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EFFECT OF SURFACTANTS ON THE SEPARATION OF PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. NON-IONIC SURFACTANTS (TWEEN)

J. P. CHANG
Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031 (China)

SUMMARY

The retention of a number of proteins on diphenyl-bonded silica coated with non-ionic surfactants (Tween) was investigated. It was found that a hydrophilic surface is formed by the interaction of Tween with the diphenyl groups on the packing surface. The hydrophilic stationary phase generated can be used for the size-exclusion chromatography of proteins.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is superior to most other methods for the separation of proteins in terms of speed and resolution. However, an important limitation is the denaturation of proteins during chromatography with organic solvents in the mobile phase. A number of studies attempting to preserve the biological activity of proteins have been reported[1,2], but an organic solvent is still necessary for eluting most of the proteins from reversed-phase columns.

Barford *et al.*[3] suggested that small amounts of surfactants could be added to the mobile phase to lower the surface tension and to control the retention of proteins in reversed-phase columns. The detailed results, however, have not been reported. Ghaemi and Wall[4,5] investigated the effects of ionic or non-ionic surfactants added to aqueous methanolic eluents with silica or zirconia as a adsorbents. They showed that a hydrophobic surface was dynamically generated by the interaction of the polar groups of the surfactant dissolved in the mobile phase with the hydroxy groups on the gel surface. The non-polar terminal function of the surfactants on the packing surface exhibits similar properties to those of alkyl groups on a bonded silica surface.

The above results led to interest in whether non-ionic surfactants, such as alkyl sorbitan polyoxyethylene polyols (Tweens), can interact with non-polar hydrocarbon groups, bonded on the silica, to produce a new hydrophilic surface. This may lead to a decrease in the retention of proteins on reversed-phase columns without the need for organic solvents in the mobile phase. In this work the effect of adding non-ionic surfactants (Tween and Triton X-100) to aqueous buffer eluents on the retention behaviour of proteins on diphenyl-bonded silica column was investigated. The influ-

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ence of composition, pH and ionic strength of the eluents on the retention and recovery of proteins was also studied.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Waters 6000A pump, a Rheodyne 7105 sample injector and a Jasco Uvidec 100-II variable-wavelength UV detector, operated at 230 nm for a mobile phase containing Tween and at 254 nm for one containing Triton X-100. A Waters M-730 data module was used for measurement of retention times and peak areas and for plotting the chromatograms.

Columns (25 × 0.5 cm I.D.) were slurry-packed with diphenyl-bonded silica (Protesil diphenyl 300, d_p = 10 μm, average pore diameter 300 Å; Whatman) suspended in a carbon tetrachloride–2-propanol (75:25). Small-volume tubing (0.3 mm I.D.) was used between the injector valve and the column and between the column and the detector to give a negligible dead volume. All experiments were performed at ambient temperature.

Materials and reagents

The proteins haemoglobin, β-lactoglobulin, α-chymotrypsin, myoglobin, lysozyme and ferritin were obtained from Sigma (St. Louis, MO, U.S.A.), cytochrome *c* and γ-globulin from United States Biochemical (Cleveland, OH, U.S.A.) and ovalbumin from Fluka (Buchs, Switzerland). Triton X-100 was purchased from Carl-Roth-Chem. Fabrik (Mannheim, F.R.G.) and Tweens from Sigma.

Mobile phases were prepared from analytical-reagent grade sodium acetate, phosphoric acid, potassium dihydrogen phosphate, potassium monohydrogen phosphate and Tris. All other reagents were of analytical-reagent grade from various supplier. Water was deionized and doubly distiilled from glass.

The packed diphenyl column was washed with a solvent consisting of 60% 2-propanol in water and then with pH 6.8 phosphate buffer before coating. The passage of 200 ml of phosphate buffer containing 0.5% of Tween was necessary to equilibrate a column completely. In all instances the column was then washed with the chosen eluent without surfactant until a constant elution volume was observed for the test proteins before initiation of size-exclusion chromatography. This procedure usually consisted of the passage of about 200 ml of eluent through the column.

RESULTS AND DISCUSSION

Preliminary investigations demonstrated that a number of common proteins can be eluted from the diphenyl-bonded silica column coated with Tween-80 by using aqueous buffers without organic modifier as the mobile phase. The elution volumes fell below the void volume of the column. The retention behaviour of the proteins in this system was apparently controlled by size exclusion. The results in Fig. 1 demonstrate that the elution volume (V_e) for proteins was a linear function of log MW in the range 10–160 kdaltons. Ferritin was fully excluded from this stationary phase. An excellent linear correlation plot (r = 0.9948) was obtained by using the data for all the proteins, except lysozyme and the totally excluded protein.

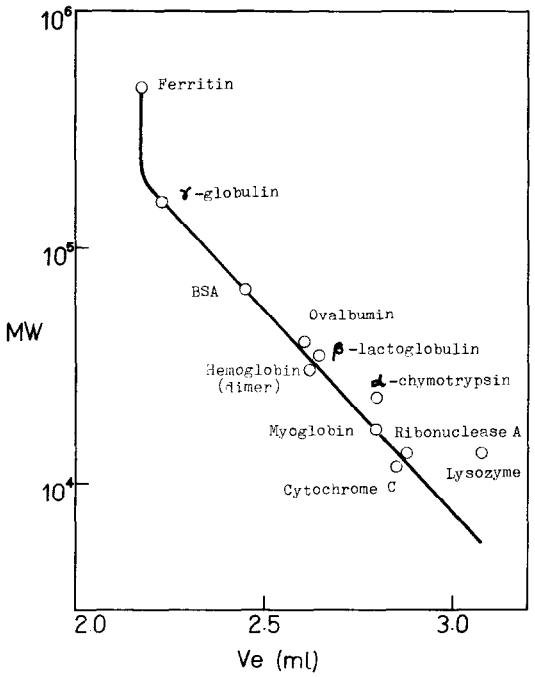

Fig. 1. Correlation of elution volume (V_e) and log MW. Column, Tween-80-coated diphenylsilica (25 × 0.5 cm I.D.); eluent, 0.02 *M* phosphate buffer (pH 6.8), containing 0.1 *M* Na_2SO_4; flow-rate, 1 ml/min.

Tween-20 and Tween-40 exhibited similar linear correlations between log MW and with only slight differences in slope and intercept:

Tween-20: $\log MW = -1.762\ V_e + 9.376;\ r = 0.9845$
Tween-40: $\log MW = -1.839\ V_e + 9.110;\ r = 0.9980$
Tween-80: $\log MW = -1.694\ V_e + 8.989;\ r = 0.9948$

As shown in Fig. 1, lysozyme and α-chymotrypsin deviated from the linear plot of log MW *vs.* V_e. This is consistent with the results obtained by Schmidt *et al.*[6] on a DIOL column. In addition, it was observed that lysozyme, with a high p*I* value, was slightly retarded at all ionic strengths in pH 6.8 buffer eluent. Such a negative molecular weight deviation may be the result of the adsorption of cationic species of lysozyme on residual silanols, which were not covered by the surfactant. This could be shown by changing the pH of the eluent to 2.4, when the elution volume of lysozyme was then close to the linear plot.

A larger scatter in the plot of log MW *vs.* V_e was observed when a phosphate buffer of lower pH was used to elute proteins from Tween-coated column. Myoglobin, haemoglobin and bovine serum albumin were fully retarded in this system.

The surfactant coating is fairly stable. In order to test the stability, a Tween-40-coated diphenyl column was subjected to an eluent of phosphate buffer (pH 6.8) containing 0.1 *M* Na_2SO_4 at a flow-rate of 0.5 ml/min for 40 h. It was found that the elution volume did not change significantly over this period. However, a slight increase in the elution volumes of most proteins occurred after 80 h (see Table I). In this instance, a linear correlation between log MW and V_e was still obtained.

TABLE I

STABILITY OF A COATED COLUMN WASHED WITH MOBILE PHASE

Column, diphenyl-bonded silica, coated with Tween-40 (25 × 0.5 cm I.D.); eluent, 0.02 *M* phosphate buffer (pH 6.8), containing 0.1 *M* Na_2SO_4; flow-rate, 0.5 ml/min.

Protein	V_e *(ml)*		
	0 h	*40 h*	*80 h*
γ-Globulin	2.12	2.14	2.20
Bovine serum albumin	2.32	2.32	2.45
Ovalbumin	2.48	2.46	2.65
β-Lactoglobulin	2.49	2.47	2.67
α-Chymotrypsin	2.65	2.68	2.92
Myoglobin	2.66	2.64	2.86
Lysozyme	2.84	3.09	3.61
Ribonuclease A	2.73	2.72	2.95
Cytochrome *c*	2.68	2.72	3.00

Fig. 2 shows the influence of ionic strength on the elution volumes of five proteins for the Tween-80-coated diphenyl column. The elution volumes of these proteins increased with increasing ionic strength in the lower ionic strength region and appeared to approach a constant value at $\mu = 0.5$–1.2. At higher ionic strength ($\mu > 1.2$), the elution volumes increased significantly. It is well known that many proteins can undergo hydrophobic interaction with the stationary phase at high salt concentration[6,7]. The results indicated that a buffer solution with an ionic strength of *ca.* 0.5–1.0 seems to be the optimum eluent for the size-exclusion chromatography of proteins on this column.

The recovery of proteins was determined by comparing the peak areas obtained at 230 nm after passage of the protein through a diphenyl column coated with

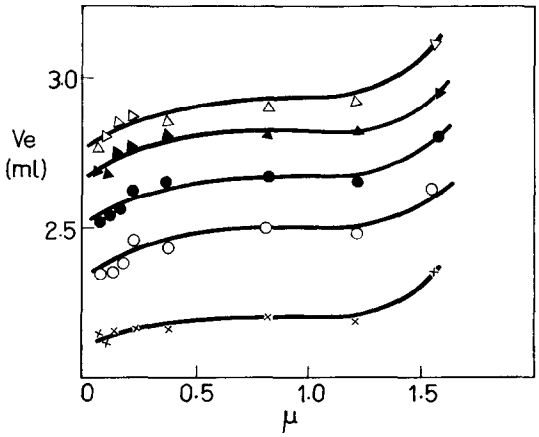

Fig. 2. Influence of ionic strength on the elution volume of proteins. Column, Tween-80-coated diphenylsilica (25 × 0.5 cm I.D.); eluent, 0.02 *M* phosphate buffer; flow-rate, 1 ml/min; ionic strength varied by addition of Na_2SO_4. ×, Ferritin; ○, BSA; ●, β-lactoglobulin; ▲, myoglobin; △, ribonuclease A.

TABLE II

RECOVERY OF PROTEINS, DETERMINED BY UV ABSORBANCE

Column, diphenyl-bonded silica, coated with Tween-80; eluent, 0.02 *M* phosphate buffer (pH 6.8) at 1 ml/min.

Protein	*Recovery (%)*
γ-Globulin	89
Bovine serum albumin	101
Ovalbumin	105
Haemoglobin	103
Myoglobin	98
Trypsin	75
Cytochrome *c*	76
Ribonuclease A	81

Tween-80 at a flow-rate of 1 ml/min with that obtained from PTFE capillary tubing with same volume as the void volume of this column. The peak areas were measured with a Waters M-730 data module. The results in Table II show that recovery of proteins obtained from the Tween-80-coated diphenyl column was comparable to that obtained from DIOL columns[6].

A comparison of the elution volumes of proteins with three different buffer systems is given in Table III. The three eluents gave comparable linear plots of log MW *vs.* V_e, although small variations in elution volume were observed for most of the proteins.

The results suggest that Tweens may be strongly attached to the packing surface. A surface layer is formed by hydrophobic interaction of the long alkyl chain of Tweens with the diphenyl groups, bonded on the silica surface. It proved impossible

TABLE III

COMPARISON OF ELUTION VOLUMES OF PROTEIN WITH DIFFERENT BUFFER ELUENTS

Column, diphenyl-bonded silica, coated with Tween-80; flow-rate, 1 ml/min.

Protein	V_e *(ml)**		
	I	*II*	*III*
Ferritin	2.02	2.10	2.04
γ-Globulin	2.06	2.15	2.15
Bovine serum albumin	2.30	2.40	2.33
Haemoglobin	2.42	2.55	2.50
Ovalbumin	2.41	2.50	2.45
β-Lactoglobulin	2.43	2.55	2.50
α-Chymotrypsin	2.67	2.80	2.70
Myoglobin	2.56	2.60	2.66
Lysozyme	2.75	2.84	2.91
Ribonuclease A	2.72	2.72	2.70
Cytochrome *c*	2.71	2.70	2.66
Glycyl-tyrosine	3.80	3.80	3.79

* I, 0.02 *M* phosphate buffer containing 0.1 *M* Na_2SO_4 (pH 6.8); II, 0.02 *M* ammonium acetate containing 0.1 *M* Na_2SO_4 (pH 7.4); III, 0.05 *M* Tris–HCl containing 0.1 *M* Na_2SO_4 (pH 7.2).

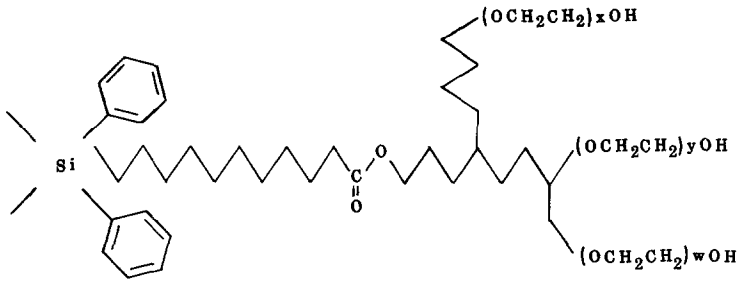

Fig. 3. Possible structure of Tween-coated packing surface.

to remove the layer with several litres of aqueous buffer eluent. The $-OCH_2CH_2OH$ groups of the Tween molecule are exposed to the mobile phase as shown in Fig. 3. These exhibit a strongly hydrophilic character, known to be useful for the size-exclusion chromatographic separation of proteins. Fig. 4 shows the separation of a mixture of three proteins and a dipeptide on a Tween-80-coated column. The result is similar to that obtained from DIOL columns with the same particle size.

Other non-ionic surfactants have also been studied. Sorbitan fatty acid esters were unsuitable because of their poor solubility in water. Triton X-100 can be dynamically coated on the diphenyl-bonded silica. Most of the proteins used in this study were eluted by the pH 6.8 buffer eluent containing 0.02% of Triton X-100, but severe peak tailing and short peaks were observed (Fig. 5). The dynamically coated layer of Triton X-100 is relatively easy to remove by washing with eluent without surfactant. One reason for this may be the weaker interaction of the shorter alkyl chain of Triton X-100 with the diphenyl groups on the silica.

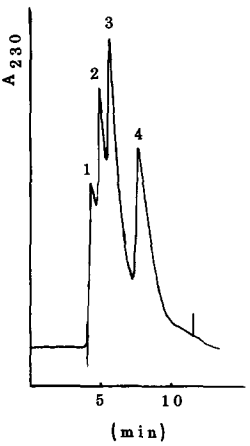

Fig. 4. Separation of a mixture of three proteins and a dipeptide. Chromatographic conditions as in Fig. 1, except flow-rate, 0.5 ml/min. 1, γ-Globulin; 2, β-lactoglobulin; 3, lysozyme; 4, glycyl-tyrosine.

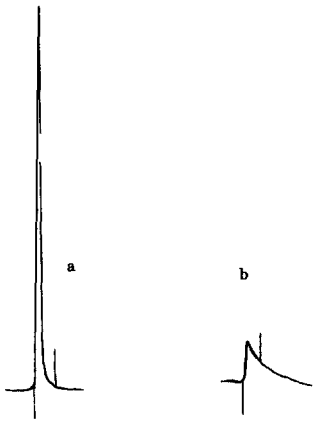

Fig. 5. Peak profiles of BSA, obtained from (a) a Tween-80-coated diphenyl column with 0.02 *M* phosphate buffer (pH 6.8) as eluent and (b) a diphenyl column dynamically coated with Triton X-100 and with 0.02 *M* phosphate buffer (pH 6.8) containing 0.02% of Triton X-100 as eluet at a flow-rate of 1 ml/min.

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