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Modification of reversed-phase columns with dyed surfactants

Preparation of mechanically resistant efficient immobilized dyes for protein purification

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ABSTRACT

The properties of reversed-phase chromatographic supports can be substantially altered by saturating the hydrophobic sites at their surface with easily prepared dyed non-ionic surfactants. The dissociation constants governing the interaction between a reversed-phase support and a model dyed surfactant were found to be in the micromolar range under several mobile phase conditions. The total amount of modified surfactant immobilized on the reversed-phase support was also measured and found to be as great as that usually immobilized on agarose supports (expressed as micromoles per millilitre of support). A reversed-phase column saturated with dyed surfactant can be used with the same aqueous mobile phases as used with immobilized dye columns prepared with agarose as a supporting matrix, but the mechanical sturdiness of the silica matrix allows the use of higher flow-rates. This methodology was used to screen several dyes to find the one best suited for a given purification. A convenient procedure (with affinity elution) was devised for the purification of pancreatic ribonuclease and chymotrypsinogen.

INTRODUCTION

Surfactants have, for several years, been added to the mobile phase for chromatography on reversed-phase supports in techniques such as ion-pair chromatography [1] and micellar liquid chromatography [2]. In both techniques, the surfactants are present at defined concentrations in the mobile phases and organic solvents are used to control the retention of injected solutes.

Surfactants can also be used to modify the surface properties of a reversed-phase column so as to be able to use it thereafter with purely aqueous mobile phases

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(with no free surfactant added to the mobile phase). Ionic surfactants can be used to prepare columns from reversed-phase supports, which perform essentially as ion exchangers [3]. An affinity chromatography support can be prepared by grafting a suitable ligand onto the polar end of a non-ionic surfactant [4].

We have used the latter approach to prepare immobilized dye columns which can withstand high flow-rates. There are several ways of preparing mechanically resistant immobilized dye columns: dyes or derivatives thereof can be directly covalently grafted onto covalently modified silica [5,6], polymer-coated silica [7,8] or mechanically resistant polymeric supports [8].

The use of dyed non-ionic surfactants to prepare immobilized dye columns is not conceptually very different from another approach in which dyes grafted onto perfluorinated alkane tails are immobilized on polytetrafluoroethylene beads [9].

EXPERIMENTAL

Materials

The non-ionic surfactants Brij 76 (decaethylene glycol *n*-octadecyl ether) and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). The reactive dyes were a generous gift from ICI France (Clamart, France). The list of dyes used and abbreviations (taken from ref. 10) used to identify them in the text are given in Table II. Bovine pancreas acetone powder and yeast RNA were purchased from Sigma. Sep-Pak C_{18} cartridges and preparative C_{18} reversed-phase packing material (55–105-μm particle size, 60 Å pore diameter; ref. No. 51 922) were purchased from Waters Assoc. (Milford, MA, USA). All other chemicals and solvents were purchased from Merck (Darmstadt, Germany).

Synthesis of dyed surfactants

A modification of the method developed by Johansson and Joelsson [11] for the synthesis of dyed polyoxyethylene derivatives was used. Dye (0.4 g) was added to Brij 76 (12.5 g) dissolved in 100 ml of 0.2 *M* potassium hydroxide solution and the mixture was stirred overnight at 50°C.

Purification of Procion Navy HE-R150-dyed Brij 76 (B4-Brij 76)

The crude synthesis mixture was passed at 3.0 ml/min through a Sephadex LH-20 column (100 cm × 5 cm I.D.) equilibrated with 5 m*M* ammonium hydrogencarbonate buffer (pH 8.3). The modified surfactant B4-Brij 76 together with unreacted Brij 76 were eluted in the void volume of the column. Deactivated and reactive B4 were washed from the column with distilled water.

The excluded peak (250 ml) from the Sephadex LH-20 column was chromatographed in 50-ml batches on a 100-ml column of specially prepared low-capacity anion exchanger (see below). The column was washed extensively with water to remove unmodified (hence uncharged) Brij 76 (the efficiency of column washing was checked by monitoring the column effluent for non-foaming when vigorously stirred, as described [4]). The B4-Brij 76 derivative was then eluted with 2 *M* sodium chloride solution, desalted by chromatography on a 6-cm bed of preparative C_{18} reversed-phase packing settled in a 1.5-cm diameter Buchner funnel: the dyed derivative was loaded onto the reversed-phase material, washed extensively with water and the dyed

Brij 76 eluted with methanol–isopropanol (3:2, v/v). The product was dried by rotary evaporation, dissolved in a small amount of water and lyophilized.

Low-capacity anion exchanger was prepared by reacting cyanogen bromide-activated Sepharose 4B [12] with 0.1 *M* ammonium chloride solution (0.046 mequiv. titratable groups per millitre of gel was found by automatic titration).

Dyed surfactants were analysed by chromatography on a Nucleosil C_{18} (100 Å) (Macherey, Nagel & Co., Düren, Germany) column (25 cm × 0.46 cm I.D.) obtained from Interchim (Montluçon, France). A 35-min linear gradient from 100% A to 100% B was used: eluent A was 0.05 *M* triethylamine containing 0.15 *M* acetic acid and eluent B was methanol–isopropanol (3:2, v/v). The absorbance of the column effluent was monitored at 607 nm and the flow-rate was 1.0 ml/min. The elution volume of unmodified surfactant was established by monitoring the ability of evaporated eluate fractions to foam after addition of water. Unmodified surfactant eluted after the dyed surfactant.

Quantitative analysis of the interaction between stationary phase and B4-Brij 76

We used a method described elsewhere [13]. A 150-mg amount of Waters preparative C_{18} reversed-phase material was packed into a Pharmacia FPLC HR 5/2 column. The mobile phase was 25 m*M* sodium phosphate–25 m*M* sodium acetate adjusted to pH 5.5, plus 0, 1 or 2 *M* sodium chloride. B4-Brij 76, dissolved in the mobile phase at $4.5 \cdot 10^{-6}$–$6.3 \cdot 10^{-5}$ mol/l, was loaded onto the column at 2.0 ml/min. When the effluent absorbance was equal to the absorbance of the incoming solution, the tubing upstream of the column was carefully rinsed with methanol–isopropanol (3:2, v/v). Dyed surfactant was stripped from the column with the same mixture. The amount of eluted dyed surfactant was determined spectrophotometrically after evaporating the organic solvents under reduced pressure and was corrected for the dead volume of the column. The dead volume was determined with tritiated water. The results were used to determine dissociation constants and the maximum amount of dyed surfactant retained by the column using the procedures of Kasai and Oda [14]. Data Desk and SAS software were used for statistical analysis of the experimental data.

Preparation of bovine pancreas acetone powder extract

Bovine pancreas acetone powder was suspended (10 mg/ml) in buffer A (25 m*M* sodium phosphate–25 m*M* sodium acetate, pH 5.5) containing 5% (w/v) PEG 6000 and shaken overnigth at 4°C. The extract was clarified by centrifugation.

Screening of dyed surfactants for ribonuclease (RNAse) purification

Crude synthesis mixtures of the dyed polyoxyethylene derivatives were diluted 15-fold with buffer A and 10 ml of each solution were injected on to Sep-Pak C_{18} cartridges with a syringe. Each cartridge was then connected to a pump and a sample injector (fitted with a 7-ml loop). Buffer A was flushed through the cartridge at 1 ml/min until no colour was eluted from the cartridge. Extract (1 ml) was then injected (note: some color was always desorbed during this first sample loading) and the cartridge was developed with 7 ml of buffer C (same composition as buffer A but adjusted at pH 7 and containing 2 *M* sodium chloride) and re-equilibrated with buffer A. A second injection of extract was done and the cartridge treated likewise (no

further dye bleeding was ever observed). The cartridge was then connected to a fraction collector and a third aliquot of pancreas extract was loaded onto it. The cartridge was developed successively with 5 ml of buffer A, 7 ml of buffer B (of the same composition as buffer A but adjusted to pH 7), 7 ml of buffer B containing 5 m*M* cytidine 5'-monophosphate (CMP) and 7 ml of buffer C. RNAse activity and protein concentration were assayed in the fractions of the column eluate.

Purification of RNAse A

Preparative C_{18} reversed-phase packing material was packed to a height of 2 cm in a 1.5-cm I.D. glass column and the column was washed extensively with methanol and deionized water. Dye-modified surfactant (0.18 m*M* in buffer A) was pumped through the column at 4 ml/min. When the effluent dyed surfactant concentration reached a constant value, the column was washed extensively with buffer A.

The column was loaded with 4 ml of bovine pancreas extract. Some colour was desorbed during this initial sample loading. The column was then washed with buffer C, re-equilibrated with buffer A and used for RNAse purification as follows: the flow-rate was 4 ml/min, 4 ml of crude extract were loaded at a time and then the column was washed with buffer A (5 min) followed by buffer B (4 min). Thereafter RNAse A was specifically eluted with 5 m*M* CMP dissolved in buffer B, then column was rinsed with buffer C, re-equilibrated with buffer A and subsequently reused in the same way several times.

Purification was also performed using a column loaded with 15-fold diluted crude synthesis mixture. The conditions for column pretreatment and for RNAse chromatography were identical with those given above.

Partially purified RNAse eluted from the immobilized dye column was loaded on a Nucleosil 100 C_4 (Macherey, Nagel & Co.) column (250 mm × 4.6 mm I.D.) equilibrated with 0.1% trifluoroactic acid (TFA) in water and the column was developed at 1 ml/min with a 1-h linear gradient between this latter mobile phase and 0.1% TFA in acetonitrile–water (1:1, v/v).

Other procedures

The activity of RNAse A was determined by the spectrophotometric method of Kunitz [15]. An activity unit is defined as the amount of enzyme causing a change of 1.0 absorbance unit/min. Chymotrypsinogen was assayed after trypsin activation using the chromogenic substrate benzyltyrosyl *p*-nitroanilide [16]. Proteins were assayed by the Bradford method [17]. Eluate fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [18] in 15% slab gels and stained with Coomassie Brilliant Blue. Elemental analyses were performed by the Centre de Microanalyse (CNRS, Vernaison, France).

RESULTS AND DISCUSSION

Purification and characterization of dyed surfactant

Most of the dyed surfactants were used as crude synthesis mixtures without further purification. B4-Brij 76 was purified by a combination of hydrophobic and ion-exchange chromatographic steps. The specially prepared ion exchanger with a low concentration of ion-exchange groups was used because B4-Brij 76 could not be eluted from several commercial anion exchangers.

The formula of Procion Navy HE-R150 is given in ref 19. As it contains two monochlorotriazine rings, dyed surfactants can conceivably be produced containing either one or two surfactant molecules. B4-Brij 76 was synthesized under different reaction conditions and the products were analysed by reversed-phase liquid chromatography (RPLC). The RPLC results for several molar ratios of dye and surfactant showed two species of dyed surfactants. The derivative with a single surfactant molecule per dye molecule largely predominates under the reaction conditions given above. Purified B4-Brij 76 was free from unmodified surfactant as appreciated from results of the analytical high-performance liquid chromatographic procedure described above.

Adsorption of purified dyed surfactant molecules on the preparative C_{18} support

Fig. 1 shows a double reciprocal plot which is similar to that described by Kasai and Oda [14]. The intercept with the ordinate gives the reciprocal of the maximum amount, Q_m, of dyed surfactant retained by the column. The intercept with the abscissa is the reciprocal of the dissociation constant between the dyed surfactant and sites which interact with it.

Statistical treatment of the experimental data indicated that K_d values measured with B4-Brij 76 are not significantly different. Even though interactions between the organic coverage of the reversed-phase support and the dyed surfactant are undoubtly hydrophobic in nature, the experimental results failed to prove any influence of the mobile phase ionic strength on the dissociation constant values. Dissociation constants are in the micromolar range.

The calculated values for Q_m (the amount of surfactant bound to the column from a mobile phase containing an infinite concentration of surfactant) are shown in Table I. The observed values for Q_m were compared with the amount of alkyl chains grafted onto the silica support. Assuming that all the carbon present in the reversed-

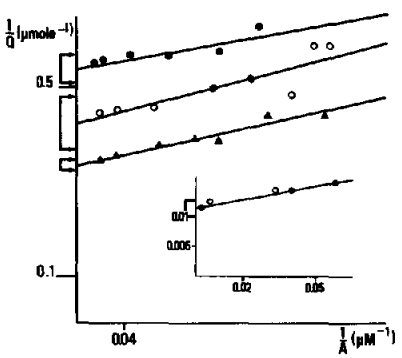

Fig. 1. Double reciprocal plot of the interaction between dyed surfactant and reversed-phase chromatographic support. Ordinate, reciprocal of the amount of dyed surfactant retained by the column; abscissa, reciprocal of incoming dyed surfactant concentration. Results in the main figure were obtained with Procion Navy HE-R150-Brij 76 and those in the inset with Triton X-100. Mobile phases for loading were (●) buffer A (○) buffer A plus 1 *M* NaCl and (▲) buffer A plus 2 *M* NaCl. The double arrows on the ordinate indicate the 0.95 confidence intervals for $1/Q_m$ values.

TABLE I

VALUES OF Q_m AND K_d OBTAINED BY GRAPHICAL ANALYSIS OF THE CURVES SHOWN IN FIG 1

Q_m is the total amount of dyed surfactant retained by the reversed-phase support. Results for Q_m are given with 0.95 confidence limits. K_d is the dissociation constant of the interaction between B4-Brij 76 and the reversed-phase support.

Parameter	Procion Navy HE-R150-Brij 76 dissolved in			Triton X-100 dissolved in buffer A
	Buffer A	Buffer A + 1 *M* NaCl	Buffer A + 2 *M* NaCl	
Q_m (μmol retained by the column)	1.85 ± 0.10	2.36 ± 0.36	2.99 ± 0.23	83.6 ± 7.7
Q_m (μmol/ml of reversed-phase support)	6.17 ± 0.33	7.87 ± 1.20	9.97 ± 0.77	278.7 ± 25.6
K_d (*M*)	$0.45 \cdot 10^{-6}$	$1.46 \cdot 10^{-6}$	$1.41 \cdot 10^{-6}$	$6.6 \cdot 10^{-6}$

phase support is derived from the alkyl chains, the microanalysis data indicate that there are 288 μmol of C_{18} chain per millitre of support. The value obtained with Triton X-100 is similar, suggesting that all the alkyl chains are involved in binding to Triton X-100 molecules, presumably via hydrophobic interactions with the hydrophobic tail of the non-ionic surfactant. A similar situation probably existed in the experiments described by Torres *et al.* [4], who obtained a very high level of adsorbed modified surfactant.

The Q_m values measured with B4-Brij 76 were much lower, 6.17–9.97 μmol/ml of support. This is probably because the substituent grafted onto the polar end of the surfactant is bulky compared with the pyridinium ring of the modified surfactant of Torres *et al.* [4] or the polar head of Triton X-100. Some steric hindrance would preclude crowding of dye molecules at a density similar to that of the alkyl chains grafted onto the reversed-phase support.

The composition of the mobile phase influenced the amount of dyed surfactant retained by the reversed-phase support (observed differences are significant, $p < 0.05$). A high ionic strength allowed more dyed surfactant molecules to be retained on the reversed-phase support. A similar effect was observed in the interaction of reversed-phase supports with ionic surfactants [20] and was attributed to a diminution of electrostatic repulsion between like charges at high ionic strength. The same explanation probably holds for dyed surfactants. The stacking of dyes is known to be favoured at high ionic strength [21].

When the amount of retained dye is expressed as micromoles per millitre of column volume, as is usually done for dyed soft gels, the figures are in the range considered satisfactory for agarose-based immobilized dyes (1–10 μmol/ml of gel [22]).

Rapid screening of dyed surfactant-loaded Sep-Pak cartridges for purification of RNAse (Table II)

B4-Brij 76 is satisfactory for RNAse purification: no enzyme was eluted during sample loading or during washing with buffer B. The enzyme is eluted with CMP at a

TABLE II

RESULTS WITH SEP-PAK C_{18} CARTRIDGES LOADED WITH SEVERAL DIFFERENT DYED SURFACTANTS

Abbreviations used to identify the different dyes are the same as used before [10]; i = irrelevant.

Dye	Unretained RNAse		RNAse eluted with buffer B		RNAse eluted with buffer B + 5 m*M* CMP	
	Purification factor	Yield (%)	Purification factor	Yield (%)	Purification factor	Yield (%)
Procion Navy HE-R150 (B4)	i	0	i	0	25.6	98
Procion Blue MX-R (B8)	0.9	53	11.5	50	i	0
Procion Blue HE-GN (B16)	1.1	69	5.2	32	i	0
Procion Brown H-3R (C3)	1.9	42	6.1	14	i	0
Procion Orange HE-R (O3)	0.6	40	27.3	71	i	0
Procion Red HE-3B (R1)	0.9	90	i	0	i	0
Procion Turquoise P-GX (T4)	1.3	47	5.7	46	2.5	4
Procion Green HE-4BD G1	1.3	71	2.1	14	2.5	13
Procion Yellow MX-GR (Y13)	1.4	100	i	0	i	0

satisfactory yield. O3-Brij 76 is also adequate as the enzyme is eluted from the column under mild conditions (pH 7) with a fairly high specific activity, but the capacity of the dyed column is lower than that of B4-Brij 76.

Other dyes were much less satisfactory. The cumulative yield obtained with the C3-Brij 76 column was low (an additional 5% of the total deposited activity could be eluted with buffer C).

The screening procedure was conducted with only a small number of dyes, which is probably why no immobilized dye suitable as a negative column for RNAse purification *i.e.*, absorbing most of the unwanted proteins but not RNAse, was identified [23].

Purification of RNAse

A representative chromatogram of the purification of RNAse on a dyed surfactant-loaded reversed-phase column is shown in Fig. 2. Fig. 3 shows the SDS-PAGE patterns of the eluate. Yields and purification factors are shown in Fig. 4.

The yield was almost quantitative after the column had been used six times (column prepared with purified B4-Brij 76) or four times (column prepared with crude synthesis mixture).

The low yields obtained during early use of the columns suggest that some

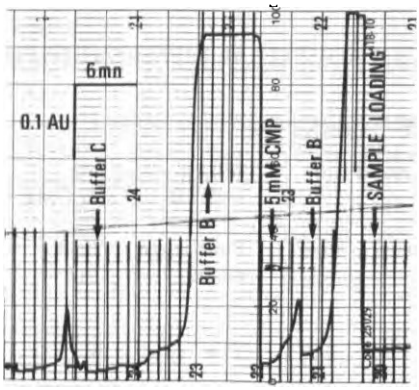

Fig. 2. Chromatography of extract of bovine pancreas acetone powder on a B4-Brij 76-loaded reversed-phase column. The dyed surfactant was loaded as a crude synthesis mixture. Column, 2.5 cm × 1.5 cm I.D.; flow-rate, 4.0 ml/min; chart speed, 12 cm/h.

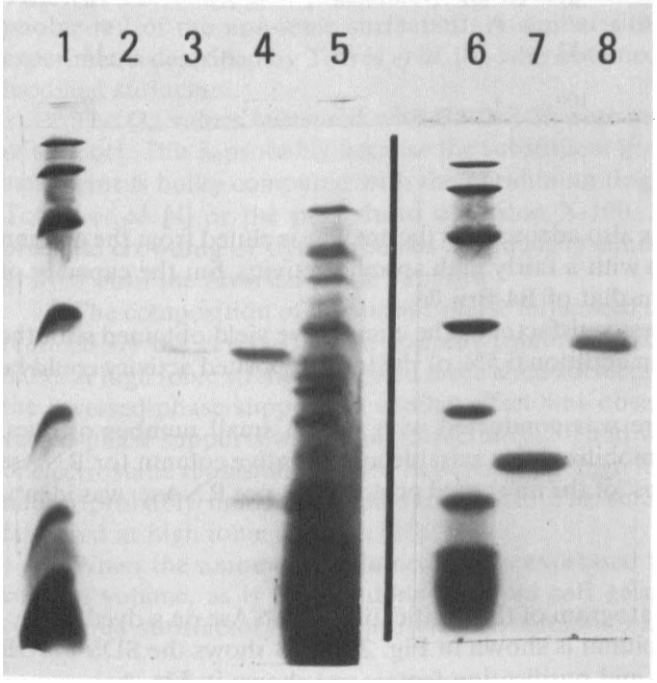

Fig. 3. SDS-PAGE on a 15% polyacrylamide gel [17]. Lanes 1 and 6 contain molecular weight standards (from top to bottom, 94, 67, 43, 30, 20.1, 14.4 and 6.6; lane 5 contains bovine pancreas acetone powder extract (prepared without PEG); lanes 3 and 4 contain aliquots of the proteins eluted from a B4-Brij 76-loaded reversed-phase column with 5 m*M* CMP; lanes 7 and 8 contain RNAse and chymotrypsinogen purified by subsequent reversed–phase chromatography.

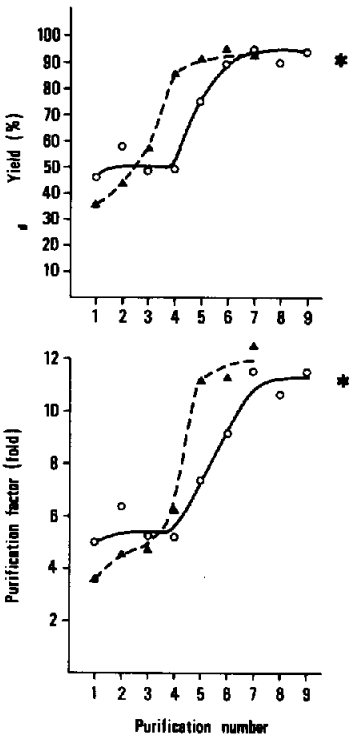

Fig. 4. Yields (top) and purification factors (bottom) for successive purifications of RNAse on (○) a purified B4-Brij 76-loaded column or (▲) a crude synthesis mixture-loaded column. The yield and purification factor obtained with a B4 agarose column are indicated by the asterisks.

RNAse is retained by interactions that are not reversed either by adding a ligand for the enzyme to the mobile phase or by increasing the ionic strength. These sites are quickly saturated, especially if the column is prepared using a crude synthesis mixture. These unwanted affinity sites are probably of a reversed-phase nature. Non-ionic surfactants are retained by the reversed-phase support and the crude synthesis mixture of dyed surfactant contains unmodified surfactant. Hence, a significant proportion of the alkyl chains of the reversed-phase support which cannot interact with the dyed surfactant (for the reasons given above) will interact with unmodified surfactant molecules, lowering the amount of reversed-phase sites later available to RNAse. Dyed surfactant-loaded columns were stripped of their modified surfactant layer by methanol–isopropanol (3:2, v/v) after several uses. Although the dyed surfactant molecules in the eluate precluded direct assay of RNAse activity, RNAse was detected in the eluate by SDS-PAGE (data not shown).

Although the yield of RNAse is not quantitative in the early runs with a dyed surfactant-loaded column, RNAse could be eluted by a known ligand of the enzyme (CMP has been used in the past to elute RNAse from a Cibacron Blue F3GA agarose column [24]). CMP-eluted enzyme was retained on the dyed surfactant loaded column only by a biospecific mechanism.

Purification factors also increased to a plateau during the first few uses of the column (Fig. 4). Thus RNAse probably contributes more than other proteins in the crude extract to coverage of the suspected remaining reversed-phase sites. The purification factor plateau values are equal to values obtained with the same dye immobilized on an agarose matrix (Fig. 4).

The SDS gels (Fig. 3) show that another protein was eluted from the dyed column by CMP. This protein was found to be chymotrypsinogen by specific assay using a chromogenic substrate after activation with trypsin.

Elution of chymotrypsinogen from an immobilized dye column by a phosphorylated nucleotide is not surprising. It has been shown [25] that it is worth trying many different eluents to elute a protein retained on an immobilized dye, without limiting such a screening to the known natural ligands of the protein. The two proteins were readily separated by reversed-phase chromatography: RNAse was pure, chymotrypsinogen was contaminated by a small amount of impurities which are barely visible on the gel (Fig. 3).

Because unmodified dye is retained on a reversed-phase material from a purely aqueous mobile phase, we checked the behaviour of RNAse injected onto a reversed-phase column loaded with unmodified or only deactivated Procion Navy HE-R150. When crude pancreatic extract was loaded on such a column large amounts of dye were desorbed, and no RNAse could be eluted with CMP. Hence the satisfactory results obtained with dyed surfactant-loaded reversed-phase columns were obtained because the non-polar part of the surfactant moiety anchored the dyed surfactant to the reversed-phase silica and the polar part acts as a spacer arm presenting proteins in the mobile phase with a ligand molecule free from other interactions with the support.

General comments and conclusion

This work has demonstrated that reversed-phase material can be modified by loading it with dyed surfactants and used in the same way as a conventional immobilized dye column with agarose as the supporting matrix. The flow-rates which can be used with these columns are fairly high because of the mechanical strength of the silica. Reversed-phase materials are readily available in a range of porosities and particle sizes, in contrast to the polytetrafluoroethylene beads used in a similar application [9]. Dyes can be very easily grafted onto non-ionic surfactants, and unfractionated synthesis mixtures can be used to screen rapidly several dyes to find the one best suited to a purification using ready-to-use reversed-phase cartridges. Also, one can plan to load successively the same reversed-phase column with different dyed surfactants, each suitable for purifying one given protein.

Nevertheless, the use of reversed-phase supports to immobilize dyed surfactants is not free from problems. First, the reversed-phase nature of the packing is not totally suppressed by the dyed surfactant-loading, which may explain the low yield of RNAse from a newly loaded column.

Second, although it had been claimed [4] that this approach allows the preparation of chromatographic supports with a very high density of immobilized affinity ligands, our data show that this is not so if the ligands are as bulky and charged as the reactive dyes. Nevertheless, the amounts of B4-Brij 76 retained by the reversed-phase material are about the same as amounts of dye grafted onto agarose-based supports. It should be remembered that an increased ligand loading is not always associated

with an increased column capacity, as soon as a limit value is reached [26], and it may be difficult to elute proteins from highly substituted affinity chromatographic supports [27]. Hence a very high level of substitution is not necessarily desirable.

Third, the dye derivative is not covalently bound to the reversed-phase material. Its affinity for the stationary phase is high, but dye is retained on the column packing because of a dynamic equilibrium. Thus, when incoming mobile phase contains no dissolved dyed surfactant, some dyed surfactant will be desorbed from the column, even though the loss may be too low to be seen by the naked eye or by standard spectrohotometric measurements (this was checked by adsorbance readings at 607 nm). Changing the mobile phase composition from one which strongly promotes hydrophobic interactions to one which is less favourable may speed up dye leakage, and dyed surfactant should therefore be loaded using the mobile phase of the purification process, which favours hydrophobic interactions less strongly.

Despite its limitations, this approach successfully allows the purification of proteins by immobilized dye chromatography at high flow-rates. It has two great advantages: straightforward chemistry is all that is needed to prepare the dyed surfactants, and the satisfactory results obtained with columns loaded with crude synthesis mixtures make the preparation of efficient immobilized dye columns simple and rapid.

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