STABLE AND HIGH CAPACITY SEPHAROSE DERIVATIVES FOR AFFINITY CHROMATOGRAPHY

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1. Introduction

Affinity chromatography has recently been introduced as a method for purification of biologically active compounds [1]. The method depends on the affinity of a protein towards its specific hapten or inhibitor, covalently coupled to an insoluble matrix. In order to use this method successfully, the essential group for interaction with the molecules to be purified must be sufficiently distant from the polymer surface to minimise steric interference. Such a distance can be obtained by introducing a spacer molecule between the solid matrix and the molecule bound to it. Since the only good method available for binding various molecules to Sepharose is by its activation with cyanogen bromide [2], the spacer must contain a free amino group through which the binding is performed.

Two different approaches of introducing the spacer were used. One has been suggested by Cuatrecasas [3] using amino alkyl-Sepharose as a starting material. The amino derivative was then reacted with different functional groups, to which ligands were coupled and used for protein purification. All of these derivatives have strong ion-exchange properties due to incomplete coupling of the ligands to the amino or carboxyl groups of succinylated derivatives. An alternative approach has been suggested by us [4] for the preparation of the same derivatives, free of ion exchange properties. It involves reaction of aliphatic diamine compounds or ϵ-aminocaproic acid with the ligands, followed by coupling to Sepharose, as shown in the scheme below.

Since in both of these methods the spacer is coupled monovalently to Sepharose, these columns suffer from a small but constant leakage of the ligands. This phenomenon would limit the use of affinity chromatography for the isolation of very small amounts of proteins.

In this communication we describe the preparation of stable and high capacity Sepharose derivatives, achieved by coupling polylysine, polyornithine or polyvinylamine to Sepharose. The high stability results from the multipoint attachment of polylysine to Sepharose. The polylysyl Sepharose thus obtained can be used for further ligand substitution in a variety of ways.

$$NH_2-(CH_2)_X-NH_2 + Z-Cl \xrightarrow{NaOH} Z-NH_2-(CH_2)_X-NH-Z \xrightarrow[AcOH]{HCl}$$

$$Z-NH_2-(CH_2)_X-NH_3^+Cl^- + R-X \longrightarrow Z-NH_2(CH_2)_X-NH-R \xrightarrow[AcOH]{HBr} Br^+NH_3-(CH_2)_X-NHR + \text{Sepharose}$$

$$\xrightarrow{CNBr} \text{Sepharose}-NH-(CH_2)_X-NHR$$

Z = benzyloxycarbonyl

R = dinitrofluorobenzene, indole acetic acid, p-nitrobenzoic acid, p-hydroxymercury benzoate and bromoacetic acid, p-hydroxyphenylpropionic acid.

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2. Materials

Sepharose 4B was obtained from Pharmacia. Cyanogen bromide was purchased from Fluka AG. Polylysine and the *N*-carboxyanhydride of alanine were products of Miles-Yeda, Rehovot.

3. Methods and results

Sepharose (25 ml) was activated with cyanogen bromide (2.5 g), as previously described [1, 2]. The activated gel was washed with 0.1 M sodium bicarbonate and added to an equal volume of polylysine (1 g) in the same solvent. After 16 hr at 4°C the gel was washed with water, 0.1 N hydrochloric acid and again with bicarbonate and water. Polylysyl-Sepharoses, containing up to 30 mg of polylysine per ml of Sepharose were obtained (corresponding to about 250 μmoles of lysine). More than 90% of the lysines were available for further derivatization, as determined by reaction with fluorodinitrobenzene, followed by estimation of the ϵ-DNP-lysine formed after total hydrolysis. The amount of free amino groups were assayed by the Van-Slyke method for primary amino groups [5].

Ligands containing free carboxyl groups can be coupled directly to the polylysyl-Sepharose at pH 4.8 using a water soluble carbodiimide. Under these conditions not more than 25% of the available amino groups were blocked. Higher yields, of up to 80% were obtained if the coupling was performed in dioxane using dicyclohexylcarbodiimide or an active ester, such as *N*-hydroxysuccinimide esters. In order to achieve better coupling in aqueous solutions, the pK of the amino groups should be lowered. This was obtained by polymerization of D,L-alanine *N*-carboxyanhydride to the polylysyl-Sepharose in dioxane. Side chains of an average 8–10 alanyl residues per lysyl amino group, were obtained when 400 mg of the anhydride was added per ml of polylysyl-Sepharose. Alternatively the same derivative was also prepared directly by coupling multi-poly-D,L-alanine–polylysine to Sepharose. The polyalanine attached to polylysyl-Sepharose also serves as a very long spacer between the solid matrix and the ligand coupled to it. More than 50% coupling of ligands containing carboxyl groups to the multi-polyalanyl–polylysyl-Sepharose was achieved at pH 4.8 using a water soluble carbodiimide and an almost quantitative substitution using dioxane as solvent.

The polylysyl-Sepharose conjugate is remarkably stable. No leakage from polylysyl-Sepharose after reaction with fluorodinitrobenzene (DNP) or dansyl-chloride (DNS) was observed, even after storage for 3 months in a solution of sodium bicarbonate (pH 8) at room temp. No fluorescence due to DNS could be detected in the supernatant. On the other hand, ϵ-DNP lysine coupled directly to cyanogen bromide activated Sepharose, lost about 15% of the DNP groups during the same period of time.

The polylysyl-Sepharose and the multi-poly-D,L-alanyl–polylysyl-Sepharose were the basis for the preparation of other agarose derivatives, similar to those prepared from aminoethyl-Sepharose. The methods used for their preparation are essentially those described by Cuatrecasas [3].

4. Discussion

This study was initiated due to a suggestion by Tesser et al. [6] that the difficulties we encountered with a *c*AMP-Sepharose column [7] may be due to a leakage of *c*AMP from the column, similarly to a leakage they observed with a different *c*AMP-Sepharose column. They suggested that using a polyvalent spacer may alleviate the problem. We have prepared N^6-succinyl *c*AMP and coupled it to the multivalent polylysyl-Sepharose and have obtained the same results as previously described. A complete study on these *c*AMP columns will be described elsewhere.

The polylysine used in this study was either poly-D-lysine or poly-L-lysine. The poly D-lysine is preferred in cases where proteolytic enzymes are present. The alanine used was of the DL configuration to achieve structureless straight chains of polyalanine. The degree of polymerization of polylysine is of little importance, since polymers ranging from 100 to several thousand lysine residues were equally effective.

The polylysyl-Sepharose gel has one disadvantage in that not all the amino or the carboxyl groups in the succinylated derivatives can be completely blocked. In several cases residual amino groups can be blocked with an excess of acetic anhydride or by reaction with

nitrous acid. However, the numerous successful applications with monovalent-coupled derivatives, possessing charged groups, may indicate that this is not too serious a problem.

The efficiency of many of the derivatives prepared from polylysyl-Sepharose was checked by the purification of bovine pancreatic ribonuclease, staphylococcal nuclease, chymotrypsin and anti-DNP antibodies (unpublished results). The polylysyl-Sepharose should find wide application in the preparation of stable, high capacity chemisorbents for the immobilization of peptides and proteins and may be especially useful in the study of the interaction between hormones and intact cells.

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