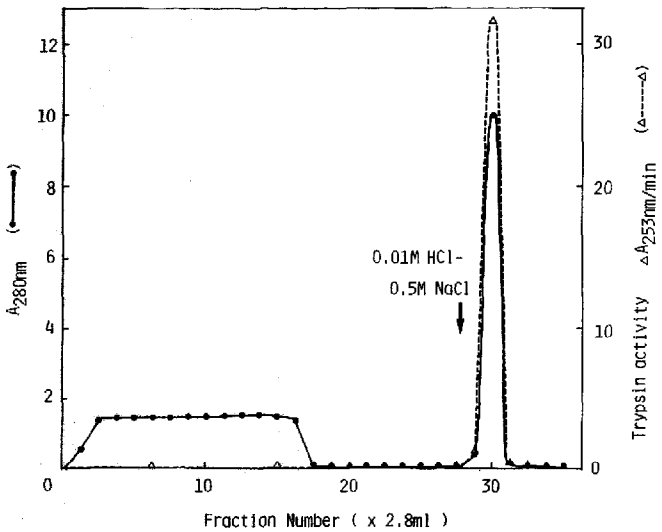



Fig. 1. Affinity chromatography of trypsin on a column of ABA-Sepharose 4B (I). A 32-ml volume of crude trypsin ($A_{280nm}$ = 2.2) was loaded on the column (1.6 cm × 0.75 cm) at a flow-rate of 6 ml/h at 4°C. After washing with buffer 1, elution of the adsorbed trypsin with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride was started at the point indicated by the arrow.

Affinity chromatography of trypsin was also performed on a column of ABA-Toyopearl HW-65 (I) (Fig. 2). The trypsin activity was observed not only in eluted fractions but also in the flow-through fractions, even though a smaller amount of sample was loaded on a longer column than in the case of the Sepharose derivatives.

The adsorption capacities of the adsorbents are listed in Table II. Adsorbents prepared with Sepharose gels showed very high capacities for trypsin despite their lower ligand concentrations compared with the Toyopearl derivatives. The capacity was not affected by the presence of the long spacer derived from glucose.

HPLAC of trypsin was performed on a column of ABA-Toyopearl HW-65 (I) as shown in Fig. 3. Tryptic activity was found in the second peak, but not in the first. The chromatography was completed in 90 min at a flow-rate of 0.5 ml/min, though a slight peak tailing was observed.

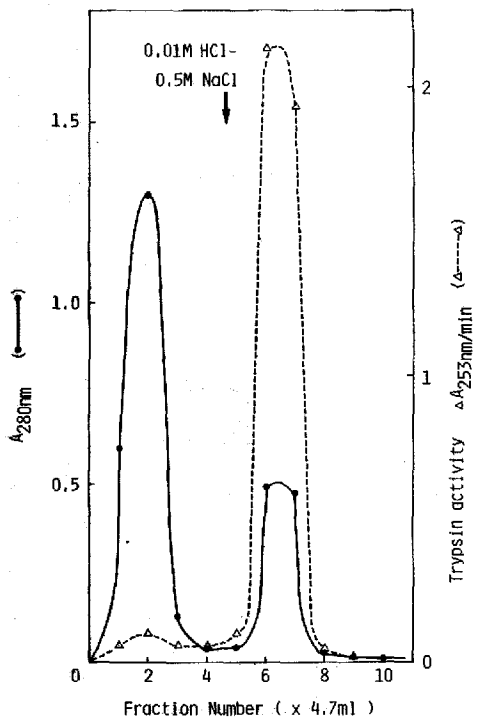



Fig. 2. Affinity chromatography of trypsin on a column of ABA-Toyopearl HW-65 (I). A 5-ml volume of crude trypsin ($A_{280nm}$ = 2.3) was loaded on the column (4.6 cm × 0.75 cm). Elution was performed as in Fig. 1.

TABLE II

ADSORPTION CAPACITY OF TRYPSIN ADSORBENTS

| *Adsorbent* | *Column size (cm)* | *Adsorption capacity (mg trypsin/ml gel)* |
|---|---|---|
| ABA-Sepharose 4B (I) | 0.75 × 1.7 | 28 |
| ABA-Sepharose 4B (II) | 0.75 × 1.5 | 33 |
| ABA-Toyopearl HW-65 (I) | 0.75 × 4.6 | 1.2 |
| ABA-Toyopearl HW-65 (II) | 0.75 × 5.1 | 2.2 |

*Affinity chromatography of pronase E*

*Streptomyces griseus* trypsin was efficiently separated from pronase E on a column of ABA-Sepharose 4B (I) as shown in Fig. 4. Almost no pronase activity was retained on the column, while all the trypsin activity was specifically adsorbed and could be recovered by elution with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride. A similar affinity chromatogram was obtained with a column of ABA-Sepharose 4B (II). The recovery of tryptic activity was as high as 87%.

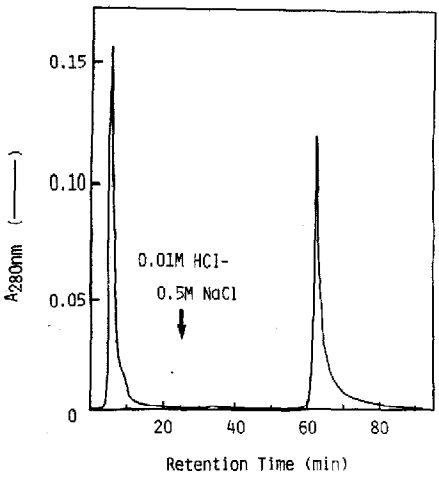



Fig. 3. HPLAC of bovine trypsin on a column of ABA-Toyopearl HW-65 (I). A 0.1-ml volume of crude trypsin ($A_{280nm}$ = 2.6) was loaded on the column (7.5 cm × 0.75 cm) at a flow-rate of 0.5 ml/min at room temperature. After washing with buffer 1, elution of adsorbed trypsin with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride was started at the time indicated by the arrow.

*Affinity chromatography of serine proteases in urine*

When a urine sample was applied onto an ABA-Sepharose 4B (I) column, not only serine proteases but also yellow dyes were adsorbed on the column. The dyes were removed with 1.4 *M* sodium chloride, then the adsorbed proteases were eluted with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride as a sharp peak as shown in Fig. 5. Urine serine proteases such as thrombin, kallikrein and urokinase[7], which have been shown to bind to adsorbents having ABA as a ligand[8–12], seemed to be

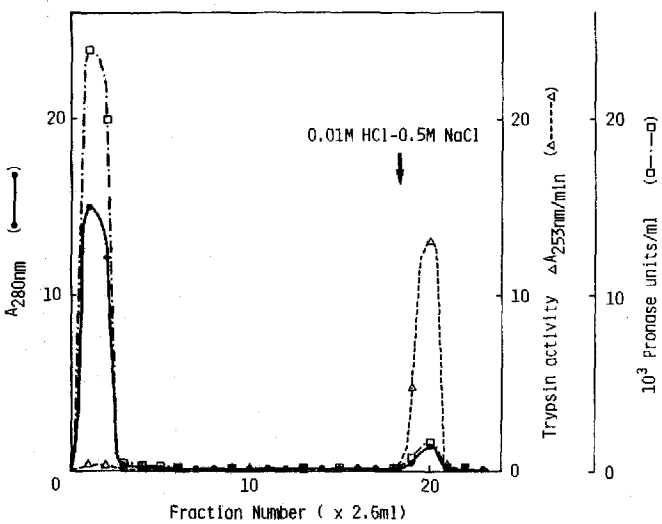



Fig. 4. Affinity chromatography of pronase E on a column of ABA-Sepharose 4B (I). A 1.6-ml volume of pronase E ($A_{280nm}$ = 48.5) was loaded on the column (1.6 cm × 0.75 cm). Elution was performed as in Fig. 1.

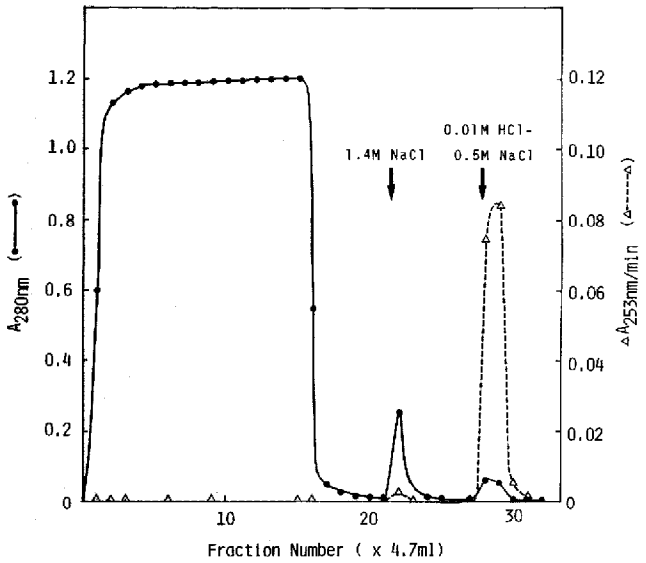



Fig. 5. Affinity chromatography of serine proteases in urine on a column of ABA-Sepharose 4B (I). A 66-ml volume of urine ($A_{280nm}$ = 1.2) was loaded on the column (1.6 cm × 0.75 cm) at a flow-rate of 6 ml/h at 4°C. After washing with buffer 2, the adsorbed dyes were eluted with 1.4 *M* sodium chloride (indicated by the first arrow) and then the adsorbed enzymes with 0.01 *M* hydrochloric acid–0.5 *M* sodium chloride (second arrow).

recovered in this peak. The recovery of the activity was unusually high (over 1000%), suggesting that inhibitors or protein substrates in the urine sample were removed by the affinity chromatography. A similar affinity chromatogram was obtained with a column of ABA-Sepharose 4B (II), though its adsorption capacity was 75–80% of that of ABA-Sepharose 4B (I).

## DISCUSSION

Affinity ligands having an amino group can be immobilized on carriers having formyl groups by reductive amination under very mild conditions. Formyl carriers are most conveniently prepared by periodate oxidation of cellulose carriers[13] or mild acid treatment of agarose gels[14]. However, these methods possess some disadvantages. In the former, the oxidation weakens the structure of the carriers, and in the latter the coupling of ligands takes a long time because of the low reactivity of the formyl groups in hemiacetal forms. On the other hand, carriers having formyl spacers seem to lack these problems. We prepared formyl-spacer carriers by immobilization of glutaraldehyde on 3-amino-2-hydroxypropyl carriers derived from epoxy-activated carriers, and successfully used them for the efficient immobilization of protein ligands. In this study we prepared new types of formyl carriers having hydrophilic spacers (formyl carriers I), by periodate oxidation of glucose carriers obtained by immobilization of glucose on epoxy-activated carriers. Chemically stable glucose carriers can be stored for a long time and converted into formyl carriers I just before

use. The formyl carriers I may contain only a few spacers different in structure from the one shown in Scheme 1. Upon immobilization of methyl α-D-glucoside on 1,4-butanediol diglycidyl ether-activated Sepharose 4B, 92% of the linkage to the carrier was formed via the hydroxymethyl group at the C-6 position of the sugar[15]. In the immobilization of D-glucosamine, 85% was through the amino group and 15% through the primary alcohol at C-6. Immobilization of D-glucose on epichlorohydrin-activated carriers also seems to proceed through the primary alcohol at C-6, and the major product of the oxidation should therefore be the one shown in Scheme 1.

Ohlson *et al.*[16], Fischer[17] and Ito *et al.*[1] have reported the immobilization of protein ligands on formylmethyl carriers (formyl carriers II). These can easily be prepared by hydrolysis of epoxy-activated carriers and subsequent periodate oxidation of the hydrolyzates, glyceryl carriers.

High concentrations of ABA could be efficiently immobilized on both formyl carriers I and II. The adsorbents thus prepared were successfully used for the affinity chromatography of trypsin-family proteases.The Sepharose adsorbents showed especially high adsorption capacities. Various affinity adsorbents having ABA as a ligand are listed in Table III. By use of the procedures reported in this study, high concentrations of ABA are efficiently immobilized and the remaining formyl groups are reduced to hydroxymethyl groups. On the other hand, most of the other adsorbents contain carboxyl groups, which may allow electrostatic non-specific adsorptions or disturb the specific interactions[18].

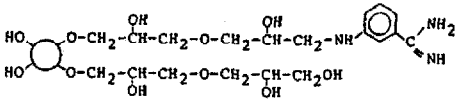
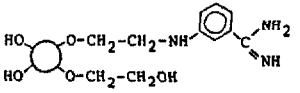
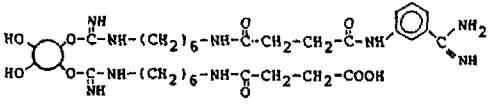
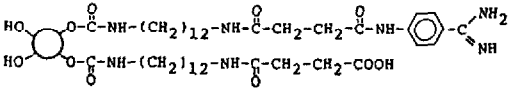
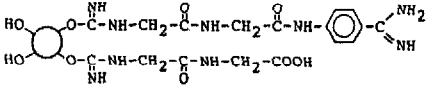
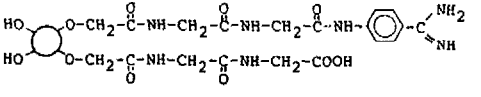
The adsorbents prepared with Sepharose gel in this study are among those having the highest capacities for trypsin. Those prepared with Toyopearl gel have a lower capacity despite having the highest concentrations of ABA. This may be due to the hydrophobic properties of the carriers. Similar differences were observed between ABA-Sepharose 4B and ABA-Sepharon HEMA[19].

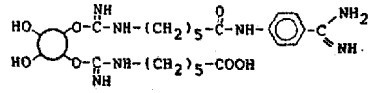
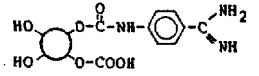
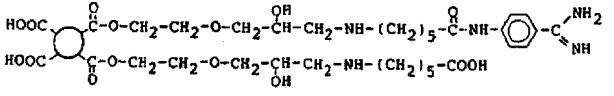
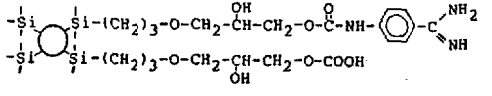
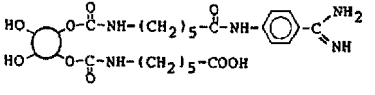
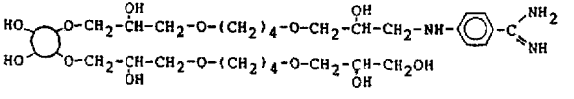
Although a Toyopearl adsorbent was used for HPLAC, the flow-rate could not be increased above 0.5 ml/min, and some peak tailing was observed as found previously[19]. Use of TSK G3000PW gel, which is an hydrophobic vinyl polymer matrix suitable for high-performance gel permeation liquid chromatography, may improve these problems.

The introduction of a spacer between the ligand and carrier has resulted in successful affinity adsorption of various biological substances, such as *Escherichia coli* β-galactosidase[22] and *Staphylococcus* nuclease[23]. Trypsin adsorbents having arginine as a ligand required a dipeptidyl spacer for the highest association constant[24]. Most trypsin adsorbents prepared with ABA, except for ABA-Reacti-Gel[19], also have long spacer arms as shown in Table III. In this study, ABA carriers II having a short spacer were found to have almost the same adsorption capacities as ABA carrier I having long spacers. The amounts of trypsin adsorbed on Sepharose gels are calculated to be 12–14% of the amounts of ABA on the gel. When carriers are activated with epichlorohydrin and hydrolyzed, polyglyceryl as well as monoglyceryl groups are produced, and some ABA could be immobilized on formyl carrier derived from such long spacers. If it is only such ABA that contributes to the specific adsorption of trypsin, the adsorption capacity of the Toyopearl adsorbent will be improved by the introduction of polyglyceryl spacers. ABA was immobilized on formyl-Toyopearl gel obtained by repeating three times the epoxy activation and subsequent hydrolysis of the epoxy group followed by periodate oxidation. However,

TABLE III

TRYPSIN ADSORBENTS HAVING ABA AS AN AFFINITY LIGAND

| *Structure of adsorbent** | *Carrier* | *Concentration of ligand* | *Adsorption capacity for bovine trypsin* | *Ref.* |
|---|---|---|---|---|
|  | Sepharose 4B (agarose) | 16 μmol/g wet gel | 28 mg/ml | This study |
| | Toyopearl HW-65 (polyvinyl alcohol) | 21 μmol/g wet gel | 1.2 mg/ml | |
|  | Sepharose 4B (agarose) | 15 μmol/g wet gel | 33 mg/ml | This study |
| | Toyopearl HW-65 (polyvinyl alcohol) | 20 μmol/g wet gel | 2.2 mg/ml | |
|  | Bio-Gel A-5m (agarose) | 60 or 12 μmol/ml | 6–7 mg/ml | 12 |
|  | Cellulose | 90–110 μmol/ml | Unknown | (Merck) |
|  | Agarose | >20 μmol/ml (estimated) | 7–10 mg/ml | 21 (Sigma) |
|  | Sepharose CL-4B (agarose) | Unknown | Unknown | 20 |

| | | | | |
|---|---|---|---|---|
|  | CH-Sepharose 4B (estimated) (agarose) | 31 μmol/g dry gel | 8.4 mg/g dry gel (batch methods) | 19 |
|  | Reacti-Gel 6X (agarose) | 21 μmol/g dry gel | 5.4 mg/g dry gel (batch method) | 19 |
|  | ACA-Separon HEMA $E_{max}$ (acrylate) | 46 μmol/g dry gel | 10.7 mg/g dry gel (batch method) | 19 |
|  | CDI-Glycophase (glass-bead) | 10 μmol/g dry gel | 1.3 mg/g dry gel (batch method) | 19 |
|  | Pierce 4A-XL (agarose) | Unknown | 5–7 mg/ml | (Pierce) |
| | Asahipak (polyvinyl alcohol) | 13 μmol/g dry gel | Unknown | 18 |
|  | Sepharose 6B (agarose) | Unknown | 13 mg/ml | (Pharmacia) |

* Spacers carrying free functional groups not used for ligand immobilization are also shown.

the adsorbents thus prepared also had a low adsorption capacity (1.6 mg trypsin per ml gel). These results indicate that a long spacer is not necessary for immobilized ABA to adsorb trypsin.

ABA-Sepharose 4B (II) seems to be the most suitable adsorbent for trypsin because of its convenience of preparation, high adsorption capacity and high chemical stability (90% of the original capacity remained after 6 months of use). The adsorbents prepared in this study may also be useful for purification of trypsin-family enzymes other than bovine trypsin.

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