A Novel Support with Artificially Created Recognition for the Selective Removal of Proteins and for Affinity Chromatography

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Summary

Acrylamide and N,N'-methylenebisacrylamide were copolymerized in the presence of a protein to form a gel which was pressed through a sieve. The gel particles obtained were packed into a chromatographic tube. The experimental conditions for the polymerization are such that the pores of the gel particles are large enough to permit the protein to diffuse out of the particles, so that the entrapped protein can be removed from the bed by washing with an aqueous solution. However, the interaction with the matrix is so strong that the protein can be desorbed only by a buffer containing 0.5 M sodium chloride or by a 10 % solution of acetic acid containing 10 % SDS. When a sample containing the protein present during the polymerization was applied to the column along with other proteins this protein was the only one adsorbed. The technique worked selectively with hemoglobin, cytochrome C and transferrin.

Introduction

Affinity chromatography is based on selective adsorption that occurs naturally in biological systems or can be artificially created. In the latter case the selectivity can be accomplished by imprinting a molecular template within the solid phase, i.e. ligands are introduced into a matrix in a spatial arrangement such that they bind specifically to functional groups in the target molecule. For recognition of proteins the ligands are often charged groups, such as amino groups, which can interact electrostatically with carboxyl groups in the protein [1–3].

In this paper we describe a very simple method for the preparation of a chromatographic bed designed for selective recognition of proteins. No ligands are introduced to create selectivity, so the recognition mechanism is uncertain. Until we understand the mechanism better we prefer not to give the method any particular name. However, we feel that the results obtained so far are sufficiently interesting to warrant publication.

Experimental

Materials

Bovine cytochrome C and bovine trypsinogen were obtained from Sigma (St. Louis, MO; USA). Human transferrin was purchased from KABI/Pharmacia (Stockholm, Sweden). Human hemoglobin from diabetic patients was obtained from the University Hospital (Uppsala, Sweden).

Acrylamide, N, N'-methylenebisacrylamide, ammonium persulfate, N, N, N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), a protein standard [equine myoglobin (1.0 mg mL^{-1}), ribonuclease A (4 mg mL^{-1}) and cytochrome C (1.0 mg mL^{-1})] were from Bio-Rad Laboratories (Hercules, CA, USA). A continuous bed column was disigned for cation-exchange chromatography [4]. This "CB-S HPLC column" had the length 20.3 mm and i.d. 7.9 mm.

The Preparation of the Affinity Bed

Cytochrome C (10 mg) was dissolved in 1 mL of deionized water. Acrylamide (57 mg), N,N'-methylenebisacrylamide (3 mg) and 10 µL of a 10 % (w/v) solution of ammonium persulfate were added. The solution, having a total monomer concentration (T) of 6 % (w/v) and a crosslinking concentration (C) of 5 % (w/w), was deaerated for 1 min before 10 µL of 5 % (v/v) TEMED was added. The polymerization proceeded overnight at room temperature. The gel was granulated by pressing it through a 100-mesh sieve and the particles obtained were then packed into a Pasteur pipette fitted with a

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glass wool plug at the constriction [5]. (It should be noted that water will not flow through the non-granulated gel.) A "hemoglobin column" and a "transferrin column" were prepared in a similar way.

Prior to the selectivity test (see below) all the affinity columns were washed with a 10 % (v/v) solution of acetic acid containing 10 % (w/v) SDS until the column was colorless, i.e. the embedded protein had been removed. If washing was incomplete, the selectivity decreased.

Test for selectivity of the bed

About 50 µl of the protein standard was applied following equilibration of the column with 10 mL of 0.01 M sodium phosphate (pH 6.2). The proteins were eluted with the same buffer. The non-adsorbed proteins were collected in a 1 mL fraction and analyzed by high-performance cation-exchange chromatography (see next section). The selectivity of the "hemoglobin column" and the "transferrin column" were tested in the same way.

None of the proteins were adsorbed when the polyacrylamide gel was exchanged for a 1 or 6 % (w/v) low-melting agarose gel.

Analysis by cation-exchange chromatography of the non-adsorbed proteins in the eluates from the affinity columns

The CB-S HPLC column was equilibrated with 0.01 sodium phosphate (pH 6.2). About 75 µl of the fraction containing the non-adsorbed proteins from each of the affinity columns was applied to the CB-S column. The chromatogram was developed with a 5 mL linear gradient from 0 to 0.5 M sodium chloride. Detection was done at 220 nm. Any protein adsorbed specifically on the affinity column will not appear in the chromatogram.

Desorption of specifically adsorbed myoglobin, hemoglobin, cytochrome C and transferrin

100 µL of cytochrome C (5 mg mL^{-1}) was applied to the "cytochrome C" column equilibrated with 6 mL of 0.01 M sodium phosphate (pH 6.2). For elution we used 6 mL of each of the following three solutions: the equilibration buffer, the equilibration buffer containing 0.5 M sodium chloride, and a 10 % (v/v) solution of acetic acid containing 10 % (w/v) SDS. 0.5 mL fractions were collected and analyzed for cytochrome C at 415 nm. The experiment was then repeated on the "hemoglobin column" with hemoglobin and myoglobin as samples. Only cytochrome C could be eluted with 0.01 M sodium phosphate (pH 6.2) containing 0.5 M sodium chloride. The other two proteins (hemoglobin and myoglobin) required the HAc/SDS solution for desorption. Transferrin could only be desorbed by this detergent solution.

Results and Discussion

Many reactions in biological systems take place with great specificity, for instance that between antigens and antibodies. The bonds involved are of the same kind as in non-living systems (electrostatic, hydrophobic, etc.). Therefore, the specificity must be based on correct distance between the reacting groups. For instance, a target protein reacting with its receptor has a spatial arrangement of some negative, positive and hydrophobic groups, etc. in the protein molecule which fits specifically certain positive, negative and hydrophobic groups, etc. in the receptor molecule. Artificial recognition systems are designed according to the same principle [1–3].

Polyacrylamide gel matrices are known to be very inert and biocompatible [5, 6] and significant interactions with proteins are not to be expected. In fact, a gel column prepared in the absence of a protein did not adsorb any of the proteins used in this study (compare chromatograms a and b in Figure 1). However, upon polymerization in the presence of a protein and subsequent removal of the protein from the gel formed, the gel had acquired a "memory" for selective interaction with that protein. We need additional experimental data to be able to formulate a more detailed hypothesis about the recognition mechanism and will not discuss it further here.

Figure 1c (for additional experimental details see the legend) shows that a gel prepared in the presence of cytochrome C shows a specific interaction with this protein following removal of the embedded cytochrome C molecules.

From Figure 2b it is evident that a column with a specific adsorption of hemoglobin can be designed in a similar way. Myoglobin, which has a structure similar to that of hemoglobin, also showed selective interaction with this column.

The selectively adsorbed hemoglobin could not even be desorbed at high ionic strength (0.01 M sodium phosphate, pH 6.2, containing 0.5 M sodium chloride) indicating that the adsorption was not caused by electrostatic interaction. Desorption required severe conditions with an eluent of 10 % (v/v) acetic acid containing 10 % SDS. The interaction is, accordingly very specific.

We have also prepared a "transferrin column" and adsorbed selectively human transferrin (not shown herein).

The first "hemoglobin columns" were prepared as described above but with the difference that we also tried to incorporate acrylic acid groups in the gel at sites corresponding to the position of the amino groups in the hemoglobin molecule. The adsorption of hemoglobin to this bed was weaker than that to the bed used in the experiment presented in Figure 2, indicating that the adsorption is not due to traces of charged groups in the polyacrylamide gel bed.

The chromatographic behavior of transferrin on a "transferrin column" was very similar to that of

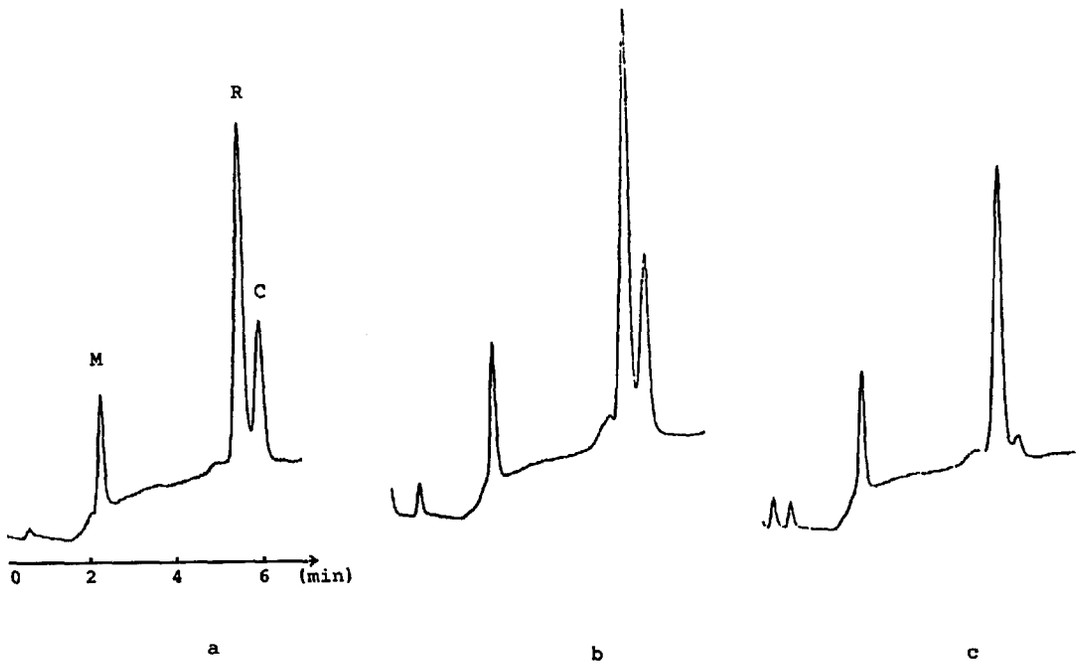

Figure 1

Ion-exchange chromatograms to test the selectivity for cytochrome C of a gel bed prepared by polymerization in the presence of this protein. The sample consisted of myoglobin (M), ribonuclease (R) and cytochrome C (C). This sample was applied to a high-performance cation-exchanger (see the chromatogram in Figure 1a) and to two polyacrylamide beds, one prepared in the absence (blank) and one in the presence of cytochrome C. The non-adsorbed proteins from the two polyacrylamide beds were analyzed on the same ion-exchange column under the same experimental conditions and gave the chromatograms (b) and (c), respectively. A comparison between (c) and the blank in (b) shows that cytochrome C was adsorbed only to the bed synthesized in the presence of cytochrome C (the cytochrome C peak is much smaller). The similarity between chromatograms (a) and (b) indicates that no proteins were adsorbed onto the blank column (the matrix itself).

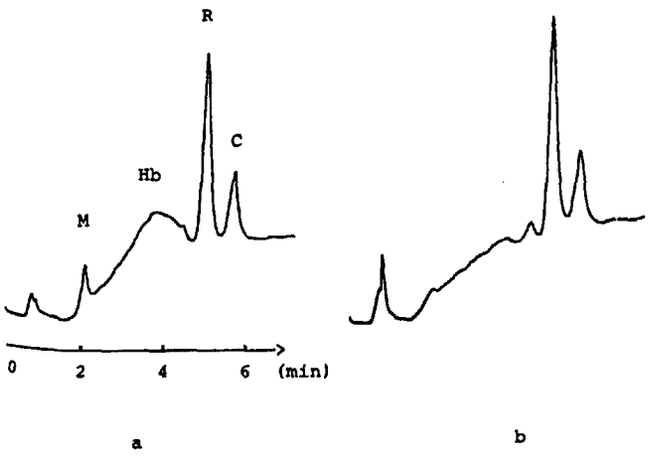

Figure 2

Ion-exchange chromatograms to test the selectivity for hemoglobin of a gel bed, prepared by polymerization in the presence of hemoglobin. The experimental conditions were the same as in Figure 1, with the exceptions that the sample also contained hemoglobin (Hb) and that the gel bed prepared by polymerization in the presence of cytochrome C was replaced by a bed prepared in the presence of hemoglobin. Chromatogram (a) was obtained when the original sample was analyzed by ion-exchange chromatography. Chromatogram (b) corresponds to a similar analysis of the non-adsorbed protein fraction from the "hemoglobin column". The absence of the hemoglobin peak in (b) shows that hemoglobin had become bound specifically to the bed synthesized in the presence of this protein.

cytochrome C on a "cytochrome C column" and a "horse myoglobin column" adsorbed horse myoglobin but not whale myoglobin (the chromatograms are not presented herein).

Conclusions

Although much remains to be done in order to clarify the mechanism of selectivity of the beds described and to find the optimal experimental conditions for adsorption and desorption, it is obvious that the beds exhibit an unusually high specificity which can be utilized not only for chromatographic purification, but also for specific extraction and removal of target substances. A practical advantage of these beds is that they are easily prepared.

The very strong and specific interaction between proteins and the gel bed has some similarities to that between antigens and antibodies and may, possibly, be utilized for clinical purposes. The application range will be still broader if beds can be designed for specific removal of other substances and particles, such as viruses and bacteria.

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