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# Hydrophobic Chromatography: Use for Purification of Glycogen Synthetase

**(protein purification/ω-aminoalkyl-agaroses/glycogen phosphorylase/lipophilic membrane proteins/affinity chromatography)**

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**ABSTRACT** **A homologous series of ω-aminoalkyl-agaroses [Sepharose-NH$(CH_2)_nNH_2$] that varied in the length of their hydrocarbon side chains was synthesized. This family of agaroses was used for a new type of chromatography, in which retention of proteins is achieved mainly through lipophilic interactions between the hydrocarbon side chains on the agarose and accessible hydrophobic pockets in the protein.**

**When an extract of rabbit muscle was subjected to chromatography on these modified agaroses, the columns with short "arms" ($n$ = 2 and $n$ = 3) excluded glycogen synthetase (EC 2.4.1.11), but the enzyme was retained on δ-aminobutyl-agarose ($n$ = 4), from which it could be eluted with a linear NaCl gradient. Higher members of this series (e.g., $n$ = 6) bind the synthetase so tightly that it can be eluted only in a denatured form. A column of δ-aminobutyl-agarose, which retained the synthetase, excluded glycogen phosphorylase (EC 2.4.1.1), which in this column series and under the same conditions requires side chains 5-(or 6)-carbon-atoms long for retention. Therefore, it is possible to isolate glycogen synthetase by passage of muscle extract through δ-aminobutyl-agarose, then to extract phosphorylase by subjecting the excluded proteins to chromatography on ω-aminohexyl-agarose ($n$ = 6). On a preparative scale, the synthetase (I form) was purified 25- to 50-fold in one step.**

**This paper describes some basic features and potential uses of hydrophobic chromatography. The relevance of the results presented here to the design and use of affinity chromatography columns is discussed.**

Classical procedures for the separation of proteins are usually based on differences in their solubility, charge, size, and shape. We have recently shown (1) that proteins can be separated also on the basis of difference in the size of their available hydrophobic pockets. This approach was illustrated in the case of glycogen phosphorylase (EC 2.4.1.1): a homologous series of hydrocarbon-coated agaroses that varied in the length of their alkyl side chains [Sepharose-NH-$(CH_2)_nH$] differed remarkably in their capacity to bind the enzyme—passing from no retention ($n$ = 1), through retardation ($n$ = 3), to reversible binding ($n$ = 4), up to very tight binding ($n$ = 6)—as the hydrocarbon chain was gradually lengthened. Under the specific conditions used for the isolation of phosphorylase from crude muscle extract, very few other proteins were reversibly retained by the butyl-agarose column. This procedure afforded up to a 100-fold purification of the enzyme in one step (1).

Further study of such agarose derivatives, and of similar derivatives that contained on their hydrocarbon side chains additional functional groups, has shown that the case of phosphorylase is not unique, and that this type of chromatography is widely applicable.

We describe here the synthesis of a homologous series of ω-aminoalkyl-agaroses, illustrate their use in the purification of glycogen synthetase (EC 2.4.1.11), and glycogen phosphorylase, discuss some of the features of this new type of chromatography, and point out several of its potential uses.

Abbreviations: Seph-$C_n$-$NH_2$, Sepharose 4B activated with CNBr and reacted with an α,ω-diaminoalkane $n$-carbon-atoms long (see also Fig. 1).

## MATERIALS AND METHODS

*Synthesis of ω-Aminoalkyl-Agaroses.* Sepharose 4B (Pharmacia) was activated at pH 10.5–11 and 22° by addition of 1 g of CNBr to 10 g(wet weight) of Sepharose (2, 3). The reaction was allowed to proceed for 8 min and the pH was maintained between 10.5 and 11 by addition of 5 N NaOH. Activation was terminated by filtration and washing of the gel with ice-cold deionized water. The activated Sepharose was suspended in cold 0.1 M $NaHCO_3$ (pH 9) (about twice the settled volume of the gel) and mixed with an equal volume of water containing 4 mol of the appropriate α,ω-diaminoalkane [$NH_2(CH_2)_nNH_2$] per mol of CNBr used for the activation of the Sepharose. The pH of the diamine was adjusted to 9 (with 6 N HCl) before mixture with the activated Sepharose. Coupling was performed at 4° for 24 hr, while the reaction mixture was gently swirled. Subsequently, the ω-aminoalkyl-agarose was washed with water, 0.1 M $NaHCO_3$, 0.05 M NaOH, water, 0.1 M $CH_3COOH$, then water again. Finally, the columns were equilibrated with a buffer composed of 50 mM β-glycerophosphate–50 mM 2-mercaptoethanol–1 mM EDTA (pH 7). All the agarose derivatives thus prepared were identical in the number of their amino groups per unit weight, as determined by reaction with 2,4,6-trinitrobenzenesulfonate (4).

*Preparation of Rabbit Muscle Extract.* Minced skeletal muscle from rabbit (1 kg) was suspended in 4 liters of 50 mM Tris·HCl–50 mM EDTA (pH 8.1) and homogenized in a large-capacity Waring Blendor for 1 min. The homogenate was then centrifuged at 5860 × $g$ and the supernatant was filtered through glass wool. An equal volume of neutralized saturated $(NH_4)_2SO_4$ solution was added and, after 24 hr at 4°, the supernatant was decanted. The settled precipitate was then centrifuged for 20 min at 16,300 × $g$. The resulting pellet was suspended (and partially dissolved) in twice its volume of 50 mM β-glycerophosphate–50 mM 2-mercaptoethanol–1 mM EDTA (pH 7). The suspension was dialyzed twice against 20 times its volume of the β-glycerophosphate buffer, then centrifuged at 35,000 × $g$ for 3 hr. The resulting partially clarified solution

contained 70% of the glycogen synthetase activity and 40% of the glycogen phosphorylase activity found in the glass-wool filtrate before precipitation with $(NH_4)_2SO_4$.

*Assay of Enzymatic Activities.* Glycogen synthetase was assayed in the presence and in the absence of glucose 6-phosphate, as described by Soderling *et al.* (5). One unit of activity is defined as the amount of enzyme that catalyzed (under the standard assay conditions) the incorporation of 1 μmol of glucose per min from UDP-glucose into glycogen.

Glycogen phosphorylase was assayed in the presence of AMP by the method of Hedrick and Fischer (6). One unit of activity is defined as the amount of enzyme causing the release of 1 μmol of inorganic phosphate from glucose-1-phosphate per min.

*Acrylamide Gel Electrophoresis.* The electrophoresis was performed with 5% gels in the presence of 0.1% sodium dodecyl sulfate–10 mM 2-mercaptoethanol (pH 7.2) as described by Dudai *et al.* (7).

*Protein Concentrations* were determined by the Biuret method (8).

## RESULTS AND DISCUSSION

*Elution Patterns of Muscle Extract on ω-Aminoalkyl-Agaroses.* A homologous series of ω-aminoalkyl-agaroses, which varied in the length of their ω-aminoalkyl side chains (Seph-$C_n$-$NH_2$, Fig. 1), was synthesized by activation of Sepharose 4B with CNBr (2, 3) and reaction with the appropriate α,ω-diamino-alkane. A large excess of the diamine was used (4 mol/mol of CNBr used for activation) to reduce the probability of crosslinking. Rabbit muscle extract was passed through short columns of each of these agarose derivatives. The emerging fractions were assayed for glycogen phosphorylase and glycogen synthetase, and their absorption at 280 nm was monitored. As seen in Fig. 2, both the synthetase and the phosphorylase were excluded from Seph-$C_2$-$NH_2$ and from Seph-$C_3$-$NH_2$. However, while glycogen phosphorylase was excluded also from Seph-$C_4$-$NH_2$, glycogen synthetase was retained by this column, from which it could be eluted by a linear NaCl gradient (Fig. 2, *III*). Higher members of this homologous series of agarose derivatives

| Abbreviation | Structure |
|---|---|
| Seph-$C_2$-$NH_2$ | -NH-$CH_2$-$CH_2$-$NH_2$ |
| Seph-$C_3$-$NH_2$ | -NH-$CH_2$-$CH_2$-$CH_2$-$NH_2$ |
| Seph-$C_4$-$NH_2$ | -NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$NH_2$ |
| Seph-$C_5$-$NH_2$ | -NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$NH_2$ |
| Seph-$C_6$-$NH_2$ | -NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$NH_2$ |
| Seph-$C_8$-$NH_2$ | -NH-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$NH_2$ |

FIG. 1. Structure of ω-aminoalkyl agaroses used in this work.

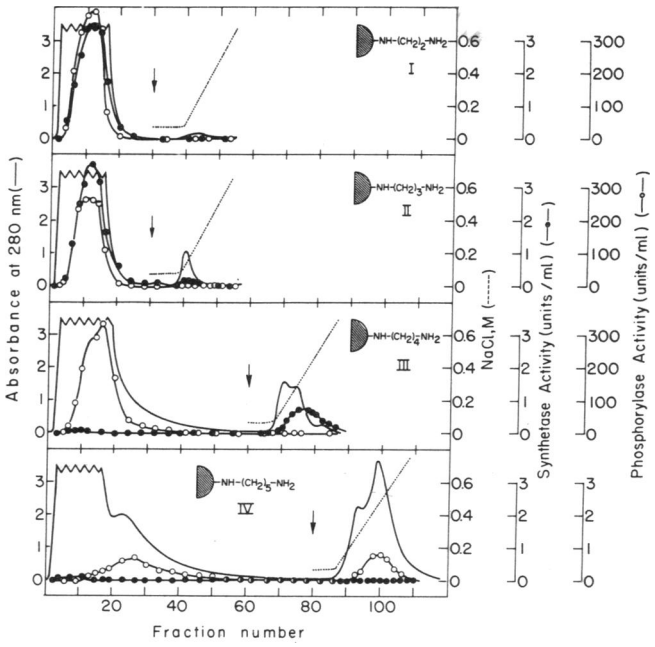


FIG. 2. Preferential adsorption of glycogen synthetase and glycogen phosphorylase on ω-aminoalkyl-agaroses that vary in the length of their hydrocarbon chains. 10 ml of muscle extract (protein concentration: 26 mg/ml) were applied on each of four ω-aminoalkyl-agarose columns (8 × 0.9 cm) equilibrated at 22° with 50 mM β-glycerophosphate–50 mM 2-mercaptoethanol–1 mM EDTA (pH 7). Unadsorbed protein was washed off until the absorbance at 280 nm (——) dropped below 0.05, then a linear NaCl gradient (in the same buffer) was applied. 1.7-ml Fractions were collected and their synthetase (●——●), as well as their phosphorylase (○——○), activities were monitored. The concentration of NaCl in the various fractions (. . . .) was determined by conductivity measurements, by use of a calibration curve.

($n = 5$–8) bound the synthetase so tightly that we have only been able to elute it in a denatured form.

It should be noted that in the ω-aminoalkyl series, glycogen phosphorylase is not retained on columns having side chains less than 5-carbon-atoms long. Therefore, it is possible to isolate the synthetase by passage of crude muscle extract through Seph-$C_4$-$NH_2$, then to extract phosphorylase by subjecting the excluded proteins to chromatography on Seph-$C_6$-$NH_2$.

*Preparative-Scale Purification of Glycogen Synthetase (I Form).* Fig. 3 depicts the purification of glycogen synthetase on a preparative scale. 70 ml of muscle extract containing 1750 mg of protein and a synthetase activity of 0.2 units/mg were applied on a short column (10 × 2.4 cm) of Seph-$C_4$-$NH_2$. The column excluded a large amount of protein, including glycogen phosphorylase, but no synthetase activity was detected in the excluded fractions. Upon application of a NaCl gradient, glycogen synthetase was eluted (Fig. 3). The specific activity of the synthetase obtained in this experiment was 5 units/mg, representing a 25-fold purification in one step. In another experiment, starting with muscle extract that had a synthetase activity of only 0.09 units/mg, a 48-fold purification was achieved. The synthetase prepared by the procedure described above is not homogeneous on acrylamide gels in the presence of sodium dodecyl sulfate and

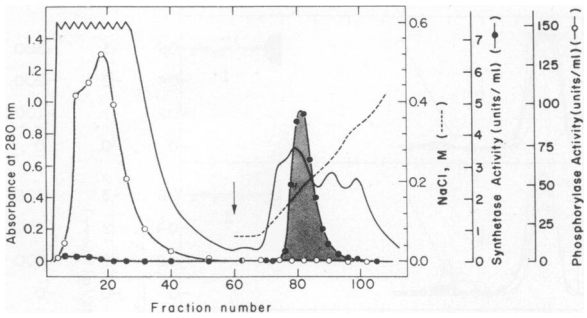


FIG. 3. Preparative-scale purification of glycogen synthetase from muscle extract on δ-aminobutyl-agarose (Seph-$C_4$-$NH_2$). 70 ml of the extract containing 1750 mg of protein and a synthetase activity of 0.2 units/mg of protein were applied on a Seph-$C_4$-$NH_2$ column (10 × 2.4 cm) equilibrated at 22° with the β-glycerophosphate buffer described in the legend to Fig. 2. After the unadsorbed protein [monitored by the absorbance at 280 nm (——)] was washed off, a linear NaCl gradient (in the same buffer) was applied. Note that the gradient in this case is less steep than the one used in Fig. 2. 7-ml Fractions were collected and their synthetase (●——●), as well as their phosphorylase (○——○), activities were monitored. The concentration of NaCl (– – –) was determined as described in Fig. 2.

2-mercaptoethanol (Fig. 4). However, by preparing a muscle extract as described, then subjecting it to chromatography on Seph-$C_4NH_2$, a purification of 350-fold was achieved, with 50% yield of activity.

*Types of Interactions Involved.* The members of the homologous series of columns described in this work are similar in structure and contain the same number of charges (amino groups) per unit weight. Yet, under identical conditions of pH, ionic strength, buffer composition, and temperature they differ remarkably in their capacity to bind glycogen synthetase (or glycogen phosphorylase). This result excludes the possibility that we are dealing here merely with ion-exchange chromatography. The above mentioned columns do differ in the length of their hydrocarbon side chains, onto which the amino groups are bound, and their capacity to retain the synthetase depends on the length of these "arms"—passing from no retention, through reversible binding, up to very tight binding—as the length of the hydrocarbon "arms" grows from 2 to 4 and to 6 carbon atoms.

It seems plausible to assume that the retention power of these columns involves hydrophobic interactions between the hydrocarbon "arms" and accessible hydrophobic pockets or regions of the enzyme. The fact that a similar gradation in the retention power was also observed with a homologous series of alkyl agaroses (1) further supports this suggestion.

We would like to emphasize that ionic interactions also affect the elution pattern of these columns. For example, while in the alkyl agarose series a side chain 4-carbon-atoms long sufficed to retain glycogen phosphorylase (1), a 6-carbon-atom side chain was needed to retain this enzyme in the ω-aminoalkyl-agarose series. Nevertheless, the results presented above suggest that hydrophobic interactions must contribute dominantly to the retention and discrimination power of these columns. Therefore, we propose to call this separation method "hydrophobic chromatography."

*Optimizing the Resolution Power for a Specific Protein.* One of the advantages of the above-mentioned family of columns is that they constitute a homologous series, in which each member has hydrophobic side chains one-carbon-atom longer than the preceding one. In fact, they contain "hydrophobic yardsticks" of different lengths. Thus, it becomes possible to optimize the isolation of a protein by choosing the member in the series that will be the most efficient for the purpose.

The separation possibilities are greatly amplified by the synthesis of other families of hydrocarbon-coated agaroses that have, on their hydrocarbon side chain, one or more functional groups, such as amines, carboxyls, hydroxyls, imidazoles, phenolic groups, etc. The resulting columns will derive their discriminating power from hydrophobic, ionic, and perhaps other types of interactions. However, it will be possible to systematically increase the contribution of hydrophobic interactions within the series to achieve optimal resolution of a specific protein.

Other variables that can be adjusted during hydrophobic chromatography involve loading and eluting conditions, such as ionic strength, buffer composition, pH, temperature, addition of a small amount of an organic solvent, etc. For example, in the present work we have shown that as the ionic strength is gradually increased, glycogen synthetase will not bind to Seph-$C_4$-$NH_2$; thus, it can be eluted from this column by application of a NaCl gradient. In a recent publication, we have shown (1) that the binding of glycogen phosphorylase to butyl-Sepharose is greatly diminished in the presence of

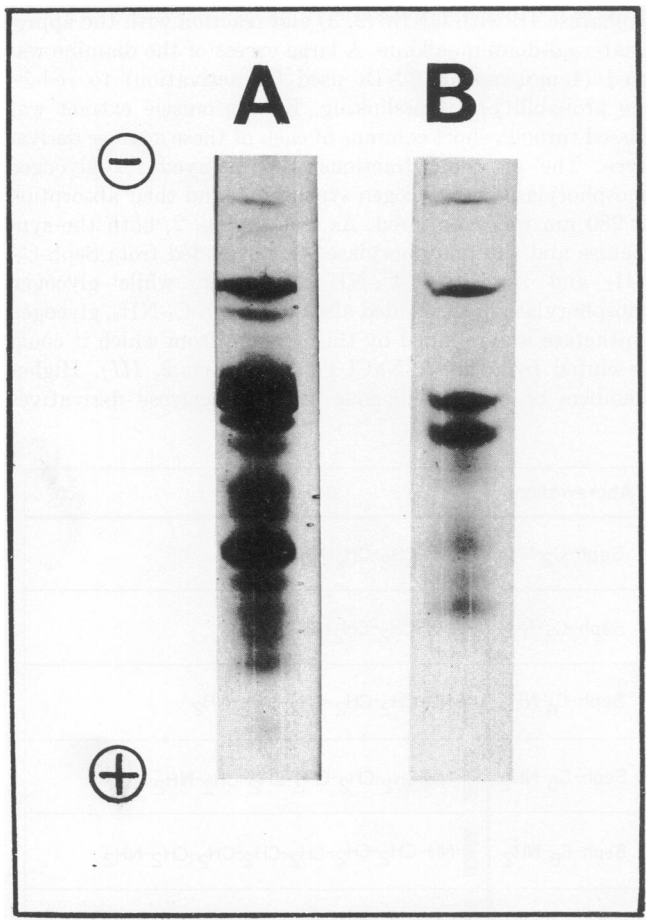


FIG. 4. Acrylamide gel electrophoresis of (*A*) crude muscle extract (25 μg of protein) and (*B*) glycogen synthetase purified on Seph-$C_4$-$NH_2$ (12 μg of protein).

an imidazole–citrate buffer (pH 7). This effect had to be attributed to specific ion effects, since a buffered NaCl solution of the same pH and ionic strength did not elute the enzyme from this column. Phosphorylase could also be eluted from butyl-Sepharose by lowering the pH to about 5.5 (Er-el and Shaltiel, in preparation).

Thus, when reporting the use of hydrophobic chromatography columns, it is important to specify the exact loading and eluting conditions (pH, ionic strength, buffer composition, temperature). A change in one of these variables may determine whether a protein will be retained or excluded by a specific member of the homologous series.

*Relevance to Affinity Chromatography.* Introduction of hydrocarbon extensions ("arms") between ligand molecules and agarose beads has been recently recommended (4, 9, 10) and extensively used in affinity chromatography (9–16). The usefulness of these arms was attributed to a relief of steric restrictions imposed by the matrix backbone and to an increased flexibility and mobility of the ligand when it protrudes further into the solvent (4, 10). In some instances, this may indeed be the case. However, our results suggest that hydrocarbon extensions may themselves contribute to the binding of proteins through hydrophobic interactions, i.e., through a mechanism that does not involve specific recognition of the ligand. Such hydrophobic interactions are more liable to occur when the columns are prepared by first coating the beads with hydrocarbon arms and then attaching the specific ligand to those extensions (4, 10). In this case, some of the arms may remain ligand-free, bind other proteins through hydrophobic interactions, and thus interfere with the specificity of the column.

It seems advisable, therefore, to use the alternative method of column preparation, which involves synthesizing first a ligand with a side chain and then attaching the elongated ligand to the agarose beads (4, 10). This procedure will minimize the probability of performing unintentional hydrophobic chromatography when affinity chromatography is intended. Furthermore, elution with a substrate or inhibitor is preferable, in order to maintain specificity also in the elution step. In other cases, control columns with ligand-free arms should be run to evaluate the contribution of hydrophobic interactions to the retention capacity of affinity chromatography columns.

*Potential Uses of Hydrophobic Chromatography.* The hydrophobic columns described here can be used repeatedly: they can withstand washing with 1 M NaOH and 1 M HCl and can be stored for months at 4° in aqueous suspension containing bacteriostatic agents (e.g., 0.02% sodium azide). The columns have a high binding capacity (see the experiment described in Fig. 3) and high flow rates (1–3 ml/min). Their main advantage lies in the fact that they can be tailored to meet the requirements of specific proteins.

So far we have reported the use of hydrophobic chromatography for the purification of two enzymes: glycogen phosphorylase (1) and glycogen synthetase. Yon (17) has recently used ω-aminodecyl agarose in the purification of aspartate carbamoyltransferase (EC 2.1.3.2) from wheat germ, achieving an 8-fold purification of the enzyme in one step. Work in progress in our laboratory indicates that hydrophobic chromatography is widely applicable to protein purification, as will be shown in forthcoming publications.

An important potential use of these columns may be in the purification and study of lipophilic, membrane-bound proteins, which most likely have available hydrophobic regions used in the hydrophobic interactions within the membrane. Hydrocarbon-coated agaroses may provide various carriers with increasing hydrophobic character that could be useful for assembly of membrane-bound proteins in reconstitution experiments.

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