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PREPARATION OF HYDROPHOBIC COTTON CLOTH

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SUMMARY

Hydrophobic cotton cloths were prepared by heating cotton flannel in a mixture of alcohols or phenols, epichlorohydrin and 4 M NaOH. These cloths adsorbed as much bovine serum albumin as did a commercial preparation of phenyl agarose. β-Galactosidase and β-glucosidase adsorbed on the cloths were about 50% as active as free enzymes. Glucoamylase immobilized on naphthyl cloth in a packed bed column efficiently hydrolyzed soluble starch to glucose. These inexpensive media would be useful for commercial-scale hydrophobic chromatography and enzyme immobilization.

INTRODUCTION

Alkyl and aryl derivatives of agarose have been used to fractionate proteins on the basis of hydrophobicity and also to immobilize many enzymes (Hofstee, 1973; Hjerten et al., 1974). The linkage between hydrophobic groups and supports should not contain charges in order to avoid ionic interaction with proteins or substrates and should be stable. Neutral hydrophobic derivatives of agarose have been prepared by coupling alcohols or phenols via stable ether linkages (Hjerten et al., 1974). Since the methods call for agarose swollen in nonaqueous solvents, the agarose needs to be washed with an extensive series of solvents of decreasing polarity. The synthesis requires a series of steps and a variety of organic reagents. After these reactions, the agarose needs to be washed with a series of solvents of increasing polarity. To increase the physical and chemical stability of the gel, cross-linked agarose is used in the commercial preparation of hydrophobic media. The cost of the cross-linked agarose and preparation of these hydrophobic media limits their application to commercial biotechnological processes.

Cellulose is inexpensive, and chemically and physically stable. However, a column packed with the fibrous form of its derivatives tends to exhibit high hydrodynamic resistance due to compaction and clogging by fine fibers. It has been shown that fabric forms (cloth) of fibers, when stacked in a column, provide good flow rates (Shemer et al., 1979;

Yamazaki et al., in press). This paper demonstrates that inexpensive hydrophobic cloths which exhibit high protein adsorption can be easily prepared by heating cotton cloth in a mixture of alcohols or phenols, epichlorophydrin and 4 M NaOH.

MATERIALS AND METHODS

Materials Cotton flannel (cloth) was locally obtained. All chemicals and enzymes were obtained from Sigma Chemical Co. Octyl and phenyl sepharose were obtained from Pharmacia Fine Chemicals.

Preparation of hydrophobic cloth A 2 cm square of cloth (about 0.05 g) was soaked in 1 ml of 4 M NaOH containing 2 mg/ml $NaBH_4$ for 15 min at room temperature. Five mmoles (about 0.4 ml) of epichlorohydrin were mixed with 5 mmoles of an alcohol (hexyl, octyl, decyl or dodecyl) or a phenol (phenol or β-naphthol). This was added to the soaking cloth, mixed and heated at 100°C for 2 h for an alcohol mixture or 1 h for a phenol mixture. After cooling to room temperature, the cloth was washed with 1 N HCl, hot ethanol, and distilled water, and blotted dry. For longer storage, the cloth was kept in ethanol.

Adsorption of bovine serum albumin (BSA) A cloth was soaked in 1 ml of 10% BSA (Sigma No. A 7906) for 16 h at 25°C. The cloth was thoroughly washed with water, blotted dry, and then heated in 2 ml of 1% sodium dodecyl sulfate at 100°C for 10 min. The extracted BSA was colorimetrically assayed (Lowry et al., 1951).

Immobilization of β-galactosidase and β-glucosidase β-Galactosidase (Sigma G 6008 Grade VI) was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) to a final concentration of 20 μg/ml. β-Glucosidase (Sigma G 8625) was dissolved in 0.05 M sodium acetate buffer (pH 5.6) to a final concentration of 200 μg/ml. A 2 cm square of hydrophobic cloth was covered with 100 μl of the enzyme solutions and left for 16 h at 25°C. The cloth was then soaked in 3 ml of the buffer for 30 min at 25°C (the steep liquid was kept for assaying the unbound enzyme). The cloth was washed with the buffer and assayed for the immobilized enzyme. For enzyme adsorption to a hydrophobic agarose gel, 100 μl of an enzyme solution was mixed with 1 g of wet gel (about 0.03 g dry weight). After 16 h at 25°C, the gel was washed with the buffer and assayed for the immobilized enzyme.

Assay of β-galactosidase and β-glucosidase A cloth containing bound enzyme was shaken at 320 rpm and 30°C in 3 ml of 2 mM o-nitrophenyl β-galactoside in phosphate buffer (for β-galactosidase) or 1 mM p-nitrophenyl β-glucoside in acetate buffer (for β-glucosidase). After 5 min, 2 ml of the reaction mixture was mixed with 1 ml of 1 M Na_2CO_3 and absorbance was determined at 420 nm (for β-galactosidase) or 400 nm (for β-glucosidase).

Glucoamylase column Naphthyl cloth segments (0.5 cm square) were soaked in a 5% suspension of crude Rhizopus glucoamylase (Sigma A 7255) in 0.02 M sodium acetate (pH 4.8) for 16 h at 25°C. The segments were washed with H_2O, soaked in 1% glutaraldehyde for 30 min, washed with H_2O and packed into a jacketed column (10 mm diameter) to a bed volume of 10 ml. A 5% soluble starch suspension in H_2O was pumped into the column (equilibrated at 50°C) at various space velocities. The degree of hydrolysis was calculated from the concentration of reducing sugars in the effluent obtained after pumping through about 10 volumes of the starch solution. The reducing sugar was assayed by

3,5-dinitrosalicylate reagent (Miller, 1959).

RESULTS

The previous derivatization method (Hjerten et al., 1974) consists of two steps: i) preparation of glycidyl ethers from alcohols and epichlorohydrin; ii) coupling of the glycidyl ethers to the hydroxyl groups of a support (agarose). They have carried out these reactions in nonaqueous solvents which dissolve alcohols, epichlorohydrin and boron trifluoride etherate (moisture sensitive catalyst). Thus, the method requires agarose swollen in the nonaqueous solvents. Here, we found that these reactions can readily be performed in aqueous NaOH solution at 100°C in a single step. This greatly simplifies and economizes the preparation of hydrophobic media from cellulose as well as other polyhydroxy compounds. Here, hydrophobic media were prepared from cotton flannel (cloth) which is much less expensive than agarose derivatives.

Since 1 ml of concentrated NaOH solution can dissolve about 5 mmoles of epichlorohydrin at 100°C, our derivatization mixture contained, per ml of NaOH solution, 5 mmoles of epichlorohydrin and 5 mmoles of ROH (alcohols or phenols). Excess ROH was found to reduce derivatization, presumably because it reacts with the glycidyl ether and thus lowers the level of this coupling reactant. The optimal derivatization conditions were examined in terms of the capacity of hydrophobic cloth to adsorb bovine serum albumin (BSA). Fig. 1A shows that use of 4 to 5 M NaOH in the derivatization mixture produced octyl and phenyl cloths with the highest BSA adsorption. Thus, 4 M NaOH was used in the derivatization mixture. Fig. 1B shows that the optimal reaction time was 1 h for phenyl cloth and 2 h for octyl cloth. Thus 1 h and 2 h were used in derivatizations involving phenols and alcohols, respectively. Longer reaction times reduced the BSA adsorption capacity. It is conceivable that excess derivatization encourages interaction between the introduced hydrophobic groups rather than these groups and proteins.

Table 1 shows that the resulting hydrophobic cloths exhibit BSA adsorption capacities comparable to those obtained by commercial octyl and phenol agarose (Sepharose). These hydrophobic cloths also adsorbed β-galactosidase and β-glucosidase efficiently and immobilized enzymes were nearly 50% as active as free enzymes. On the other hand, commercial hydrophobic agarose did not adsorb β-galactosidase in an active form, and the activity of adsorbed β-glucosidase was less than

that observed on the hydrophobic cloth.

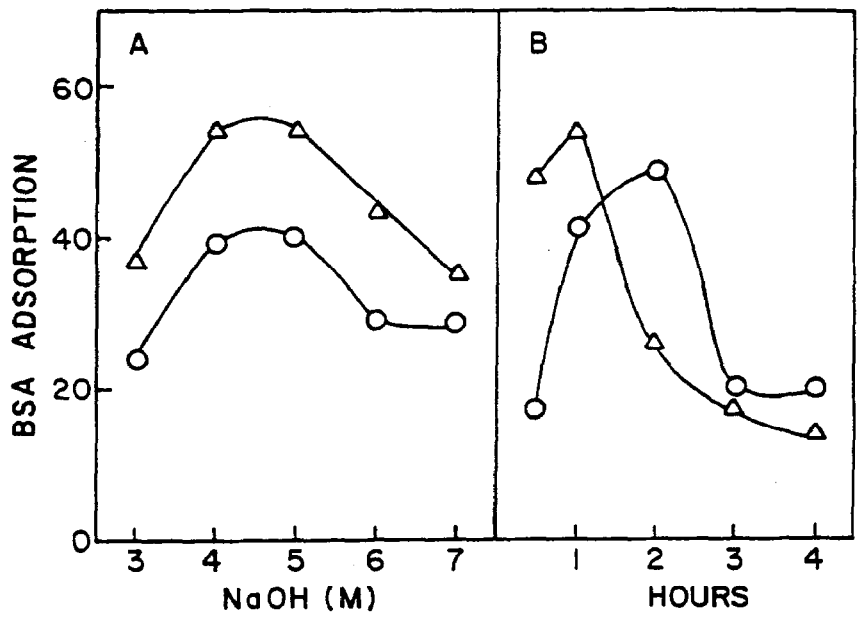

Fig. 1. Effect of NaOH concentration (Part A) and reaction time (Part B) on BSA adsorption capacity of resulting hydrophobic cloth. Octyl (○) and phenyl (△) cloths were prepared as described in METHODS except for the following conditions. In Part A, derivatization mixtures consisting of various concentrations of NaOH were heated for 1 h. In Part B, derivatization mixtures described in METHODS were heated for various lengths of time. The resulting hydrophobic cloths were assayed for BSA adsorption (mg BSA/g cloth). The data are the average of the triplicate samples.

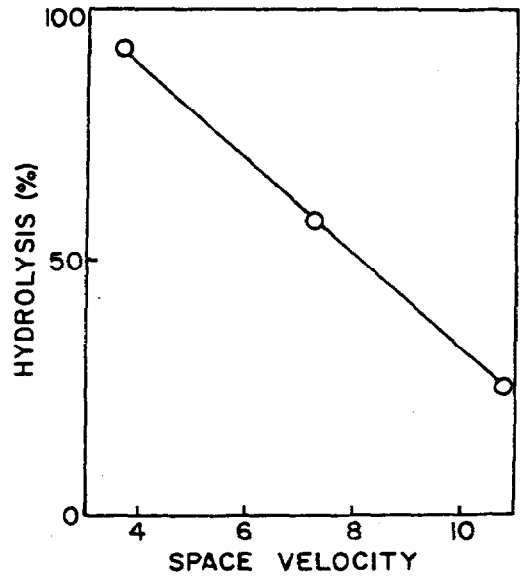

Fig. 2. Hydrolysis of soluble starch in a column packed with glucoamylase naphthyl cloth.

Table 1: Adsorption of BSA, β-galactosidase and β-glucosidase[a]

Hydrophobic media	BSA	β-Galactosidase	β-Glucosidase
Hexyl cloth	24	19 (86)[b]	37 (76)
Octyl cloth	46	22 (88)	43 (84)
Decyl cloth	51	22 (88)	51 (92)
Dodecyl cloth	35	26 (88)	48 (97)
Phenyl cloth	51	28 (95)	56 (96)
Naphthyl cloth	51	28 (96)	49 (96)
Octyl Sepharose	60	ND[c]	21 (55)
Phenyl Sepharose	50	ND[c]	22 (60)

[a] BSA and enzymes were adsorbed to 2 cm squares of hydrophobic cloths as described in METHODS. BSA adsorption is expressed as mg BSA per g dry cloth or Sepharose. Enzyme adsorption is expressed as activity (nanomoles of substrate hydrolyzed per min) of enzymes immobilized on a cloth square. The enzyme activities added to a square were 54 nanomoles/min of β-galactosidase and 105 nanomoles/min of β-glucosidase (activities of free enzymes).

[b] The numbers in the parentheses indicate the percentage of enzyme adsorption which was calculated from the activities of applied enzyme and unadsorbed enzymes.

[c] No activity was detected on the hydrophobic Sepharose nor in the steep liquid.

Enzyme immobilization on hydrophobic cloth would be particularly suited for transformation of large-sized substrates as an enzyme is exposed on the surface of cellulose. We have found that Rhizopus and Aspersillus glucoamylases adsorb to naphthyl cloth and their activity can be stabilized by glutaraldehyde treatment. To evaluate column performance of the immobilized enzyme, 0.5 cm squares of naphthyl cloth containing Rhizopus glucoamylase were tightly packed into a column. Fig. 2 shows that nearly complete hydrolysis of soluble starch could be achieved at a space velocity of 3 to 4. Higher space velocities resulted in a linear reduction of the degree of hydrolysis.

DISCUSSION

We have demonstrated that alkyl and aryl derivatives of cotton cloth can be easily prepared by aqueous reactions in a single batch operation. This derivatization method is simpler and less costly than the previous method (Hjerten et al., 1974). Hydrophobic cloth is much less expensive than hydrophobic agarose and yet adsorbs as much protein. Therefore,

hydrophobic cloth will be useful for commercial-scale hydrophobic chromatography and enzyme immobilization. The absence of charges on the cloth permits fractionation based solely on hydrophobic interactions. We have demonstrated the advantage of enzyme immobilization on hydrophobic cloth for transformation of large-sized substrates.

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