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MODIFICATION OF CELLULOSIC
FABRICS FOR CHROMATOGRAPHY AND
IMMOBILIZATION OF BIOMACROMOLECULES:
APPLICATION TO BIOTECHNOLOGY.

by

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A thesis submitted to the faculty of
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ABBREVIATIONS

A ₅₀₀ or 540	absorbance at 500 nm or 540 nm
<u>A.niger</u>	<u>Aspergillus niger</u>
BDGE	1,4, butanediol-diglycidyl ether
BSA	bovine serum albumin
¹⁴ C	carbon-14
cm	centimeter
CNBr	cyanogen bromide
cpm	count per minute
DNS	dinitrosalicylic acid
<u>E.coli</u>	<u>Escherichia coli</u>
g	gram, gravity
h	hour
L	liter
M	molar
uCi	microcurie
ug	microgram
ul	microliter
mg	milligram
min	minute
ml.	milliliter
mM	millimolar
mmoles	millimole
NH ₂	amino group
nm	nanometer
0°C	degree celsius

o.d.	optical density
OH	hydroxyl group
O/N	overnight
ONPG	O-nitrophenyl β -D-galactopyranoside
PAA	polyacrylic acid
PEI	polyethylenimine
PVP	polyvinylpyrrolidone
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
^{35}S	sulphur-35
Tris	tris(hydroxymethyl)aminomethane

GENERAL INTRODUCTION

Biotechnology aims for efficient and economic production of materials of practical value by controlling the activities of living cells, particularly microorganisms, or their enzymes. Therefore, biotechnology is not a new science. Aside from the ancient arts of brewing and baking, biotechnology has for decades been providing us with a variety of other foods and beverages, flavoring agents, nutritional supplements such as amino acids and vitamins, and pharmaceuticals such as antibiotics and hormones. The potential of biotechnology has been greatly increased by recent developments in genetic engineering such as recombinant DNA, protoplast fusion and hybridoma technologies.

A major project for future biotechnology is the efficient use of renewable biomass for production of compounds which are currently derived from non-renewable petroleum which is rapidly declining. Since Canada is rich in biomass, it has the resources to fuel such a biotechnology industry. The most effective way of converting biomass into practical compounds is through use of enzymes and cells which catalyze highly specific reactions at moderate pH and temperature ranges.

Bioprocesses employing these biocatalysts generally generate higher yields of products with the expenditure of less energy and chemicals than conventional chemical methods.

Immobilization of these biocatalysts on insoluble supports will facilitate their separation from products and their re-use. Immobilized biocatalysts packed in a column permit continuous and controlled processes and use of the biocatalysts. Although still not widely used, the immobilization of biocatalysts offers a diversity of new opportunities for their industrial applications.

The cost of immobilized enzyme systems depends on the cost of the catalysts, support, immobilization method and stability of immobilized enzymes. Since biological catalysts are generally of microbial origin, their cost is relatively low. Methods which have been used for enzyme immobilization are classified according to the chemical nature of binding.

(i) Adsorption: Biocatalysts are adsorbed on a suitable support by ionic, hydrogen or hydrophobic interactions. Adsorption is a simple and mild procedure. However, adsorbed enzymes may be desorbed under conditions which weaken these interactions. Immobilization by adsorption has the advantage of easy desorption and recharging with fresh enzyme when the substrate conversion power of the enzyme reactor has dropped below the lowest tolerable level.

(ii) Entrapment: Biocatalysts are physically entrapped in cases which are composed of highly cross-linked polymers such as polyacrylamide and polysaccharide gels.

Entrapped enzymes often leak out and the network around the enzymes often imposes severe diffusional resistance to the transport of large substrate molecules and products. Microencapsulation is a form of entrapment in which biocatalysts are enclosed in spherical polymer membranes.

(iii) Covalent coupling: Biocatalysts are bound to a suitable support by a covalent bond which permanently immobilizes the enzyme. However, due to chemical reactions involved in immobilizing the enzyme activity is often lost or reduced.

(iv) Chemical aggregation: Biocatalysts (usually cells) are cross-linked to each other by a bifunctional reagent such as glutaraldehyde to produce an insoluble complex which can be used in a granular or pellet form in a bioreactor column.

General characteristics of these immobilization methods are compared in Table 1.

Recently, insoluble supports carrying hydrophobic groups have been used to immobilize several enzymes (Hofstee, 1973., Caldwell et al, 1975, 1976., Cashion et al, 1982). These hydrophobic supports were originally developed to fractionate substances on the basis of hydrophobicity on their surface. As many proteins have hydrophobic groups exposed on their surfaces (Brand, 1972., Klotz, 1970), the proteins can be fractionated by hydrophobic chromatography (Hjerten et al, 1974).

Table 1. Comparison of Biocatalyst Immobilization Methods

Characteristic	Adsorption	Entrapment	Covalent coupling	Chemical aggregation
Activity loss	low	low to moderate	high	high
Specificity change	little	moderate	moderate to large	moderate
Retention*	weak to strong	moderate	strong	moderate
Regeneration	possible	impossible	impossible	impossible
Cost	low	low to moderate	high	moderate to high

* Retention of catalysts

Often, the interaction between hydrophobic groups in proteins and hydrophobic sites on the insoluble support become so large, that the proteins tightly bind to the hydrophobic support. Enzymes immobilized by hydrophobic interaction have been shown to exhibit almost unaffected activity. This simple and mild immobilization has not yet been used in biotechnological processes because inexpensive hydrophobic supports are not available.

In contrast to enzyme immobilization, the status of affinity chromatography in biotechnology has not yet been established. Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is physically and reversibly adsorbed by a complementary binding substance (ligand) immobilized on an insoluble support (Pazur, 1981). In most cases, the ligand is attached to the support by a structure known as "spacer arm". This arm allows the ligand to have an access to the binding site (Scouten, 1981). Applications of this technique have resulted in the purification of a large and still increasing number of enzymes, antigens and antibodies, hormones and other biologically active substances (Hoffman, 1978., Lanzillo, 1980., and Pillion, 1980). Even though it has simplicity and efficiency desired by industry, currently available affinity chromatography supports are too expensive for large scale commercial operation.

Industrial applications of these immobilization

and chromatographic methods have met with difficulty as the supports used are either expensive or lack appropriate characteristics such as good flow for large scale column operations.

Agarose, is one of the most widely used supports for enzyme immobilization, hydrophobic and affinity chromatography. The main reason for its popularity is its macroporosity combined with low non-specific adsorption, chemical stability and availability of hydroxyl groups for chemical attachment of spacers, ligands, or other binding groups (Cuatrecasas, 1970). However, there are several disadvantages to its use. The first is cost, as the purification of agarose from agar makes it expensive. Furthermore, agarose is unstable at temperatures above 40°C. Although this problem has been resolved by cross-linking, such a thermostable agarose is even more expensive. The gel structure of agarose creates diffusional resistance to the movement of large sized substrates and products to and from an enzyme immobilized within the gel matrix. In addition gels in a tall bed in a column operation are deformable and compressible, giving rise to large pressure drops across the bed and poor flow rates.

Derivatives of Sephadex (cross-linked dextran) and cellulose fibers have also been used for chromatography and immobilization of macromolecules in laboratory scale operations. Sephadex derivatives are costly. Although

derivatives of cellulose fibers are less costly, cellulose fiber products packed in a column suffer hydrodynamic resistance (poor flow) which makes them less ideal for large scale column operations. Therefore, appropriate supports for industrial application still remain to be developed.

The purpose of my thesis research was to develop supports for enzyme immobilization, hydrophobic and affinity chromatography which are suitable for large-scale column operations as used in commercial processes. The development of such a support should take into account three major criteria: (i) low cost, (ii) ease of preparation and (iii) ease of use of products.

I have chosen cellulosic materials in the form of fabric to produce these supports. The reason for the choice is discussed below.

Cellulose has a number of characteristics which makes it a suitable matrix for use in an enzyme reactor. Cellulose is inexpensive and readily available. It is chemically and mechanically stable, and is compatible with most organic solvents. Because of its heat stability, it can be easily heat sterilized. Although cellulose fibers are relatively non-porous, enzyme immobilized on the external surface of the fiber is in immediate contact with the substrate solution, minimizing the diffusional resistance, experienced in gels. Although cellulose has these characteristics suitable for industrial use,

the hydroxyl groups of cellulose are less amenable to chemical modification than the hydroxyl groups in polysaccharide gels.

In cellulose, 1,4 hydroxyl groups are used to form β 1-4 linkages between glucose residues. Hydroxyl groups at the C-2, C-3 and C-6 positions are engaged in hydrogen bonding between glucose polymers resulting in "crystalline" regions which occupy more than 85 % of cellulose. These hydroxyls are not readily modified unless the crystalline regions are disrupted, for example, by soaking in concentrated alkali solutions. Local irregularities in the alignment of the glucose polymers creates some amorphous regions where the hydroxyl groups are less involved in hydrogen bonding and thus are more reactive than the hydroxyl groups in the crystalline regions. However, the amorphous region represents less than 15 % of cellulose. Since cellulose fibers are flexible (ascribed to the amorphous regions) and have irregular structures, they are compressible under pressure. Furthermore, fine fibers present in the preparation are difficult to remove and tend to clog the column.

To circumvent this problem of flow rate, two types of cellulose modifications have been suggested. The first is microcrystalline cellulose which is prepared by controlled acid digestion of the amorphous regions of the cellulose. It exhibits more satisfactory flow properties (Goldstein and Manecke, 1976). However,

microcrystalline cellulose powder contains less reactive hydroxyl groups and its preparation is costly. Although it is less compressible than fibers, the flow rate is not high enough to be used in a tall column. Industrial use of cellulose fibers and microcrystalline products necessitates the construction of series of shallow columns in which the effect of compaction on the flow rate is minimized (Olson and Korus, 1977., Lilly, 1976). This special column is expensive and creates channelling effect (non-uniform localized flow).

To improve flow characteristics of cellulose products, Chen and Tsao (1976), developed porous cellulose beads. Cellulose acetate was dissolved in an organic solvent and the solution then dispersed into water to obtain cellulose acetate beads. The acetate groups were then removed by alkaline hydrolysis which generates free hydroxyl groups for chemical modification. The beads no doubt improved the flow rate and caused less clogging in comparison to microcrystalline cellulose powder. However, the series of steps involved make its large scale preparation difficult and costly.

Cotton cloth has an open and compact structure devoid of fines. Yamazaki et al (1984), developed a simple way to convert cotton cloth to an anion exchanger by coating the cloth with polyethylenimine (PEI). This support was used to produce stable and efficient immobilized yeast invertase preparations. Columns

packed with stacks of such a fabric exhibited excellent flow. Yamazaki's group is now developing a variety of other anion and cation exchangers using cotton cloth, which are useful as chromatographic and enzyme immobilization media.

The present thesis mainly describes preparation of hydrophobic cloth and its applications to the immobilization of enzymes and immunoglobulins. The thesis also demonstrates the potential of deacetylated cellulose acetate cloth as a support for affinity chromatography.

Chapter 1

Preparation of Hydrophobic Cotton Cloth

Introduction

For a proper hydrophobic adsorbent, hydrophobic groups should not be placed too densely because they interact with each other rather than with the exogenous molecules which are to be purified or immobilized. Cotton cloth would be suitable for the preparation of hydrophobic media since the density of reactive OH groups on cotton is relatively low.

Currently, agarose is most commonly used as a support for preparing hydrophobic adsorbents. There are two major methods for introducing hydrophobic groups to agarose. The first method is an aqueous system in which cyanogen bromide-activated agarose is allowed to react with alkylamine in an aqueous solvent (Hofstee, 1973., Wilchek and Miron, 1976). However, this method introduces a positively charged linkage group. Furthermore, the linkage formed is unstable at extreme pH (eg. strong alkali), which limits regeneration of hydrophobic supports by alkali. The second method is a nonaqueous system which couples the OH group of the support with alcohols or phenols via ether-linkages which are uncharged and stable in alkali (Hjerten et al., 1974). Although the nonaqueous solvent dissolves reactants, hydrophilic agarose needs to be washed with a series of solvents of decreasing polarity to obtain agarose swollen in the nonaqueous solvent. After the coupling reaction, agarose needs to be washed with a

series of solvents of increasing polarity. To increase the stability of the gel, cross-linked agarose is used in the commercial preparation of hydrophobic adsorbents. The high cost of such a support and the nonaqueous method limit the application of resulting hydrophobic adsorbents to commercial biotechnological processes.

This chapter describes a simple and economic method for preparing hydrophobic adsorbents from cotton cloth.

Results and Discussion

and boron trifluoride ethyl etherate (moisture sensitive catalyst). This method required agarose swollen in nonaqueous solvents.

We have reasoned that these reactions should occur in an alkaline aqueous solution in which epichlorohydrin and phenols show considerable solubility. Even though higher alcohols are sparingly soluble, epichlorohydrin should react with the soluble portion of the alcohols and mediate their immobilization onto an insoluble support. As solubilized alcohols are withdrawn by reaction with epichlorohydrin more alcohols dissolve and react with the epichlorohydrin. A higher temperature would facilitate the rates of solubilization and reaction. Indeed I found that these reactions can readily proceed in an aqueous NaOH solution at 100°C in a single batch reaction. 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 cloth which is much less expensive than agarose.

Since 1 ml of concentrated NaOH solution was found to dissolve about 5 mmoles of epichlorohydrin at 100°C, our derivatization mixture contained per ml of NaOH solution, 5 mmoles of alcohols or phenols (R-OH). Excess ROH was found to lower derivatization presumably because it reacts with the glycidyl ether and thus reduces the level of this coupling intermediate. The

optimal derivatization conditions were examined in terms of the capacity of the hydrophobic cloth to adsorb bovine serum albumin (BSA). Fig. 1A shows that the 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 likely that excess derivatization encourages interaction between the introduced hydrophobic groups rather than between these groups and protein. Different alkyl and aryl derivatives of cotton cloth were prepared and assayed for BSA adsorption. Table 2 shows that the resulting hydrophobic cloths exhibit BSA adsorption capacities comparable to those obtained by commercially available octyl and phenyl agaroses (Sephacrose).

These results clearly indicate 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 much less costly than the previous nonaqueous method (Hjerten *et al.*, 1974).

Fig. 1. Effect of NaOH concentration (Part A) and reaction time (Part B) on BSA adsorption capacity of resulting hydrophobic cloth. Octyl (O) and phenyl (Δ) 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 triplicate samples.

Table 2 Adsorption of bovine serum albumin (BSA)^(a)

<u>Hydrophobic media</u>	<u>BSA adsorption</u>
Butyl cloth	38
Hexyl cloth	62
Octyl cloth	92
Decyl cloth	102
Dodecyl cloth	70
Phenyl cloth	102
Naphthyl cloth	102
Untreated cloth	7.0
NaOH treated cloth ^(b)	7.2
Epichlorohydrin cloth ^(c)	8.4
Octyl Sepharose	120
Phenyl Sepharose	100

(a) BSA was adsorbed to 2 cm squares of hydrophobic cloths as described in Methods. BSA adsorption is expressed as mg BSA per gram dry cloth or Sepharose (agarose). The data are the average of duplicate samples.

(b) Cotton cloth was heated in 4 M NaOH at 100°C for 1 h.

(c) Cotton cloth was heated in 4 M NaOH plus 5 mmoles/ml of epichlorohydrin at 100°C for 1 h.

Fractionation of *E. coli* proteins on hydrophobic cloth

Alkyl and aryl derivatives of agarose have been used to fractionate proteins on the basis of hydrophobicity (Hofstee, 1973., Hjerten et al., 1974). Hydrophobicity of alkyl derivatives increases with their chain length, and proteins bound to longer hydrophobic chains are increasingly difficult to elute. Thus, butyl derivatives have been applied to protein fractionation (Hjerten et al., 1973). Butyl cloth prepared by the present method was examined for chromatographic application. Hydrophobic interaction is known to increase with increasing ionic strengths (Pahlman et al., 1977, 1978) and decrease with increasing concentrations of detergents or polarity reducing agents such as ethylene or propylene glycol (Hjerten et al., 1973).

Here, [^{35}S]-labeled *E. coli* proteins were adsorbed to butyl cloth in the presence of 2 M $(\text{NH}_4)_2\text{SO}_4$. Fig. 2 shows the chromatographic resolutions of polypeptides eluted when the $(\text{NH}_4)_2\text{SO}_4$ concentration was linearly lowered to 0 M and then the propylene glycol concentration was linearly increased to 100 %. Although the eluted proteins were resolved as distinct peaks, these represented only 5 % of the radioactivity adsorbed to the column. Therefore, butyl cloth is too hydrophobic to be used for protein fractionation. Although the remaining proteins were eluted with hot 1 % SDS, the eluted proteins were not native.

Fig. 2 Chromatography of [^{35}S]-labeled E. coli protein on butyl cloth. Butyl cloth pieces (0.5 cm squares) were packed to a 5 ml bed volume in a column (1 cm diameter). The column was equilibrated at 25°C with 2 M $(\text{NH}_4)_2\text{SO}_4$ in 0.01 M Tris-HCl (pH 8.0). 0.5 ml of [^{35}S]-labeled E. coli proteins (2.7×10^6 cpm) was added to the column. After 1 h the column was washed with 2 ml of 2 M $(\text{NH}_4)_2\text{SO}_4$. Elution was carried out at 25°C by 80 ml of a linearly decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient from 2 M to 0 M (Part A) and followed by 80 ml of a linearly increasing propylene glycol gradient from 0 to 100 % (Part B) as indicated by arrows. Each fraction contains about 1.2 ml for $(\text{NH}_4)_2\text{SO}_4$ gradient and about 2 ml for propylene glycol gradient.

Before considering the preparation of ethyl cloth, hydrophobicity of unmodified cotton cloth was examined by adsorbing E.coli proteins. Table 3 shows that about 30 % of total bound E.coli proteins were eluted in H_2O . 55 % of the total proteins were eluted with 100 % propylene glycol, and the remaining proteins were eluted with 1 % hot SDS. This adsorption and elution behavior are typical of hydrophobic interaction. This led to the conclusion that cotton cloth itself is already too hydrophobic. In cellulose, most of the hydrophilic OH groups are not available for interaction with H_2O and the property of the hydrocarbon predominates (thus cellulose does not dissolve in H_2O even at higher temperatures, unlike agarose or starch). This conclusion is consistent with undocumented observations (Personal communication., K. Matsumoto) that a considerable amount of protein irreversibly binds to 'new' cellulose ion exchangers and thus 'used' cellulose ion exchangers are preferred as purification media by experienced protein biochemists.

This conclusion then strengthens the possibility that hydrophobic cloth is suitable as media for immobilizing polypeptides (eg. enzymes). This possibility will be examined in the following chapter.

Conclusions

A simple method for preparing hydrophobic cloth has been developed. Since this method eliminates the need for

nonaqueous conditions, and thus the use of non-polar solvents, it is simpler and more economical than the previous nonaqueous method. As a support, cotton cloth is physically stable and much less expensive than cross-linked agarose. The resulting hydrophobic cloth is much less expensive than hydrophobic agarose prepared by the nonaqueous method and yet adsorbs as much protein. It is concluded that hydrophobic cloth is more suitable as media for immobilizing polypeptides than for hydrophobic chromatography.

Table 3 Adsorption of E. coli proteins to unmodified cotton cloth. (a)

<u>Washing</u>	<u>Protein desorbed</u>
H ₂ O	
First	11.3
Second	1.0
Third	0
Propylene glycol	
First	11.1
Second	3.7
Third	6.7
1 % hot SDS	
First	5.0
Second	0.2
Third	0.1

(a) 0.5 ml of E. coli proteins (about 10 mg/ml) suspended in 2 M (NH₄)₂SO₄ was applied to a 2 cm square of unmodified cotton cloth. After 16 h at 25°C, the cloth was washed with 2 M (NH₄)₂SO₄ and blotted. The cloth was then eluted with three 2 ml portions of H₂O, propylene glycol and 1 % SDS (heated at 100°C). Proteins desorbed (in duplicate sample) were colorimetrically assayed (Lowry, 1951) and are expressed as mg per g of cloth.

Material and Methods

Materials

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

Methods

Preparation of hydrophobic cloth

A 2 cm square of cotton cloth (about 0.05 g) was soaked in 1 ml of 4 M NaOH containing 2 mg/ml NaBH_4 for 15 min at 25°C. Five mmoles (about 0.4 ml) of epichlorohydrin were mixed with 5 mmoles of 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 or 1 h for a phenol mixture. After cooling at room temperature the cloth was washed with aliquots of 1 M 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 (O/N) at 25°C. The cloth was washed thoroughly with water, blotted dry, and then heated in 2 ml of 1 % sodium dodecyl sulphate (SDS) at 100°C for 10 min. The extracted BSA was colorimetrically assayed (Lowry, 1951).

Preparation of [^{35}S]-labeled *E. coli* proteins

To maximize [^{35}S]-labeling, low sulphate medium was prepared by dissolving 13.6 g KH_2PO_4 , 2.0 g NH_4Cl and 0.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.9 L H_2O . After the pH was adjusted to 7 with KOH, H_2O was added to give a final 1.0 L volume. After autoclaving, 1 ml of sterile 1 M MgSO_4 and 10 ml of sterile 50 % glucose was added. *E. coli* Crooke's strain was grown to absorbance of 2 at 500 nm in the above medium containing 1 μCi per ml of [^{35}S] H_2SO_4 . The cells were harvested and washed by centrifugation and resuspended in 0.01 M Tris HCl (pH 8.0) buffer containing 0.01 M MgCl_2 . The washed cells were frozen, ground with alumina, and extracted with the above buffer (twice wet weight of the cells). The extract was centrifuged at 15,000 g for 10 min. The supernatant was centrifuged at 30,000 g for 30 min. The final supernatant was dialyzed against the Tris buffer. The specific activity of S-30 *E. coli* proteins was 2.7×10^5 cpm per mg of proteins.

Chapter 2

Immobilization of Enzymes on

Hydrophobic Cloth

Introduction

Several enzymes have been immobilized onto hydrophobic agarose (Hjerten et al., 1974 , Caldwell et al., 1976). Immobilized enzymes for commercial processes should excel in the following criteria (i) low cost; (ii) ease of immobilization; (iii) good activity; (iv) good flow (in a column operation); and (v) stability. Hydrophobic cloths are much less expensive than hydrophobic agarose and yet bind as much protein. Hydrophobic interaction provides a simple enzyme immobilization technique. Enzymes are not exposed to the denaturing chemical conditions as in covalent binding or chemical aggregation methods. Studies in this chapter examine the applicability of hydrophobic cloth for immobilization, of enzymes and immunoglobulins.

Results and Discussion

Effect of hydrophobic groups on activity of immobilized enzymes :

After an enzyme is immobilized, it is inevitable that the specific activity of the enzyme decreases significantly as the diffusion of the enzyme is restricted. The activity of the immobilized enzyme therefore greatly depends on the rate of diffusion of substrate to the enzyme as well as the conformational and/or chemical modifications caused by immobilization. These effects can be expressed in terms of the efficiency

of an immobilized enzyme which is the activity of the immobilized enzyme expressed as the % of the activity as free enzyme of the enzyme immobilized. β -galactosidase and β -glucosidase were chosen to compare the efficiency of immobilization of various hydrophobic cloths. These enzymes and their chromogenic substrates are available at reasonable costs. Use of the chromogenic substrate make their assays simple. Table 4 shows that all hydrophobic cloths adsorbed both enzymes efficiently and immobilized enzymes were nearly 50 % as active as free enzymes. On the other hand, commercial hydrophobic Sepharose (agarose) did not adsorb β -galactosidase in an active form, and the efficiency of adsorbed β -glucosidase was less than that observed on the hydrophobic cloth.

There was no marked difference in performance between decyl, dodecyl, phenyl and naphthyl cloths. However, naphthyl cloth was chosen for further studies, because arylation required only 1 h heating at 100°C instead of 2 h for alkylation, and phenol is corrosive and more expensive than β -naphthol. Before naphthyl cloth was examined for immobilization of industrial enzymes, an attempt was made to economize the preparation of naphthyl cloth. Fig. 3 shows that BSA binding capacity decreased as lower concentrations of reactants (naphthol and epichlorohydrin) were used. Therefore, the original concentration of reactants (5 mmoles added to 1 ml of 4 M NaOH) was used throughout.

Table 4. Immobilization of β -galactosidase and β -glucosidase onto hydrophobic cloth^(a)

Hydrophobic media	β -galactosidase ^(c)		β -glucosidase ^(c)	
	free	immobilized	free	immobilized
Hexyl cloth	46	19 (41) ^d	80	37 (46) ^d
Octyl cloth	48	22 (46)	88	43 (49)
Decyl cloth	48	22 (46)	97	51 (53)
Dodecyl cloth	48	26 (54)	102	48 (47)
Phenyl cloth	51	28 (55)	101	56 (55)
Naphthyl cloth	52	28 (54)	101	49 (49)
Octyl Sepharose		ND ^(e)	58	21 (36)
Phenyl Sepharose		ND	63	22 (35)

(a) To a 2 cm square of each hydrophobic cloth or 0.05 g of hydrophobic Sepharose, were added 54 nanomoles/min of β -galactosidase or 105 nanomoles/min of β -glucosidase activities (expressed as nanomoles of substrates hydrolyzed per min). The activities of adsorbed and unadsorbed enzymes were assayed. The data shown above represent the average of duplicate samples.

(b) The activities as free enzymes of enzymes adsorbed were calculated by subtracting the activities of unadsorbed enzymes measured as described in Methods from the activities added to the hydrophobic media.

(c) The activities of immobilized enzymes in nanomoles/min.

(d) The efficiency (%) of immobilized enzyme = the activities of immobilized enzymes expressed as the % of the activities as free enzymes of enzymes adsorbed.

(e) No activity was detected on the hydrophobic Sepharose nor in the steep liquid.

Fig. 3. Effect of concentrations of β -naphthol and epichlorohydrin on BSA adsorption. Naphthyl cloth was prepared as described in Methods except that smaller amounts (in millimoles) of β -naphthol and epichlorohydrin in an equal molar ratio were added per ml of 4 M NaOH. BSA adsorption by resulting naphthyl cloth is expressed as mg of BSA bound per g of the cloth.

Immobilization of glucoamylase on naphthyl cloth

Enzymes immobilized on hydrophobic cloth are attached on the surface of cellulose fibers and accessible to large sized substrates (eg. starch and proteins). On the other hand enzymes immobilized on hydrophobic agarose are largely embedded inside the gel which causes diffusional resistance to large-sized substrates. To confirm this advantage of hydrophobic cloth, Aspergillus niger glucoamylase was adsorbed onto naphthyl cloth. Glucoamylase hydrolyzes starch or dextrans to glucose and is currently used in free (unimmobilized) form for commercial production of glucose. Since polyhydroxy compounds such as starch are known to weaken hydrophobic interaction, the enzymes adsorbed to the naphthyl cloth need to be cross-linked in order to prevent desorption by the substrate. Therefore, the adsorbed glucoamylase was cross-linked with glutaraldehyde. This bifunctional reagent has been used to cross-link many enzymes (Richards and Knowles, 1968) via amino groups and probably other groups (Habeeb et al, 1976). Unmodified cloth also adsorbed significant amount of enzyme but the enzyme was desorbed immediately upon contact with the substrate (starch). Fixation with glutaraldehyde did not prevent this desorption. Fig. 4 shows the effect of various glutaraldehyde treatments on the activity of glucoamylase immobilized on naphthyl cloth. Although treatment with 2 % glutaraldehyde for 2 h at

Fig. 4 Effect of glutaraldehyde treatment on the stability of Aspergillus niger glucoamylase adsorbed on naphthyl cloth. A. niger glucoamylase was adsorbed on naphthyl cloth and treated with glutaraldehyde and assayed at one week intervals for production of reducing sugar from soluble starch as described in Methods. After immobilization at 0 time, the immobilized enzyme was continuously incubated at 50°C in 5 % soluble starch (periodically changed) for 4 weeks. The specific rate of hydrolysis is expressed in g of reducing sugars (equivalent to glucose) produced per min per g of glucoamylase bound cloth. In part A, adsorbed enzymes were treated for 2 h at 25°C with glutaraldehyde at 0.2 % (○); 0.5 % (●); 1 % (▲); 2 % (▲); and not treated with glutaraldehyde (□). In part B, adsorbed enzymes were treated with 0.2 % glutaraldehyde at 25°C for the periods of 30 min (○); 60 min (●); and 120 min (Δ).

at 25°C is commonly used (Avrameas et al, 1967., Broun et al, 1969). Fig. 4 A shows that the activity of glutaraldehyde treated enzyme cloth showed stable activity over 4 week period, whereas the activity sharply declined without the glutaraldehyde treatment. This decrease is at least partly ascribed to enzyme desorption. However, enzyme denaturation may also occur without cross-linking, which supposedly restricts enzymes from taking inactive conformations (denaturation), thus increasing stability. Fig. 4 A also shows that treatment with 0.2 % (rather than 2 %) of glutaraldehyde was sufficient for stabilizing the enzyme activity. The effects of treatment times at 0.2 % were studied. Fig. 4 B shows that a 30 min rather than 2 h treatment was sufficient for stabilizing the enzyme for 4 weeks.

Optimal pH of immobilized glucoamylase

Immobilization often causes a significant change in the optimal pH of an enzyme if it modifies the microenvironment of amino acid side chains responsible for enzyme catalysis. Glucoamylase was adsorbed to naphthyl cloth and treated with 0.2 % glutaraldehyde for 30 min at 25°C. The activity of the immobilized glucoamylase was determined at various pH and compared to the free enzyme. Fig. 5 shows that the optimal pH of the immobilized enzyme was about 4.4 and this was similar to that of free enzyme. This result suggests that immobilization did not markedly affect the micro-

Fig. 5 Effect of pH on the activity of Aspergillus niger glucoamylase immobilized on naphthyl cloth. A.niger glucoamylase was adsorbed on naphthyl cloth and treated with 0.2 % glutaraldehyde for 30 min at 25°C. The activity of immobilized and free enzymes was assayed using 5 % soluble starch solution in 0.01 M sodium acetate buffers having different pHs. The activity of immobilized enzyme (○) is the amount (in g) of reducing sugars (equivalent to glucose) produced per min per g of the immobilized enzyme cloth. The activity of free enzyme (●) is expressed as the amount of reducing sugar produced per min per mg of enzymes.

environment of the active site.

Hydrolysis of starch in a glucoamylase column

An immobilized enzyme preparation packed in a column permits controlled and continuous enzymic processes which are desired in industry. To evaluate column performance of the immobilized glucoamylase, 0.5 cm squares of naphthyl cloth containing immobilized glucoamylase were packed in a column (bed volume of 10 ml) and starch hydrolysis was determined at different space velocities. The space velocity is the flow rate (in h^{-1}) divided by the bed volume. Fig. 6 shows that complete hydrolysis of 5 % soluble starch could be achieved at a space velocity between 3 to 4. Higher space velocities resulted in a linear reduction of the degree of hydrolysis.

These results show that immobilization on hydrophobic cloth is suitable for transformation of large-sized substrates.

Immobilization of aspartase on naphthyl cloth

Several enzymes have been immobilized by adsorption to ion-exchangers such as diethylaminoethyl (DEAE) cellulose or Sephadex (Sober et al, 1956., Peterson et al 1956). However, adsorbed enzymes tend to be desorbed at high ionic strengths. If a substrate is an ionic substance, higher substrate concentrations cannot be used unless adsorbed enzymes are cross-linked to minimize

Fig. 6 Hydrolysis of soluble starch in a column packed with glucoamylase naphthyl cloth. A. niger glucoamylase was adsorbed on naphthyl cloth segments (0.5 cm square), treated with 0.2 % glutaraldehyde for 30 min and packed into a jacketed column (10 mm diameter) to a bed volume of 10 ml. A 5 % soluble starch suspension (pH 4.4) was pumped into the column (equilibrated at 50°C) at various space velocities. The degree of hydrolysis was calculated from the concentration of glucose in the effluent obtained after pumping through about 10 bed volumes of the starch solution at each space velocity.

desorption. Since hydrophobic interaction increases with increasing ionic strength (Pahlman et al, 1977,1978), enzymes adsorbed to hydrophobic supports should not be desorbed at high concentrations of ionic substrates. To confirm this advantage of hydrophobic cloth, Escherichia coli L-aspartase adsorbed to naphthyl cloth was used as a model.

Aspartases catalyze the conversion of ammonium fumarate to L-aspartic acid. The enzymatically produced L-aspartic acid is used for the production of Aspartame (N-L-aspartyl-L-phenylalanine 1-methyl ester) which is an artificial sweetener for diet products. Commercially, E. coli cells (containing aspartase) entrapped in a carrageenan gel are used to convert 1 M ammonium fumarate (pH 8.5) to L-aspartate (Nishida et. al., 1979).

E. coli grown in ammonium fumarate (as sole carbon and nitrogen source) induced a high specific activity of aspartase (S. Sontakke, personal communication). Here, cell free extracts of such E. coli cells were used without purification. Fig. 7 shows that aspartase adsorbed to naphthyl cloth exhibited an optimal pH of 8.5 as free aspartase does. Table 5 (column 1) shows that E. coli aspartase adsorbed to naphthyl cloth exhibited stable activity during incubation at 37°C in the substrate solution. After 12 weeks, the immobilized enzyme still retained about 80 % of the original activity. Immobilized enzymes are known to be further stabilized by cross-linking. However,

Fig. 7 Effect of pH on the activity of aspartase on naphthyl cloth. E. coli aspartase was immobilized on naphthyl cloth as described in Methods. The conditions of the enzyme assay were the same as described in Methods except that substrate solutions of different pH values were used. Enzyme activity is expressed in 10^{-4} moles of substrate converted per min per g of cotton cloth. The broken lines represent the effect of pH on free aspartase (Tosa et al, 1973).

Table 5. Stability of E. coli aspartase immobilized on naphthyl cloth^(a)

Incubation time (weeks)	Activity (10^{-4} mole aspartic acid produced per min per g cloth)			
	Treatment with cross-linking agents			
	None	PEI	PAA	Purifloc C31
0	3.6	3.6	3.6	3.6
1	3.6	3.6	3.6	3.6
2	3.5	3.5	3.5	3.5
3	3.5	3.4	3.5	3.5
4	3.4	3.3	3.4	3.4
5	3.2	3.2	3.2	3.2
6	3.2	3.2	3.2	3.2
7	3.2	3.1	3.2	3.2
8	3.2	3.1	3.2	3.2
9	2.8	2.7	2.8	2.9
10	2.8	2.7	2.8	2.9
11	2.8	2.7	2.8	2.9
12	2.8	2.7	2.8	2.9

(a) E. coli aspartase was adsorbed to naphthyl cloth, treated with polyethylenimine (PEI), polyacrylic acid (PAA) or Purifloc and assayed every week for 12 weeks as described in Methods. The immobilized enzymes were always kept at 37°C in 1 M ammonium fumarate (pH 8.5).⁴ A significant amount of the enzyme activity (3.1×10^{-4} mole/min/g cloth) was adsorbed to unmodified cotton cloth but the activity was decreased to nearly zero after one week.

glutaraldehyde could not be used because it even at 0.2 % it was found to inactivate aspartase, which is in agreement with the previous observation (Nishida et al, 1979). Yamazaki's laboratory is developing a cross-linking method for glutaraldehyde sensitive enzymes. Polycationic polymers such as polyethylenimine and purifloc C31 (Dowex Chemicals Co.) have been used to flocculate macromolecules (eg. proteins) containing negative charges. Polyacrylic acid is a polyanionic polymer which should flocculate macromolecules containing positive charges. Treatment with these polymers was found to prevent desorption of E. coli aspartase adsorbed to DEAE- cloth (P. Park, personal communication). When the aspartase adsorbed to naphthyl cloth was treated with these polymers, little change in the activity was observed (Table 5).

Production of aspartic acid in an aspartase column

To evaluate column performance of the immobilized aspartase, 0.5 cm squares of naphthyl cloth containing immobilized aspartase were packed in a column and conversion of 1 M ammonium fumarate was determined at different space velocities. Fig. 8 shows that 85 % conversion was achieved at a space velocity of 1 and 100 % conversion would be achieved at about 0.5 space velocity.

These results confirm that hydrophobic cloth is a suitable adsorbent for enzymes which need to be

Fig. 8 Conversion of ammonium fumarate to L-aspartic acid in a column packed with aspartase naphthyl cloth. E. coli aspartase was adsorbed to naphthyl cloth (0.5 cm squares) and the cloths packed into a jacketed column (10 mm diameter) to a bed volume of 10 ml, as described in Methods. The ammonium fumarate solution was pumped into the column (equilibrated at 37°C) at various space velocities. The conversion rate (%) was calculated from the concentration of aspartic acid in the effluent obtained after pumping through about 10 bed volumes of the substrate solution at each space velocity.

operated in a reaction mixture of high ionic strength. The column showed good flow characteristics; a faster flow rate of 10 could be easily obtained in this column.

Regeneration of hydrophobic cloth

The activity of enzymes immobilized onto hydrophobic cloth should eventually decrease to an unacceptable level. Although hydrophobic cloths are relatively inexpensive, regeneration of cloths is advantageous for industrial processes where large scale enzyme reactors are used. Preliminary studies showed that A. niger glucoamylase adsorbed to naphthyl cloth was very slowly desorbed by soaking in concentrated NaOH solution at room temperature but heating in 4 M NaOH for 1 h at 100°C caused complete desorption. Fig. 9 A shows that heating in 2 M NaOH (instead of 4 M) at 100°C for 1 h was sufficient to regenerate capacity of naphthyl cloth to adsorb the original activity of glucoamylase. Fig. 9 B shows that the capacity of the cloth to immobilize glucoamylase was unchanged even after 3 regenerations of the same cloth. This suggests that this treatment causes complete desorption of glucoamylase and little effect on hydrophobic properties of the naphthyl cloth. The recharged glucoamylase showed little change in activity over 4 weeks incubation with the substrate. Table 6 also shows that the naphthyl cloth containing immobilized E. coli aspartase was also regenerated by the same procedure. This relatively simple and economic regeneration makes

Fig. 9 Regeneration of glucoamylase naphthyl cloth.

A. niger glucoamylase was adsorbed to 2 cm squares of naphthyl cloth and treated with 0.2 % glutaraldehyde and assayed for enzyme activity as described in Methods. The enzyme activity is expressed as the amount of reducing sugar (equivalent to glucose) produced per min per g of naphthyl cloth. In Part A, cloths were heated at 100°C for 1 h in various concentrations of NaOH solutions, washed with 1 N HCl and H₂O and, allowed to immobilize glucoamylase as above and assayed for enzyme activity. In Part B, regeneration of glucoamylase cloth and immobilization were repeated and enzyme activity was measured after each immobilization.

Table 6. Regeneration of E. coli aspartase naphthyl cloth with NaOH (a)

No. of regeneration times	Enzyme activity
0	3.6
1	3.5
2	3.5
3	3.5

(a) E. coli aspartase was adsorbed to 2 cm squares of naphthyl cloth and assayed for enzyme activity as described in Methods. The cloth was heated in 2 M NaOH at 100°C for 1 h, washed with 1 N HCl and H₂O, allowed to adsorb aspartase and assayed as above. Regeneration of the cloth and adsorption of aspartase was repeated and enzyme activity was determined after each adsorption. The enzyme activity is expressed as 10⁻⁴ moles of aspartase produced per min per g of cloth.

use of hydrophobic cloth in commercial enzyme reactors.

Immobilization of immunoglobulin G

Currently cyanogen bromide activated insoluble polysaccharides such as agarose gel or cellulose fiber are most commonly used to prepare immunoabsorbents (Reeves et al., 1981., Zoller et al., 1976). Industrial advantages of cotton cloth over these materials have been discussed in the preceding sections.

Since immunoglobulins are known to be hydrophobic and bind to the surface of polystyrene or polyvinyl chloride film, they should tightly bind to hydrophobic cloth. This will then provide a simple and mild method for preparing immunoglobulin adsorbents which can be used for purification or concentration of antigens in a packed column. Such a possibility was examined in the following preliminary investigations.

First, adsorption of human IgG to naphthyl cloth and unmodified cloth was determined. Table 7 (Part A) shows that naphthyl cloth exhibited greater IgG adsorption than unmodified cloth though saturating amounts of IgG were not used due to the expense. A greater difference between the modified and unmodified cloth is likely to be observed if the naphthyl cloth were saturated with IgG. To test whether adsorbed human IgG is immunologically active, IgG bound cloth was incubated with goat anti human IgG-alkaline phosphatase conjugate (antibody prepared against human

Table 7. Adsorption of human immunoglobulin G (IgG) to naphthyl cloth (Part A) and its ability to interact with its antibody (Part B).

<u>Part A</u> ^(a)	<u>IgG adsorbed</u>
Naphthyl cloth	60
Unmodified cloth	7

(a) Human IgG was adsorbed to naphthyl cloth and unmodified cloth and adsorbed IgG was assayed as described in Methods. IgG adsorption is expressed as mg IgG adsorbed per g of cloth.

<u>Part B</u> ^(b)	<u>Alkaline phosphatase activity</u>
Naphthyl cloth	2.4×10^{-7} (65 %) ^(c)
Unmodified cloth	1.6×10^{-7} (43 %)

(b) Human IgG was adsorbed to 0.5 cm squares of naphthyl cloth and unmodified cloth. To each cloth was added 100 μ l of goat anti human IgG-alkaline phosphatase conjugate, which in a free form hydrolyzed 3.7×10^{-7} moles of p-nitrophenyl phosphate per min. The enzyme activity bound is expressed as moles of p-nitrophenyl phosphate hydrolyzed per min when the cloth was shaken in 2.5 ml of the substrate solution.

(c) The activity of adsorbed enzyme activity expressed as % of the activity as free enzyme added to the cloth.

IgG in goat and subsequently covalently linked to alkaline phosphatase). If adsorbed IgG is immunologically active, it will bind the antibody having alkaline phosphatase activity. Table 7. (Part B) shows that the naphthyl cloth exhibited 65 % of the enzyme activity (as free enzyme) of the conjugate. Since an immobilized enzyme is generally half as efficient as a free enzyme, this suggests that all anti human IgG was bound to human IgG adsorbed on cloth. The concentration of anti human IgG-enzyme conjugate was not specified in the preparation used. However, this result suggests that the amount of the conjugate added was less than the binding capacity of the human IgG adsorbed to naphthyl cloth. The greater difference between the modified and unmodified cloth should be observed if naphthyl cloth were treated with larger amounts of the conjugate. Encouraged by this preliminary results, scientists at the Animal Diseases Research Institute, Agriculture Canada are now developing immunoabsorbents from bovine IgG prepared against Brucella abortus (cattle pathogen) to concentrate this pathogen or its antigens from cattle for diagnostic purposes.

Conclusions

All the enzymes tested (β -galactosidase, β -glucosidase, glucoamylase and aspartase) were easily immobilized onto hydrophobic cloth by incubation at 25°C. The results with β -galactosidase and β -glucosidase shows

that the adsorbed enzymes were nearly 50 % as effective as free enzymes. The column packed with glucoamylase and aspartase cloth showed good flow characteristics: space velocity of 10 could be readily obtained without any difficulty. Immobilized glucoamylase and aspartase showed little or no change in activity after 4 weeks exposure to reaction conditions. The results with glucoamylase and aspartase showed that hydrophobic cloth is ideal for immobilizing enzymes whose substrate is either large-sized or highly ionized. Hydrophobic cloth showed its potential as media for immunoadsorbent. A simple and economic regeneration of hydrophobic cloth was developed.

Materials and Methods

Materials

Octyl and phenyl Sepharose were obtained from Pharmacia Fine Chemicals. Glutaraldehyde (25 % w/w) was obtained from Aldrich Chemical Co; and 3, 5 Dinitro-salicylic acid (DNS) from Eastman Organic Chemical Co. Aspergillus niger glucoamylase was a gift from Miles Laboratories. Human immunoglobulin G (IgG) (I-4506), antihuman IgG (γ -chain specific): alkaline phosphatase conjugate (A-3150), polyvinylpyrrolidone (pVp-40) and Tween 20 were obtained from Sigma Chemical Co.

Methods

Immobilization of β -galactosidase and β -glucosidase

E.coli 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 $\mu\text{g}/\text{ml}$. Almond β -glucosidase (Sigma G8625) was dissolved in 0.05 M sodium acetate buffer (pH 5.6) to a final concentration of 200 $\mu\text{g}/\text{ml}$. A 2 cm square of hydrophobic cloth was covered with 100 μl of enzyme solutions and left for 16 h (O/N) at 25°C. The cloth was then soaked in 3 ml of buffer for 30 min at 25°C and the steep liquid was removed for assaying the unbound enzyme. The cloth was washed with the buffer and assayed for the immobilized enzyme. For enzyme adsorption to agarose gel 100 μl of an enzyme solution was mixed with 1 g wet gel (about 0.03 g dry weight). After 16 h, (O/N) at 25°C, the gel

was washed with 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 -galactosidase in the 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) (Craven *et al.*, 1965) or 400 nm (for β -glucosidase) (Conchie *et al.*, 1967).

Immobilization of glucoamylase (from *Aspergillus niger*)

A. niger glucoamylase (Miles lab) was found to have the specific activity of 0.27 g glucose produced per min per mg proteins. A 2 cm square of naphthyl cloth was soaked in 0.5 ml (about 170 mg protein) of *A. niger* glucoamylase preparation for 16 h (O/N) at 25°C. The cloth was washed with H_2O to remove unbound enzyme, soaked in 0.2 % glutaraldehyde at 25°C for 30 min and then washed with H_2O . To remove any starch elutable enzyme the cloth was shaken at 50°C in 2 ml of 5 % soluble starch for 10 min and washed with H_2O . This procedure was repeated and the washed cloth was blotted and assayed for immobilized glucoamylase.

Assay of immobilized glucoamylase

A cloth containing immobilized glucoamylase was shaken at 320 rpm and 50°C in 2 ml of 5 % soluble starch

(pH 4.4) in a test tube (16 mm diameter). After 10 min a 100 μ l sample was withdrawn and added to a mixture containing 5.9 ml of H_2O (to dilute sample) and 4.0 ml DNS. The mixture was heated for 10 min in boiling water for development of colour due to reduced DNS (Miller, 1959). After cooling, a 1 ml sample was mixed with 4.0 ml H_2O and absorbance at 540 nm was determined against a blank. DNS reagent was prepared as follows : To 5.0 g of 3,5, di-nitrosalicylic acid; 8 g of NaOH and 150 g of sodium potassium tartarate was added. Water was added to make volume upto 500 ml, and the mixture warmed with continuous stirring till a homogenous solution was obtained. A standard curve correlating absorbance to glucose concentration(%) was constructed (Fig. 10). The specific rate of glucose production by the immobilized glucoamylase was calculated according to the following equation.

Specific rate = amount (in g) of glucose produced per min per gram of cotton cloth.

$$= \frac{X}{100} \times (\text{dilution factor}) \times \frac{(\text{ml of starch})}{\text{solution}} \times \left(\frac{1}{\text{weight of cotton (g)}} \right) \times \left(\frac{1}{10 \text{ min}} \right)$$

where X is concentration of glucose (%) of a diluted sample determined from the standard curve; dilution factor is 60; the volume of the starch solution is 2 ml.

Assay of free glucoamylase

A 10 μ l of A. niger enzyme solution was added to 2 ml of 5 % starch solution (pH 4.4) and shaken for

Fig 10. Standard curve for glucose assay by the dinitrosalicylic acid (DNS) method. Glucose (%) refers to concentration of standards. 6.0 ml of each standard was mixed with 4.0 ml of DNS reagent, heated at 100°C for 10 min, mixed with 4 volumes of H_2O , and absorbance at 540 nm (A_{540}) was measured against a blank.

10 min at 320 rpm at 50°C. The rest of the experimental procedure was the same as that used for assaying immobilized enzyme.

Specific activity = amount of glucose produced per min per mg protein.

$$= \frac{X}{100} \times \frac{\text{(dilution)}}{\text{factor}} \times \frac{\text{(ml of starch)}}{\text{solution}} \times \frac{\text{(1)}}{\text{mg of protein in 10 } \mu\text{l enzyme}} \times \frac{\text{(1)}}{10 \text{ (min)}}$$

Glucoamylase column

Naphthyl cloth segments (0.5 cm square) were soaked in A.niger glucoamylase for 16 h (O/N) at 25°C. The segments were washed with H₂O, soaked in 0.2 % glutaraldehyde for 30 min at 25°C, washed with H₂O and packed into a jacketed column (10 mm diameter) to a bed volume of 10 ml. A 5 % soluble starch (pH 4.4) suspension in H₂O was pumped into the column (equilibrated at 50°C) at various space velocities. The degree of hydrolysis was calculated from the concentration of glucose in the effluent obtained after pumping through about 10 bed volumes of the starch solution. The glucose was assayed by DNS reagent (as described before).

Preparation of aspartase from E.coli Crooke's strain

The following growth medium was developed by S. Sontakke (unpublished) which was found to induce a high level of aspartase in E.coli Crooke's strain. One litre of the medium contains 11.6 g of fumaric acid, 0.12 g of MgSO₄, 0.5 mg of FeSO₄ and 6.8 g of KH₂PO₄.

The pH of the medium was adjusted to 7.3 with NH_4OH . 10 ml of the medium was inoculated with E. coli Crooke's strain. and shaken at 37°C . After overnight it was mixed with 500 ml of fresh medium and shaken at 37°C until an $A_{500} = 2$. The culture was then centrifuged at 20,000 g for 20 min at 4°C and the pelleted cells were re-suspended in 0.01 M Tris HCl buffer (pH 8.0) containing 0.01 M MgCl_2 and 1 mM sodium dithionite and centrifuged again at 20,000 g for 20 min. The pellet was transferred to a preweighed, precooled mortar and frozen at -90°C . The frozen pellet was ground with washed alumina (twice the wet weight of cells), then mixed with twice the volume of the above buffer, and given additional grinding. The mixture was centrifuged at 20,000 g for 30 min. The resulting supernatant was then centrifuged at 30,000 g for 30 min. The supernatant was stored in aliquots of 0.5 ml at -90°C . The specific activity of the preparation was 3.14×10^{-5} moles of aspartic acid produced per min per mg of proteins.

Immobilization of aspartase on naphthyl cloth

To each 2 cm squares of naphthyl cloth 200 μl of cell free extract of E.coli Crooke's strain (prepared as above) was added and left for 16 h (O/N) at 25°C . The cloth was washed with H_2O , then soaked in 2 ml of substrate (to remove any weakly adsorbed or substrate elutable enzyme) for 10 min at 25°C , washed with H_2O and then assayed for aspartase activity.

Assay of immobilized aspartase

A naphthyl cloth containing immobilized aspartase was shaken at 320 rpm and 37°C in 2 ml of the substrate solution in a test tube (16 mm diameter). The substrate solution consisted of 1 M fumaric acid, NH_4OH (added to obtain pH 8.5), 1 mM MgCl_2 and 1 mM sodium dithionite. After 10 min a 100 μl of sample was transferred to a 20 ml scintillation vial containing 100 μl of 5 M NaOH. The mixture was heated (for at least 10 min) to dryness. After cooling, 100 μl of 5 N HCl was added and heated (for at least 10 min) to evaporate to dryness. After cooling, 10 ml of 0.1 M sodium borate buffer (pH 8.2) was added to suspend the residue. To 1 ml of the above suspension, 1 ml of 1 mg/ml of trinitrobenzene sulphonic acid in 0.1 M borate buffer (pH 8.2) was added and incubated at 50°C. After 1 h, 2 ml of 0.1 N HCl was added and absorbance at 340 nm was measured against blank. A standard curve correlating absorbance to concentration of aspartic acid was constructed (Fig.11). This colorimetric method was developed by S. Sontakke (unpublished). The rate of aspartic acid production was expressed as moles aspartic acid produced per min per g of cotton cloth.

Fig. 11 Standard curve for aspartic acid assay by the trinitrobenzenesulphonic acid method. Aspartic acid (10^{-6} mole/ml) refers to the concentration of standards. One ml of each standard was mixed with 1 ml of the trinitrobenzenesulphonic acid solution (1 mg/ml), heated at 50°C for 1 h, and mixed with 2 ml of 0.1 N HCl, and absorbance at 340 nm was measured against a blank.

Adsorption of human IgG on naphthyl cloth

300 μ l of 10 mg/ml human IgG in coating buffer was applied to 2 cm square of naphthyl cloth or unmodified cloth. After 6 h at 25°C, the cloth was washed with PBST buffer. The adsorbed IgG was eluted by heating at 100°C in 2 ml of 1% SDS for 10 min, and assayed for protein according to Lowry (1951), using human IgG as a standard.

Assay of adsorbed human IgG with goat antihuman IgG-alkaline phosphatase conjugate.

100 μ l of 10 mg/ml human IgG in coating buffer was applied to 0.5 cm square of naphthyl cloth or unmodified cloth. After 6 h at 25°C, the cloth was washed with PBST buffer (see below) and then soaked in 2% BSA at 25°C for 16 h (O/N). (to saturate any unoccupied hydrophobic sites). and finally washed with PBST buffer. 100 μ l of goat anti-human IgG-alkaline phosphatase conjugate in enzyme conjugate buffer (see below) was added to the cloth. After 5 h at 25°C, the cloth was washed with PBST and assayed for alkaline phosphatase activity in the conjugate adsorbed to human IgG in the cloth. For this assay, the cloth was shaken at 25°C for 5 min in 2.5 ml of 5 mg/ml p-nitrophenyl phosphate in substrate buffer. 2 ml of the reaction mixture was mixed with 0.5 ml of 3 M NaOH and absorbance at 405 nm of the resulting colour was measured against a blank. The enzyme activity is expressed as moles of

p-nitrophenyl phosphate hydrolyzed per min.

Buffers used :

PBST (pH 7.4)

NaCl	8.00 g
KH ₂ PO ₄	0.20 g
Na ₂ HPO ₄	1.15 g
KCl	0.20 g
NaN ₃	0.20 g

Make up to 1 liter.

Add 0.5 ml Tween 20 per liter.

Coating buffer (pH 9.6)

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
NaN ₃	0.20 g

Make up to 1 liter with H₂O.

Enzyme conjugate buffer

Add PVP (final 2 %) and BSA (final 0.2 %) to PBST.

Substrate buffer (pH 9.8)

Diethanolamine	97 ml
H ₂ O	.800 ml
NaN ₃	0.20 g

Make up to 1 liter with H₂O.

Chapter 3

The Potential of Deacetylated Cellulose
Acetate Cloth as an Affinity Chromatography
Support in Commercial Purification Processes.

Introduction

Affinity purification makes use of the specific affinity between a substance to be purified and a molecule (a ligand) to which it can bind (Scouten, 1981; Yamazaki, 1984^(a)). The ligand should be in a readily recoverable (e.g. sedimentable) form or immobilized onto an insoluble support. In affinity chromatography, the immobilized ligand is packed in a column. When a crude sample containing a wanted substance is passed through such a column, the substance is retained on the column while unwanted molecules are washed away. The substance can then be eluted with a solution of the ligand or its structural analogs which have the same specificity, or by changing the conditions in which the substance no longer binds to the ligand tightly. Several requirements must be met for successful affinity purification. The insoluble or readily recoverable matrix containing the ligand should not adsorb unwanted molecules and should be physically and chemically stable during the purification procedure. Binding to the ligand should be specific and relatively tight but it should be possible to elute the wanted substance without destroying it. The ligand should be attached to the matrix in such a form that its specificity and binding properties are least affected and it is readily accessible to the binding site on the wanted substance. If the substance of interest is a macromolecule it is often necessary to attach a ligand to a flexible "spacer arm" (an elongated chain consisting of C-C, C-O, and/or C-N bonds), so that the ligand can

reach into the binding site of the substance without having steric hindrance from the matrix. Ideally the spacer arm should not contain ion-exchange or hydrophobic properties which result in non-specific adsorption. The matrices should have functional groups which can (or can be activated to) form covalent bonds with a ligand or one end of spacer arm (if required). The other end of a spacer arm should have appropriate groups capable of forming covalent bonds with the ligand.

Purification procedures in affinity chromatography are relatively simple, and if successful a very high degree of purification can be achieved in a single step. Thus affinity chromatography has a great potential in commercial processes for purification of biomolecules (antibodies, hormones, enzymes, etc.). In laboratory investigations, agarose and its derivatives are used as insoluble support for affinity chromatography because they have reactive hydroxyl (OH) groups and possess a minimal charge (thus exhibiting minimal adsorption), provide reasonable flow characteristics in a short column and tolerate broad ranges of pH and ionic strength. However, as described in General Introduction, these supports are relatively expensive and deformable when used in a tall column. Commercial processes would desire an inexpensive affinity support which has appropriate physical and chemical properties.

This chapter will demonstrate the potential of deacetylated cellulose acetate cloth as affinity chromatography

support. As discussed in General Introduction, cellulose fabric such as cotton cloth has advantages over agarose derivatives as chromatographic supports for commercial processes. However, a major problem associated with cellulose is that the OH groups are hydrogen bonded to each other (thus forming chemically stable crystalline structure) and are thus not amenable to chemical modification. To disrupt the hydrogen bonding, harsh conditions are generally required while activation reactions used for affinity chromatography requires relatively mild conditions. In cellulose acetate cloth, the OH groups are about 80 % acetylated. If the removal of acetyl groups (deacetylation) does not effect the fabric strength, deacetylated cellulose acetate cloth can be used as affinity chromatography support since its OH groups would be more freely available for chemical modification than those in cellulose cloth. This chapter examines such a possibility.

Results and Discussion

Deacetylation of cellulose acetate (about 80 % acetylated) can be performed in a mild alkaline solution (eg. 0.1 M NaOH) at room temperature (25°C), (Chen and Tsao, 1976). Soaking in 0.1 M NaOH at 25°C for 16 h (O/N) was used here. Preliminary study showed that cellulose triacetate (100 % acetylated) cloth even after this treatment, generated little reactive OH groups when assayed by the method to be described later.

Since triacetate has its OH groups fully acetylated, it is soluble in non-polar solvents. Triacetate thread is made by extruding a solution of triacetate (in a non-aqueous solvent into hot air (Durso, 1978)). Since the thread strength results from hydrophobic interactions, aqueous NaOH solution may not be able to penetrate the inside of the thread. Cellulose acetate on the other hand is less hydrophobic and thus permeable to the NaOH solution. The soaking of cellulose acetate cloth in 0.1 M NaOH at 25°C for 16 h (O/N) did not change its fabric strength but generated the reactive OH group as demonstrated below. Therefore, the NaOH treated cellulose acetate cloth was used throughout.

Cyanogen bromide activation

Cyanogen bromide (CNBr) can activate two adjacent OH groups in polyhydroxy supports such as agarose (Fig. 12A). The activated supports can readily immobilize NH_2 containing substances such as lysine or polypeptides with lysine residues, and have been most commonly used in affinity chromatography.

If the 2- and 3-OH groups of cellulose acetate can be deacetylated, the resulting adjacent OH groups should readily react with CNBr. To test this, cellulose acetate cloth was treated with 0.1M NaOH (25°C, 16 h), and treated with CNBr. To determine the extent of activation, lysine binding to the activated supports was measured by using $[^{14}\text{C}]$ lysine of known specific activity. As control, cotton cloth was similarly activated with CNBr and assayed

for lysine binding. Table 8 shows that CNBr- activated deacetylated cellulose acetate cloth bound about 3.5 times more lysine than activated cotton cloth. Thus, deacetylated cellulose acetate cloth is more suitable as affinity chromatography support than cotton cloth. CNBr activated supports can be used to immobilize macromolecular ligands for affinity chromatography. For example, an immunoglobulin immobilized on CNBr activated agarose can be used to purify a specific antigen (Pillion et al, 1980; Hollingshead et al, 1982). A very small amount of lysine also bound to both cotton cloth and deacetylated cellulose acetate cloth which had not been activated with CNBr (Table 8). The mechanism for this binding is not known.

Butanediol diglycidyl ether activation

Since CNBr activated supports do not provide a "spacer-arm", small ligands immobilized to these supports cannot freely interact with the binding site of a macromolecule which is to be purified. A bisoxirane such as 1,4 butanediol diglycidyl ether (BDGE) has been used to introduce an activated spacer arm. As shown in Fig. 12B, it consists of a relatively hydrophilic chain having two terminal epoxy groups which can react with amino, OH or thiol groups. This bifunctional reagent forms an ether linkage when it reacts with an OH group of a support. One remaining epoxide can react with a ligand that contains a hydroxyl, an amino or thiol group to yield an affinity adsorbent (Scouten, 1981). BDGE- activated agarose derivatives are available, but too expensive to be used for

Fig 12. Chemical modifications of OH group for affinity chromatography.

(A) Cyanogen bromide (CNBr) activation

Activation

Coupling

Table 8. Lysine binding to cyanogen bromide activated deacetylated cellulose acetate and cotton cloth

Support	Lysine bound (a)
<hr/>	
<u>CNBr-activated</u>	
Cotton cloth	1.0×10^{-5}
Deacetylated cellulose acetate cloth	3.5×10^{-5}
 <u>Unactivated</u>	
Cotton cloth	3×10^{-8}
Deacetylated cellulose acetate cloth	7×10^{-8}
<hr/>	

(a) Moles of lysine bound to one gram of support. Data represent the average of triplicate samples.

commercial affinity chromatography. Therefore, BDGE activation of deacetylated cellulose acetate cloth was studied. To determine the extent of BDGE activation, lysine binding was measured as before.

A higher pH accelerates the reaction of BDGE with the OH group but the epoxide group will be hydrolyzed at extreme pH values. A preliminary experiment showed that deacetylated cellulose acetate cloth became increasingly fragile in NaOH solutions higher than 1M NaOH. First, BDGE activation of deacetylated cellulose acetate was examined in reaction mixture having different ratios of 1M NaOH and BDGE. Fig. 13A shows that the 1:1 mixture gave the best activation and the optimal reaction time was 5 h.

Pharmacia (producer of BDGE - activated agarose) recommends the use of pH 11 at 40°C for reacting the epoxy-group with an amino group of a ligand. The results in Table 9 confirmed this recommendation, that is, use of lower pH or temperature resulted in lower lysine binding to BDGE activated cloth. In this experiment lysine binding was performed at 40°C for 2 days. Fig. 14 shows that a 2 day incubation was sufficient to maximize lysine binding. Fig. 14 also shows that after BDGE activation deacetylated cellulose acetate cloth bound about 3.4 times more lysine than cotton cloth.

The results of this chapter show that deacetylated cellulose acetate cloth is rich in OH groups which are amenable to activation by CNBr and BDGE. The optimal condi-

tions for BDGE activation and reaction with NH_2 group in a ligand have been determined. While one gram of BDGE cloth bound 3.4×10^{-5} moles lysine, one gram of commercially available epoxy activated agarose has approximately 5×10^{-5} mole of epoxy group (Table 1). The 1984 price of this agarose derivative is about U.S.\$ 20 per gram.

Since cellulose acetate cloth is much less expensive than agarose, it has the potential to be used for commercial purification processes. However, final evaluation must await its actual applications to purification of variety of biomolecules.

Conclusions

Deacetylation of cellulose acetate cloth produced a cloth which had at least three times as many reactive OH groups as cotton cloth. Since CNBr activated and BDGE derivatives of such a cloth retained desirable fabric structure and strength, affinity chromatography media derived from these activated supports would be suitable for a large scale column operation. The CNBr activated derivative can be used to immobilize macromolecule ligand for affinity chromatography of compounds which specifically interacts with the macromolecule. The BDGE derivative would be suitable for affinity purification of macromolecules as it provides a spacer. Affinity media derived from deacetylated cellulose acetate cloth are less expensive than those derived from cross-linked agarose, and thus are more suitable in commercial processes.

Fig 13. Determination of conditions for BDGE activation.

Part A . Effect of ratios of 1 M NaOH to BDGE. De-acetylated cellulose acetate cloth was soaked in reaction mixtures having different ratios of 1 M NaOH (containing 2 mg of NaBH_4 per ml) to BDGE. After shaking at 25°C for 5 h, the activated cloth was reacted with lysine at pH 11 and 40°C for 1 day. Lysine bound represents 10^{-5} moles per g of cloth.

Part B. Effect of reaction time. The reaction conditions are the same as above except that the reaction time was varied.

Table 9. Effect of pH and temperature on lysine binding to BDGE-activated cloth^(a)

Cloth	Conditions for lysine binding	Lysine bound
Cellulose acetate	40°C, pH 11	3.2×10^{-5}
Cellulose acetate	25°C, pH 11	1.5×10^{-5}
Cellulose acetate	40°C, pH 10	1.5×10^{-5}
Unactivated control ^(b)	40°C, pH 11	5×10^{-8}
Cotton cloth	40°C, pH 11	1.0×10^{-5}
BDGE-agarose ^(c)	-	5×10^{-5}

(a) BDGE activation of deacetylated cellulose acetate cloth and cotton cloth (2 cm squares) was performed by shaking in a mixture of 1 ml of 1 M NaOH containing 2 mg NaBH₄ and 1 ml of BDGE. Cloth was reacted with lysine for 2 days either at 40°C or 25°C or at pH 11 or 10. Lysine bound represents 10^{-5} mole per g of cloth. The data are results of duplicate samples.

(b) Deacetylated cellulose acetate was incubated at 25°C for 5 h without BDGE and assayed for lysine binding.

(c) Epoxy activated agarose available from Pharmacia has approximately 5×10^{-5} moles of epoxy group per g of gel.

Fig 14. Effect of time on lysine binding. BDGE
activation of deacetylated cellulose acetate cloth
and cotton cloth was performed as described in
Table 3. Lysine binding was carried out at 40°C
at pH 11 for different periods of time. Lysine
bound represents 10^{-5} mole lysine per g of cloth.
Deacetylated cellulose acetate cloth (●); cotton
cloth (○).

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Materials and Methods

Cellulose acetate was obtained locally. One square cm of cotton cloth weighs 13 mg and cellulose acetate 9 mg. 1,4, Butanediol diglycidylether (BDGE) (Sigma No. B-7381) was obtained from Sigma Chemicals Co. ; Cyanogen bromide from J.T. Baker Co.; (^{14}C) lysine from New England Nuclear Corporation.

Deacetylation of cellulose acetate cloth

2 cm square pieces of cellulose acetate cloth were soaked in 0.1M NaOH at 25°C for 16 h. Cotton cloth (control) was also treated in the same manner.

Cyanogen bromide activation

CNBr activation was carried out according to the method of Hoffman et al. (1981) which is the modification of the method by March et al. (1974). One gram of deacetylated cellulose acetate or cotton cloth pieces (2 cm squares) were rinsed once with 2M Na_2CO_3 , and then resuspended in 15 ml of 2M Na_2CO_3 for 30 min. One gram of cyanogen bromide was dissolved in 0.5 ml acetonitrile (in fume hood) and then added to the suspension. The suspension was mixed vigorously for 2 min and then rapidly filtered. The activated pieces were washed quickly five times with 100 ml each of 0.01M NaHCO_3 and six times with 100 ml of cold distilled water, and stored at 4°C until use.

Lysine binding to CNBr activated cloth

To each 2 cm square pieces of the activated cloth, 0.8 ml of 0.1M NaHCO_3 (pH 8.3) and 0.2 ml (^{14}C) lysine

solution (5 mM ; specific activity of 0.01 Ci/mole) were mixed. After incubation at 25°C for 2 h, the cloth pieces were washed with water, dried, weighed and counted in 10 ml of toluene based scintillation fluid. The amount of lysine bound to the cloth was calculated by comparing radioactivity bound to the cloth with the known amount of the (^{14}C) lysine solution dried on the cloth (counted in the same manner).

BDGE activation

To each 2 cm square piece ~~of~~ deacetylated cellulose acetate or cotton cloth, 1 ml of 1 M NaOH (containing 2 mg NaBH_4) and 1 ml of 1,4 butanediol diglycidyl ether (BDGE) was added and shaken for 5 h 25°C. The pieces were then washed thoroughly with H_2O and stored at 4°C until use.

Lysine binding to BDGE- activated cloth

To each 2 cm square piece of BDGE- activated cloth, 0.8 ml of 0.2 M sodium carbonate buffer (pH 11) and 0.2 ml of (^{14}C) lysine solution (5 mM; specific activity of 0.01 Ci/mole) were added. After incubating at 40°C for 2 days, the pieces were washed thoroughly with H_2O , dried, weighed and counted in 10 ml of toluene based scintillation fluid.

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