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HYDROPHOBIC INTERACTION CHROMATOGRAPHY

THE SYNTHESIS AND THE USE OF SOME ALKYL AND ARYL DERIVATIVES OF AGAROSE

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SUMMARY

Aliphatic and aromatic alcohols in the form of glycidyl ethers have been coupled to agarose gels. These neutral agarose derivatives, which thus contain hydrophobic substituents, have been used as adsorbents in hydrophobic interaction chromatography. The coupling yield and the degree of substitution have been determined for one aliphatic and one aromatic model substance.

Different fractionation problems require different degrees of hydrophobicity of the substituents. To "tailor make" gels, the hydrophobicity can be varied in small steps by the use of aliphatic alcohols of different chain length.

The agarose derivatives described have been used for the purification of proteins, demonstrated with a plasma fractionation, viruses (STNV) and even whole cells (baker's yeast). Under suitable experimental conditions, the interactions can be very mild (enzyme activities have been recovered in a 50–100% yield).

Enzyme reactors with a high capacity can be prepared in a simple manner by applying the enzyme solution at any pH on to a suitable hydrophobically interacting bed. As the enzymes are not covalently linked to the bed, they can easily be recovered in the free form.

Contrary to ion-exchange chromatography, the adsorption in hydrophobic interaction chromatography decreases with a decrease in ionic strength and temperature.

INTRODUCTION

Previously, we have demonstrated the usefulness of hydrophobic interaction chromatography for the fractionation of biological material and discussed some general

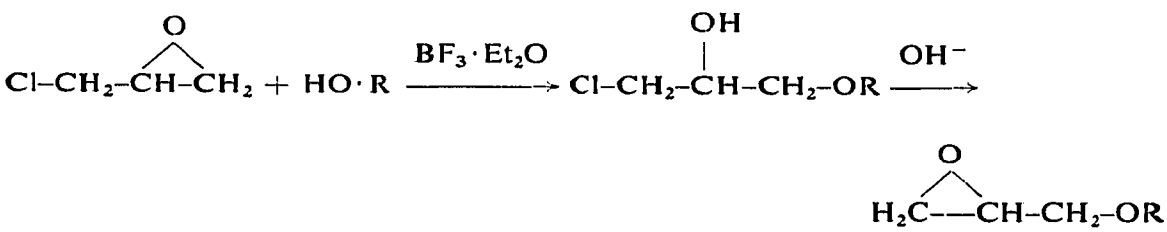
aspects of the method[1,2]; other workers who have used this technique were also referred to[2]. We also pointed out that aliphatic and aromatic amines coupled to Sepharose® by the cyanogen bromide reaction give rise to a positively charged matrix. Such gel materials therefore exhibit ion-exchange properties superimposed upon the hydrophobic character. The observations that many proteins can be desorbed from these beds by increasing the ionic strength of the buffer and that acidic proteins are preferentially adsorbed indicate the involvement of electrostatic interactions[3] (hydrophobic interactions should, in contrast, increase with increasing ionic strength[2]).

In order to increase the contribution of hydrophobic interaction, electrostatic interactions must be minimized. Therefore, we have prepared neutral adsorbents by coupling alcohols instead of amines to Sepharose, as previously reported[2]. The synthesis and some properties of these beds are described in this paper, as well as some typical areas of application. In this context, we will mention that Porath *et al.*[4] have described the preparation of a benzyl ether derivative of agarose to be used for hydrophobic adsorption.

PREPARATION OF ALKYL AND ARYL DERIVATIVES OF AGAROSE

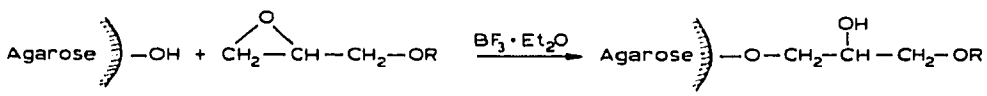
Synthesis of glycidyl ethers

Glycidyl ethers were prepared from alcohols and epichlorohydrin in the presence of boron trifluoride ethyl etherate as a catalyst, as described by Ulbrich *et al.*[5]:



Coupling with glycidyl ethers

The following reaction scheme shows the principle of the coupling method:



The agarose derivative obtained is denoted throughout the paper according to the R substituent (R is alkyl or aryl). A pentyl-Sepharose, for instance, was accordingly prepared by coupling the glycidyl ether, synthesized from *n*-pentanol, to Sepharose (an agarose gel in bead form obtained from Pharmacia, Uppsala, Sweden).

This coupling method was introduced by Ellingboe *et al.*[6] as a general method for the attachment of epoxides to polysaccharide matrices. They started by preparing a hydroxypropyl derivative of the polysaccharide in order to obtain a polymer that would swell in organic solvents, which considerably facilitates the substitution with epoxides. Their method can be simplified considerably by omitting the step involving attachment of hydroxypropyl groups, as agarose gels can be made to swell in most organic solvents by successive washings of the gel with a series of solvents of decreas-

ing polarity. These washings can be performed according to one of the following two procedures.

(*i*) A 100-ml volume of sedimented agarose gel was transferred to a glass filter-funnel and mixed with 100-ml portions of the following solvents:

(1) twice with acetone–water (1:2) (dehydration[7]);
(2) twice with acetone–water (2:1);
(3) 10 times with acetone;
(4) twice with acetone–1,2-dichloroethane (2:1);
(5) twice with acetone–1,2-dichloroethane (1:2);
(6) 10 times with 1,2-dichloroethane.

Each new suspension of gel was stirred continuously for a few minutes and the solvent was then carefully aspirated off so as to avoid the formation of aggregates of gel beads in the filter cake.

(*ii*) The following washings can be used as an alternative to those in procedure (*i*):

(1) once with water–dioxane (4:1);
(2) once with water–dioxane (3:2);
(3) once with water–dioxane (2:3);
(4) once with water–dioxane (1:4);
(5) 7 times with dioxan.

After exchanging the water in the agarose with an organic solvent according to procedure (*i*) or (*ii*), the coupling with the glycidyl ether was performed as follows. The gel was transferred to a round-bottomed reaction vessel equipped with a stirrer, and 100 ml of 1,2-dichloroethane or dioxane and 2 ml of a 48% solution of boron trifluoride etherate in diethyl ether were added. The mixture was stirred for 5 min, then a mixture of 1 ml of glycidyl ether and 10 ml of 1,2-dichloroethane or dioxane was added dropwise from a separating funnel. The reaction mixture was stirred for 40 min at room temperature. After the reaction, the gel was washed according to procedure (*i*) or (*ii*), but in the reverse order, and finally with water.

The agarose derivative used for the experiments described below were prepared from non-cross-linked agarose, but the above coupling procedure also works well with cross-linked agarose [8,13].

Determination of the degree of substitution and the coupling yield

Six 25-ml portions of well sedimented cross-linked Sepharose 4B were treated as described in procedure (*i*), the final volume of each gel suspension being 50 ml. The reactions were carried out in six erlenmeyer flasks with magnetic stirring. A 0.5-ml volume of boron trifluoride etherate was added to each flask as catalyst, followed by epichlorohydrin (0–0.27 mmole) dissolved in 1,2-dichloroethane. The experimental conditions for the coupling procedure were exactly as described above. After completion of the reaction, the gels were washed well on a glass filter with 1,2-dichloroethane, acetone, water and acetone and finally dried in a vacuum oven. The samples were weighed and then analyzed for chlorine and the degree of substitution, expressed as moles of epoxide per mole of galactose, was calculated (agarose is a polygalactose). No correction was made for the cross-links in the gel. The coupling yield, *i.e.*, the ratio between the amount of epoxide coupled and the amount added, was also calculated. The experiment was then repeated and the average values ob-

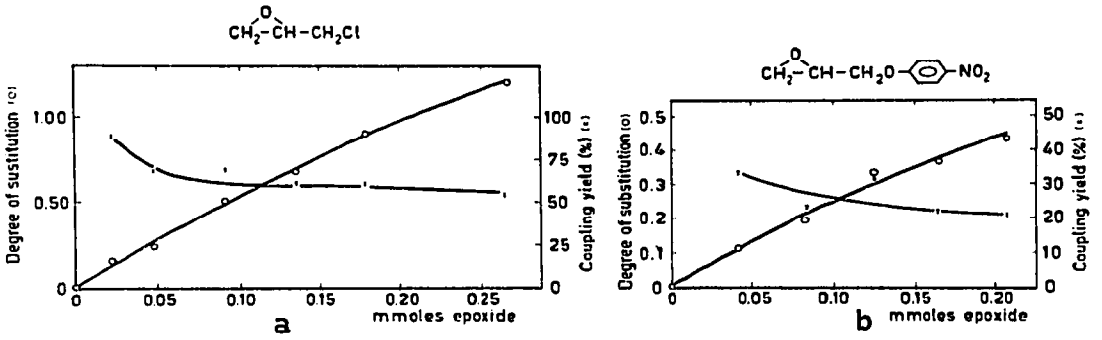


Fig. 1. Degree of substitution (moles of substituent per mole of galactose) and coupling yield as a function of the amount of epoxide added. (a) Epichlorohydrin; (b) 1,2-epoxy-3-(*p*-nitrophenoxy)-propane.

tained were plotted against the number of millimoles of epichlorohydrin added (Fig. 1a). A similar set of experiments was also performed for 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (CH_2–CH–CH_2O–C_6H_4–NO_2), analyzed for nitrogen; see Fig. 1b (this epoxide was synthesized according to Marle[9]).

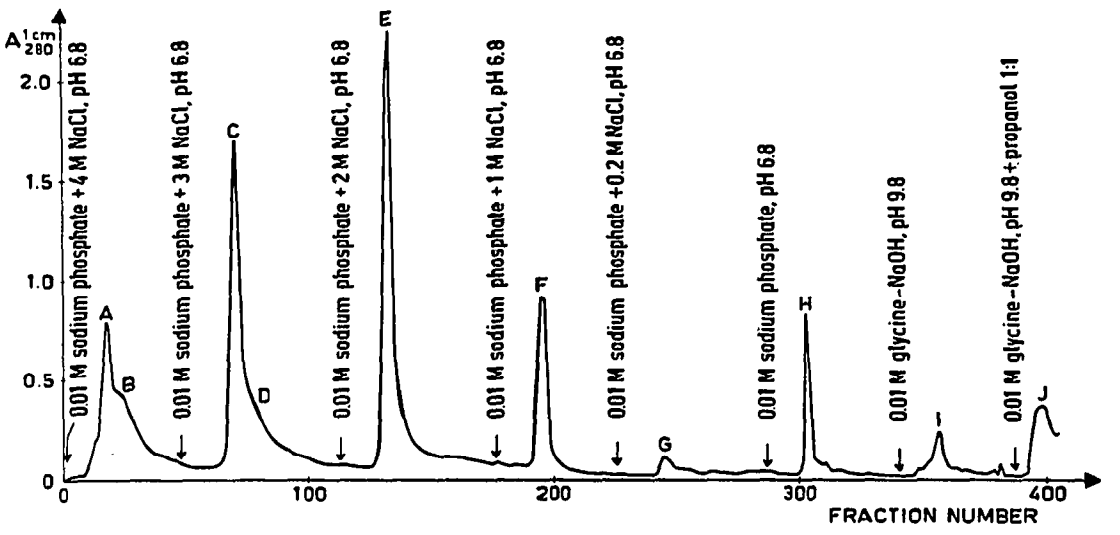
From Figs. 1a and 1b, it is evident that the degree of substitution is approximately proportional to the amount of epoxide added. The aromatic epoxide gave a lower coupling yield than the aliphatic epoxide.

APPLICATIONS

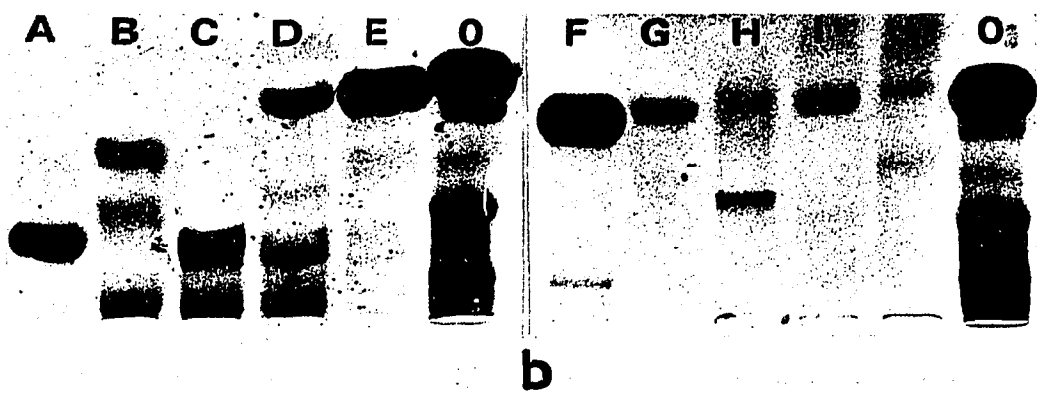
Preliminary experiments with alkyl derivatives of agarose with chain-lengths up to 12 C indicate that no amino acids or dipeptides (not even the very hydrophobic peptide leucyl-leucine) are adsorbed. As proteins (Figs. 2a and 3) and large particles (Figs. 4a and 4b) bind to these gels, it is evident that a multi-point attachment is required for adsorption to take place. The alkyl gels might therefore be used to separate low-molecular-weight compounds from high-molecular-weight substances and particles.

As plasma contains a large number of easily identifiable proteins, it is a convenient sample to use in order to test the resolving power of a proposed method for protein fractionation. We therefore submitted plasma to hydrophobic interaction chromatography. The chromatogram was developed by decreasing the ionic strength and increasing the pH[2]; the most strongly adsorbed material was displaced by propanol[2]. Some of the fractions obtained (A–J) were analyzed by agarose gel electrophoresis (Fig. 2b), which indicated an interesting fractionation. No efforts were made to isolate any particular plasma component by altering the elution conditions.

The results shown in Fig. 2 (and Fig. 3) indicate that the neutral alkyl-Sepharose beds described in this paper exhibit a high resolving power. However, a chromatographic method to be used for the fractionation of labile biopolymers must also be very mild. This requirement seems also to be fulfilled, as many enzymes have been chromatographed on this bed material without loss of activity. An example is given



a



b

Fig. 2. (a) Hydrophobic interaction chromatography on pentyl-Sepharose 4B of normal human plasma. Sample: 2 ml of plasma, dialyzed against 0.01 *M* sodium phosphate + 4 *M* NaCl, pH 6.8. Column dimensions: 36 cm × 1.5 cm I.D. Flow-rate: 6 ml/h. Temperature: 4°. (b) Analysis by agarose gel electrophoresis of the material corresponding to fractions A–J in Fig. 1a. Buffer: 0.075 *M* veronal, pH 8.6.

in Fig. 3, which shows the chromatographic behaviour of a crude extract of a cellulase system from *Trichoderma viride* on a column of hexyl-Sepharose 4B. The β-glucosidase activity[10] was recovered to the extent of 100% and the CM-cellulase activity[10] to 50–60%. The different activity peaks may correspond to iso-enzymes known to be present in the applied sample. Fig. 3 also indicates that the two enzymes have different affinities for the hexyl chains coupled to the agarose beads.

The hydrophobic beds described can be used not only for protein fractionation but also for the purification of particles as large as viruses, as illustrated in Fig. 4a for satellite tobacco necrosis virus (STNV). The material in fractions 105–115 contained virus particles of a high degree of purity, as shown by electron microscopy and the

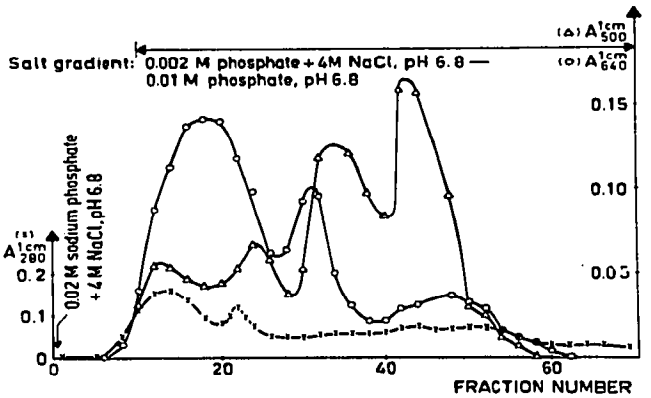


Fig. 3. Hydrophobic interaction chromatography on hexyl-Sepharose 4B of a crude extract of a β-glucosidase (△—△) and CM-cellulase (○—○) from *Trichoderma viride*. Amount of sample: 11 mg in 1.7 ml of the starting buffer. Column dimensions: 17 cm × 2 cm I.D. Flow-rate: 8 ml/h. Temperature: 4°.

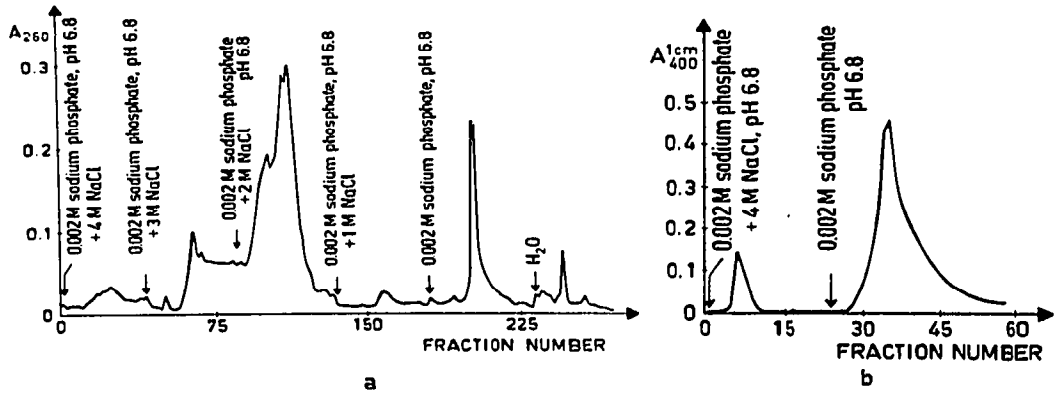


Fig. 4. Hydrophobic interaction chromatography of particles. (a) Crude extract of satellite tobacco necrosis virus on dodecyl-Sepharose 4B. Amount of sample: 250 µl of $A^{1cm}_{260} = 96$. Column dimensions: 36 cm × 1.5 cm I.D. Flow-rate: 6 ml/h. Temperature: 4°. (b) Baker's yeast on a 1% gel of naphthylagarose. Amount of sample: *ca.* 20 mg suspended in 1 ml of 0.002 *M* sodium phosphate + 4 *M* NaCl, pH 6.8. Column dimensions: 26 cm × 1.5 cm I.D. Flow-rate: 100 ml/h. Temperature: 4°.

ratio between the absorption at 260 and 280 nm (= 1.8). The columns seem to be useful for even larger particles, as illustrated in Fig. 4b by a chromatogram of baker's yeast cells. In order to chromatograph whole cells, one must use relatively large gel grains, so that the cells can pass through the channels between them[11]. The hydrophobic substituents in this chromatographic experiment were naphthyl groups. They were coupled to gel grains obtained by pressing a gel of 1% agarose (prepared according to ref. 12, method IIIb) through a 30-mesh net and removing the "fines" by decantation[11].

Fig. 5. Octyl-Sepharose 4B utilized as matrix in an enzyme reactor. Buffer: 0.01 *M* Tris–HCl, pH 7.5, + 0.01 *M* $MgCl_2$. By hydrophobic interaction, *β*-galactosidase from *Escherichia coli* was adsorbed on to the bed. Substrate solution and buffer were alternately applied to the column. The substrate (2-nitrophenyl-*β*-D-galactopyranoside) was immediately converted into a yellow product when applied to the bed.

HYDROPHOBIC BEDS UTILIZED AS MATRICES FOR ENZYME REACTORS

The neutral, hydrophobically interacting bed materials described can also be utilized with advantage as matrices in enzyme reactors (*cf.*, Hofstee[3], who used beds exhibiting both hydrophobic and electrostatic interaction for the same purpose). The covalent coupling of enzymes to a solid support can thus be replaced in several instances by simply adding the enzyme to the hydrophobic bed. In comparison with some covalent linking procedures, the attachment by hydrophobic interaction has the advantage that it can be performed at any pH if a suitable agarose derivative is chosen. Fig. 5 shows a photograph of such an enzyme reactor in operation. The experiment was repeated each week for 4 weeks, during which period the enzyme activity decreased to about 50%. A similar result was obtained when the enzyme was adsorbed on to a bed of dodecyl-Sepharose 4B.

TEMPERATURE EFFECT

We have reported earlier that the expected decrease in adsorption with a decrease in temperature could not be unambiguously demonstrated with beds obtained by coupling aromatic or aliphatic amines to Sepharose by the cyanogen bromide reaction[2]. This finding is not surprising, as these columns also have an element of electrostatic interaction which increases with a decrease in temperature. For gel beds with neutral hydrophobic chains, the expected temperature dependence was easy to demonstrate, as follows.

A gel of pentyl-Sepharose 4B was equilibrated at room temperature with 3 *M* sodium chloride, buffered at pH 6.8 with 0.002 *M* sodium phosphate. The sample, a red phycoerythrin solution, was dialyzed against the same buffer and then centrifuged. The supernatant was mixed with the gel in a centrifuge tube, which was then slowly shaken. The phycoerythrin that did not adsorb to the gel was removed by repeated centrifugations and washings with the above buffer until the supernatant was colourless. The temperature was then changed from room temperature to 0° by immersing the centrifuge tube in an ice–water bath. After 5 min, the gel was repeatedly washed with buffer at 0°, which released 25–30% of the phycoerythrin adsorbed at room temperature.

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