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INTRODUCTION OF ω-CARBOXYL SPACERS ONTO CROSS-LINKED AGAROSE GEL BEADS BY O-ALKYLATION FOR THE PREPARATION OF AFFINITY ADSORBENTS

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SUMMARY

Cross-linked agarose gel beads (Sepharose CL-4B) were O-alkylated with chloroacetylglycylglycine or N-chloroacetyl-6-aminohexanoic acid in dimethyl sulfoxide by using methylsulfinyl carbanion as a catalyst. The reaction was completed within 30 min and the degree of substitution reached 100 μmol per ml settled volume of the gel with about 50% yield. The terminal carboxyl group of the spacers can be coupled with an affinity ligand containing an amino function by the use of water-soluble carbodiimide. The ligand–matrix linkage is stable and free from undesired electric charge. *Streptomyces griseus* trypsin was purified to homogeneity from Pronase by using a column of the Sepharose derivative coupled with *p*-aminobenzamidine.

INTRODUCTION

Although CNBr activation of agarose gel beads[1] is excellent for the preparation of affinity adsorbents, there are a few drawbacks, *e.g.* the linkage is not stable at alkaline pH and undesired positive charges are inroduced[2]. Rosengren and Glad[3] reported the O-alkylation of cross-linked agarose gel beads with alkyl chloride in dimethyl sulfoxide using methylsulfinyl carbanion as a catalyst; however, the details of the experimental conditions and application of the derivatized gels to affinity chromatography were not described. Recently we reported an application of the Hakomori reaction[4] for the introduction of ω-carboxyl spacers (6-aminohexanoic acid or glycylglycine) onto hydrophilic vinyl-polymer gel particles; the product proved to be useful in high-performance affinity chromatography[5]. The spacers were linked to the matrix through an ether bond and their carboxyl groups were utilized for the immobilization of ligands. This procedure enabled us to prepare stable affinity adsorbents which are free from undesired electric charges.

In this paper, application of this procedure to cross-linked agarose gel is described. The use of *p*-aminobenzamidine as the ligand yielded an affinity adsorbent for trypsin, and this adsorbent was confirmed to be useful in the purification of *Streptomyces griseus* trypsin from Pronase.

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EXPERIMENTAL

Materials

The following materials were obtained from commercial sources: Sepharose CL-4B (Pharmacia, Uppsala, Sweden); *p*-aminobenzamidine monohydrochloride, N^{α}-benzoyl-D,L-arginine *p*-nitroanilide hydrochloride, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (Sigma, St. Louis, MO, U.S.A.); N^{α}-benzoyl-L-arginine *p*-nitroanilide hydrochloride (Protein Research Foundation, Osaka, Japan); Pronase P (Kaken Chemical Co., Tokyo, Japan); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Dojin Laboratories, Kumamoto, Japan). N-Chloroacetylglycylglycine (CAGG) was synthesized according to Fischer[6]. Synthesis of N-chloroacetyl-6-aminohexanoic acid (CAAHA) was described previously[5]. Dimethyl sulfoxide (DMSO) was dehydrated by the use of Molecular Sieve 4A. Methylsulfinyl carbanion was prepared according to Greenwald *et al.*[7].

Drying of Sepharose CL-4B

Sepharose CL-4B (200 g wet) was washed with water (2 l) on a sintered glass funnel, mixed with 100 ml of 30 m*M* NaOH containing 0.3 *M* $NaBH_4$ and allowed to settle overnight at room temperature. The beads were washed with water (2 l), then with small portions of dioxane (1 l) to exchange the solvent. After filtration, the gel cake was freeze-dried without heating in a container capped with a filter paper to prevent the loss of gel particles. An amount of 7–9 g of powder was obtained.

Alkylation of Sepharose CL-4B

Precautions were taken to maintain anhydrous conditions throughout the procedure. The dried Sepharose CL-4B (0.5 g) was weighed into a two-necked pear-shaped flask. One of the openings was coupled to a three-way stopcock and the other was sealed with a rubber septum through which liquid reagents were added by using syringes bearing 19-gauge needles. The air in the flask was replaced with nirogen in a balloon attached to the flask through the three-way cock by evacuation and flushing. DMSO (10 ml) containing 50 mg of $NaBH_4$ was introduced into the flask and the gel was swollen by gentle shaking for 10 min. Methylsulfinyl carbanion (2 mmol, 1–2 *M* in DMSO) was added and mixed. After another 10 min, CAGG or CAAHA (0.4 mmol, dissolved in 1 ml of DMSO) was added and the reaction mixture was allowed to stand for 1 h at room temperature. The content of the flask was poured into 100 ml of cold water, and the gel was washed with 100 ml each of water, 0.05 *M* NaOH containing 1 *M* NaCl, and then water to neutrality. To find the optimum conditions, a series of experiments was carried out with changes of one or two of the factors described above in each experiment. The Sepharose–spacer conjugates produced by alkylation with CAGG and CAAHA are referred to as Sepharose–AGG and Sepharose–AAHA, respectively.

Spacer content was determined by amino acid analysis after acid hydrolysis (6 *M* HCl, 110°C, 16 h, *in vacuo*) and expressed as μmol per ml settled volume of the beads. The values were corrected for the recoveries through the hydrolysis (these were determined by control experiments on free amino acids subjected to the same treatments in the presence of Sepharose CL-4B: glycine, 79%; 6-aminohexanoic acid, 72%).

Coupling of amines to Sepharose–AGG or Sepharose–AAHA

Twenty grams (wet cake) of Sepharose–AGG or Sepharose–AAHA were suspended in 20 ml of 0.2 *M* sodium morpholinoethanesulfonate buffer (pH 4.75) containing either 50 m*M* *p*-aminobenzamidine hydrochloride or 2 *M* ethanolamine hydrochloride. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.54 g) was then added, the pH was readjusted and the mixture was gently shaken at room temperature for 20 h. The gel was consecutively washed with ten volumes of water, 1% acetic acid containing 1 *M* NaCl, 0.05 *M* NaOH containing 1 *M* NaCl and then water to neutrality. The derivatives of Sepharose–AGG coupled with *p*-aminobenzamidine and ethanolamine are referred to as Sepharose–AGG–ABA and Sepharose–AGG–EA, respectively. The derivatives of Sepharose–AAHA are similarly abbreviated.

Affinity chromatography of Pronase on a column of Sepharose–AGG–ABA (Fig. 3)

Sepharose–AGG–ABA (10 g wet) was packed in a chromatographic column (10 mm I.D.) and equilibrated with the initial buffer: 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.5 *M* NaCl and 20 m*M* $CaCl_2$. Pronase (100 mg) was dissolved in 10 ml of the initial buffer and applied to the column. After addition of 50 ml of the initial buffer, elution with a pH gradient was started. The pH gradient was formed by the addition of a solution consisting of 50 m*M* HCl, 25 m*M* formic acid and 20 m*M* $CaCl_2$ (pH 1.72) to a constant-volume mixing chamber (19.5 ml) filled with the initial buffer. Chromatography was performed at 4°C at a flow-rate of 30 ml/h by using a multiple-head peristaltic pump. To avoid prolonged exposure of the enzyme to low pH, the effluent was mixed immediately with 0.17 *M* sodium acetate buffer (pH 5.0) containing 20 m*M* $CaCl_2$ (flow-rate, 8 ml/h) by using a three-way connector. The effluent was collected in fractions of 3.2 ml each.

RESULTS AND DISCUSSION

Introduction of ω-carboxyl spacers by alkylation of cross-linked agarose

Cross-linked agarose gel suspended in DMSO was treated with a strong base, methylsulfinyl carbanion, to convert its hydroxyl groups to alkoxide form[4]. CAGG or CAAHA was added to the suspension as an alkylating reagent to form stable ω-carboxyl spacers.

When Sepharose CL-4B was treated directly with methylsulfinyl carbanion in DMSO, its colour changed to brown and its mechanical rigidity was adversely affected. $NaBH_4$ treatment before drying of the gel and the addition of the reagent to the alkylation mixture, however, largely prevented such changes. Although reduction of organic halogen compounds by $NaBH_4$ was reported[8], the alkylation yield with CAGG was not affected by the addition of $NaBH_4$ up to the concentration of 0.5% (w/v) in the alkylation mixture.

The reactions proceeded very quickly. The effects of reaction time with methylsulfinyl carbanion and with alkylating reagents on the amount of spacers introduced were tested. For both steps, maximum values were reached within 15 min with either CAGG or CAAHA (data not shown). Alkylation yields with CAGG and CAAHA were determined as a function of added amounts of the anion and alkylating reagent (Fig. 1). In the presence of a sufficient amount of anion, the alkylation yield

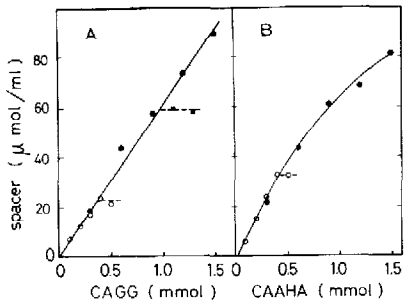

Fig. 1. Coupling of CAGG (A) and CAAHA (B) to Sepharose CL-4B as a function of the amounts of the alkylating reagents and methylsulfinyl carbanion. The procedure is described in the text. Amount of anion per 0.5 g of dried gel: ●, 5 mmol; ■, 3 mmmol; ○, 2 mmol.

was nearly proportional to the amount of the alkylating reagent added. Thus, highly substituted Sepharose can be easily obtained. This is very useful, especially for low affinity systems. Low levels of spacers content could be obtained by using smaller amounts of anion with similar yield.

Characterization of the Sepharose derivatives

Fig. 2 shows the titration curves for Sepharose–AGG and Sepharose–AAHA. The pK_a value of the ω-carboxyl group of Sepharose–AGG was about 1 unit lower than that of Sepharose–AAHA. After coupling with amines by the use of water-soluble carbodiimide, almost all the carboxyl groupos of the spacer had disappeared (Fig. 2). Thus, the affinity adsorbents prepared in this manner are free from undesirable ionic groups.

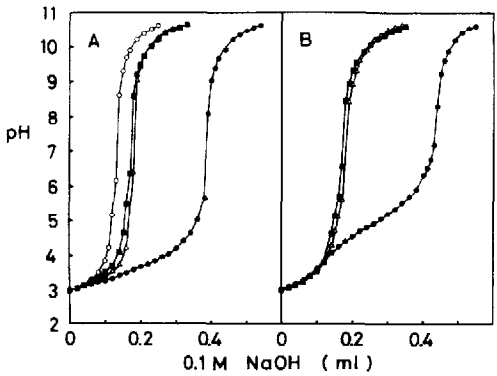

Fig. 2. Titration curves of the Sepharose derivatives containing the ω-carboxyl spacers. The Sepharose derivatives (1 g wet cake, corresponding to 1.1 ml settled volume) were suspended in 10 ml of 0.5 *M* NaCl. The pH was adjusted to 3 with 1 *M* HCl, then the gel was titrated with 0.1 *M* NaOH. (A) ○, Sepharose CL-4B; ●, Sepharose–AGG; ■, Sepharose–AGG–ABA; △, Sepharose–AGG–EA. (B) ●, Sepharose–AAHA; ■, Sepharose–AHHA–ABA; △, Sepharose AAHA–EA.

Purification of trypsin from Pronase by affinity chromatography on Sepharose–AGG–ABA

Hixson and Nishikawa[9] described the affinity chromatography of bovine pancreatic trypsin by the use of immobilized *p*-aminobenzamidine on agarose gel beads, in which spacer moieties were coupled to the beads by CNBr activation procedure. Some preparations of their affinity adsorbents showed the character of a cation exchanger due to incomplete coupling of the ligand to the spacer. According to these authors α-chymotrypsin or chymotrypsinogen are good test proteins for proving the absence of unmodified carboxyl groups of the spacer. In our affinity adsorbents, however, little carboxyl group was titrated as shown in Fig. 2.

The effectiveness of the Sepharose derivatives prepared by the present procedure was demonstrated by a purification of *Streptomyces griseus* trypsin from Pronase, a mixture of proteolytic enzymes produced by a strain of *Streptomyces*[10]. Trypsin was retained by a column of Sepharose–AGG–ABA and eluted by lowering the pH of the eluent (Fig. 3). The peak fraction containing trypsin was concentrated by Amicon PM-10 membrane filtration (Amicon, Lexington, MA, U.S.A.) and its activity was determined. Active-site titration using *p*-nitrophenyl *p'*-guanidinobenzoate[11] revealed the ratio of active site to enzyme to be 1.03 [based on a molar absorbance value at 280 nm of $3.7 \cdot 10^4\ M^{-1}\ cm^{-1}$ (ref. 12)]. Specific activity towards N^{α}-benzoyl-D,L-arginine *p*-nitroanilide (0.2 m*M*) was 8.1 μmol min^{-1} per A_{280} unit (0.05 *M* Tris–HCl, 0.02 *M* $CaCl_2$, pH 8.2, 25°C). This result indicates that the purity of this preparation is equal to that of the most highly purified preparation so far reported[12]. Essentially the same result was obtained with Sepharose–AAHA–ABA.

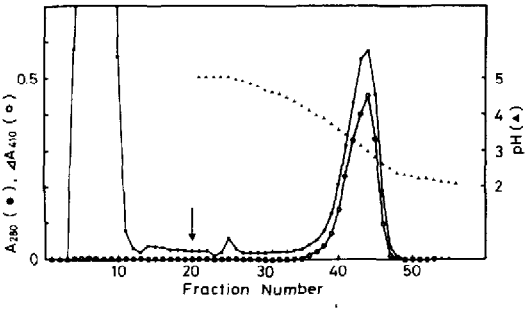

Fig. 3. Affinity chromatography of Pronase P on a column of Separose–AGG ABA. Chromatographic procedures are described in the text. Trypsin activity of each fraction was determined according to Nishikata *et al.*[13] on 200-fold diluted samples using 0.5 m*M* N^{α}-benzoyl-L-arginine *p*-nitroanilide as a substrate (pH 8.2, 10 min incubation) and the increase in absorbance at 410 nm was plotted (○). The arrow indicates the start of the pH gradient, and the pH of effluent fractions (3.2 ml each) was plotted (▲). Absorbance at 280 nm (●).

The mechanical rigidity of Sepharose beads was slightly lowered by the present procedure. However, this is not critical in usual laboratory use, and this method has many advantages, *e.g.* easy control of ligand content, attainability of high ligand concentration, and the absence of unwanted charges in the ligand–matrix linkage.

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