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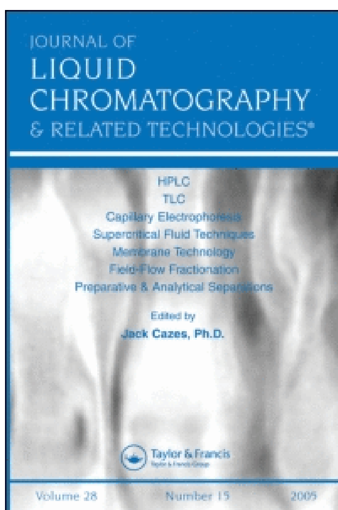

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Jeffrey R. Deschamps[a]
[a] ONT/ASEE Postdoctoral Fellow Laboratory for the Structure of Matter Code 6030, Naval Research Laboratory, Washington, D.C.

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DETERGENT MEDIATED EFFECTS ON THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

Jeffrey R. Deschamps
ONT/ASEE Postdoctoral Fellow
Laboratory for the Structure of Matter
Code 6030, Naval Research Laboratory
Washington, D.C. 20375-5000

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ABSTRACT

Present chromatographic systems for the high-performance liquid chromatography (HPLC) of hydrophobic-proteins are generally limited to size-exclusion or ion-exchange chromatography. A major stumbling block to the successful chromatography of membrane-proteins is their limited solubility. Detergent is usally required to solublize these proteins. This detergent causes some problems in size-exclusion chromatography, but does not always interfere with the separation. It is more deleterious in anion-exchange chromatography, where ionic detergents can poison the column, and reversed-phase chromatography, where strong interactions can occur between the stationary phase and detergent. Successful chromatography of membrane-proteins requires favorable detergent/stationary-phase interactions that enhance, rather than interfere with, the separation.

To study these "detergent-mediated effects" a series of protein standards were chromatographed by reversed-phase HPLC. The column was then satur-

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ated with detergent and the standards rechromatographed. To evaluate any irreversible effects (caused by detergent/stationary-phase interactions) the column was washed extensively and re-evaluated. Following this procedure a variety of stationary-phases and detergents were tested.

The results of these studies showed that resolution was enhanced by detergent. Retention time was generally uneffected, but peak width was noticeably decreassed. Proteins were separated by fast gradients and recovered in high yields (95-99%). A C-18 stationary-phase gave better resolution than a C-8 stationary-phase. In all cases studied the column was irreversibly modified.

A final test of the "detergent-modified" columns was the chromatography of membrane-proteins. Prior attempts at the reversed-phase HPLC of these proteins had resulted in either no sample recovery, or of very low yields of purified protein. An acetylcholinesterase containing sample chromatographed as series of fused peaks, two of which were found to contain cholinesterase activity. Human lymophocyte function-antigen chromatographed as a single peak and was recoved with a 95% yield.

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INTRODUCTION

At one time a protein chemist was content to study the major water soluble proteins as is evident in "A Chronology of Biochemistry" (1), but as techniques have become more refined the emphasis has shifted to less abundant proteins having either an interesting function, or a highly localized distribution, these proteins are often located on the cell-surface. These membrane-proteins require detergent to solubilize, and in some cases stabilize, the protein. Investigations of these hydrophobic proteins have been hampered by their lack of solubility, as

denaturing agents, such as 8M urea, 6M guanidine HCl, or strong detergents, are required for their solubilization. It has been noted that "...these solvent systems are useful in size-exclusion chromatography (SEC) but not in reversed-phase chromatography (RPC)" (2). Investigators have searched for alternative solvents for membrane-proteins, and both ethylene glycol and 60% formic acid have been used successfully (2,3). The purification of hydrophobic proteins is usually limited to SEC or ion-exchange chromatography (IEC) due to the solubility problems, and "... a general method [such as reversed-phase HPLC for soluble proteins] that can be applied to the purification of labile, membrane-bound enzymes, still remains an elusive goal" (4).

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Interactions between the proteins, solvent, and stationary-phase lead to various types of non-ideal behavior in the reversed- phase HPLC of proteins. It is these interactions that make a general separation method an "elusive goal". Non-ideal behavior can range from denaturation (5-8) and ghost peaks in subsequent elutions of the column (9,10) to loss of protein (9,11). Much of the non-ideal behaviour exhibited by proteins is the result of complex multisite interactions between the protein and the column packing. These multi-site interactions account for peak asymmetry, ghost peaks, and loss of protein on the column. Additionally, protein loss may be due to adsorbtion onto the frit, or active sites on the column (11), although there are some indications that this can be overcome by column conditioning (9). Denaturation is often the result

of chromatographic conditions rather than interactions between the column and protein (5-7). In some instances HPLC has been used to study the denaturation of proteins (12). The pore size of many reversed-phase columns (100 A) may limit their use to relatively small proteins (13).

Various membrane-proteins have been isolated in spite of the problems associated with the chromatography of these proteins. Bacteriorhodopsin has been solubilized in detergent and purified by size-exclusion high-performance liquid chromatography (14), but the authors also report some diffculties in the isolation of non-denatured monomeric bacteriorhodopsin from detergent micelles. Certain viral coat proteins have also been isolated by high-performance liquid chromatography. Both Sendai and Influenza viral proteins have been purified by high-performance IEC and SEC, but only the Sendai viral proteins have been purified by high-performance RPC (15-17).

The effect of certain surfactants on the high-performance RPC of proteins and peptides has been studied, and the non-ionic detergents, Brij 35 and Triton X-100, cause "significant decreases in the retention of polypeptides" (18). The reduction in retention is thought to be due to a decrease in the surface tension between the polar mobile phase and the non-polar packing material. The effects of anionic and cationic surfactants have also been studied in some detail, but only for simple compounds (18-21). The effect of these ionic-surfactants is

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complex, involving "composite pairing ion/dynamic liquid-liquid ion-exchange effects" (18,22).

Because "denaturing agents" are necessary for the solubilization of membrane-proteins, and concentrated solutions, such as 8M urea, exhibit both high viscosity and poor miscibility with organic solvents, a successful separation must tolerate detergent. Therefore the effect of detergent on the reversed-phase HPLC of proteins was systematically studied by chromatographing standard proteins in the presence of different detergents and on different stationary-phases.

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METHODS AND MATERIALS

A 4.6 x 10 mm column was slurry packed with Nucleosil C18 (Macherney-Nagel), BakerBond-Wide Pore C18 (J.T. Baker) or an experimental C8 material (Altex) using a Haskel air-induction pump. All packing materials had a particle size of 5 microns. A 5% slurry of packing material was prepared in 20% 0.02 M sodium acetate/ 80% methanol. The column blank was packed at 2000 psi, and repressurized to 2000 psi 5 times. The column was washed with 100 ml HPLC grade methanol (J.T. Baker).

Solvents were prepared from HPLC grade water, HPLC grade acteonitrile (J.T. Baker), "sequanal" grade trifluoroacetic acid (Pierce), and laboratory grade detergents, Brij 35 and Brij 99 (Sigma), and

octylglucoside (Calbiochem). The chromatographic system consisted of a Beckman 450 controller, a series 346 binary solvent delivery system, and a model 165 variable-wavelength uv-vis detector. A short gradient was used to take full advantage of the column length. The chromatography conditions were: solvent A: 0.1% trifluoroacetic acid/ water; solvent B: 0.1% trifluoroacetic acid/acetonitrile; flow rate, 1.0 ml/min; isocratic at 10% B for 1 min; a linear gradient from 10% to 70% B in 10 min; and isocratic at 70% B for 1 min.

Solutions of lysozyme, bovine serum albumin, and ovalbumin were prepared in HPLC grade water and their concentrations determined by measuring absorbance at 280 nm.

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Effect of Detergent

The HPLC columns were evaluated with a standard mixture of proteins prior to exposure to detergent. The columns were then saturated with detergent by adding 0.2% of the desired detergent to solvents A and B and pumping a mixture of these solvents (90% A and 10% B) through the column. Monitoring the UV absorbance of the column effluent allowed observation of the detergent breakthrough. After the detergent breakthrough, an additional 10 column volumes of mixed solvent were pumped through the column. The standard test mixture was chromatographed with detergent containing solvents. The chromatographic parameters were determined for each column in the presence and absence of detergent.

Column Modification by Detergent

Column saturation was monitored by monitoring the retention time of a standard protein after various amounts of detergent had been pumped across the column. After a column had been used with detergent, an attempt was made to wash the detergent off the column. This was done by extensive washing with water followed by several slow water/2-propanol gradients. The column was then equilibrated to the original buffer system and re-tested.

Membrane Proteins

In addition to the standard proteins, hydrophobic proteins were chromatographed when avaliable. Human erythrocyte acetylcholinesterase, supplied by M.B. Penno and B.J. Earls, and human lymphocyte function-antigen, supplied by J.E. Hildreth, were both chromatographed in the presence and absence of β-D-octylglucoside. Fractions collected during the chromatography of these proteins were evaluated by either SDS-PAGE or UV absorbance to determine protein recovery.

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RESULTS

Small Pore Silica (Nucleosil C18)

Preliminary experiments showed that reversed-phase HPLC on columns less than 1 cm in length yield sufficient resolution for protein purification. Previously it had been demonstrated that resolution of proteins was not dependent on column length over the range 5cm to 25cm in reversed-phase chromatography (10). Additionally, these short columns are inexpensive and easy to pack.

Early experiments on Nucleosil columns indicated that HPLC can be performed in the presence of detergent with no adverse effect on resolution (Figure 1), although the reduction in retention time noted in previous investigations was not evident. Data evaluation consisted of the calculation of peak width (based on peak height and peak area as determined by integration) and apparent plate count (N'). It is not possible to obtain a true plate count for the reversed-phase chromatography of proteins as proteins tend to elute at a specific concentration of organic solvent. Under isocratic conditions most proteins exhibit either no retention or infinite retention. Thus, it is nearly impossible to determine a plate count using proteins as the test mixture. For convenience an "apparent plate count" is used in this study to compare the effect of various detergents. The apparent plate count, N', is based on the consistent gradient used in these comparisons and calculated from the equation:

$$N' = 5.54(t_g - t_o)/w_{1/2}$$

Both of these paramters, peak width and plate count, were sensitive to detergent and showed some improvment in the presence of detergent (Table 1).

After extensive washing of the column exposed to Brij 35, both lysozyme and bovine serum albumin were chromatographed without any detergent in the solvents. Both retention time and selectivity were altered by exposure to detergent. Lysozyme, which originally eluted first, was the last protein to

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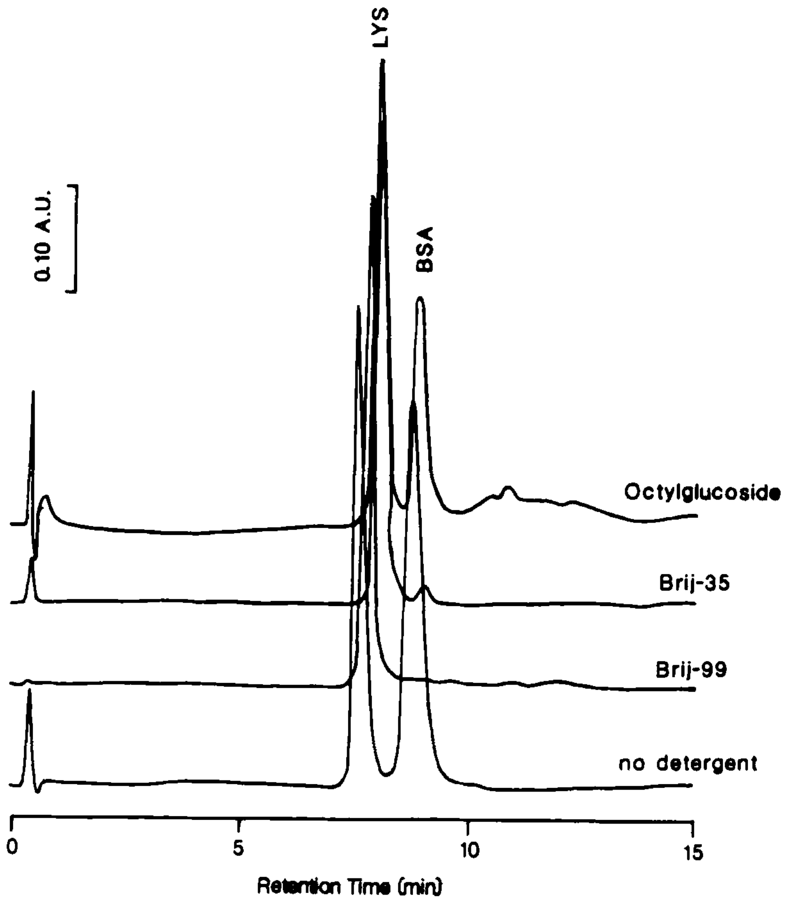

Figure 1. Comparison of the effect of detergent type on the retention times of proteins on a Nucleosil C-18 column (4.6 x 10 mm).

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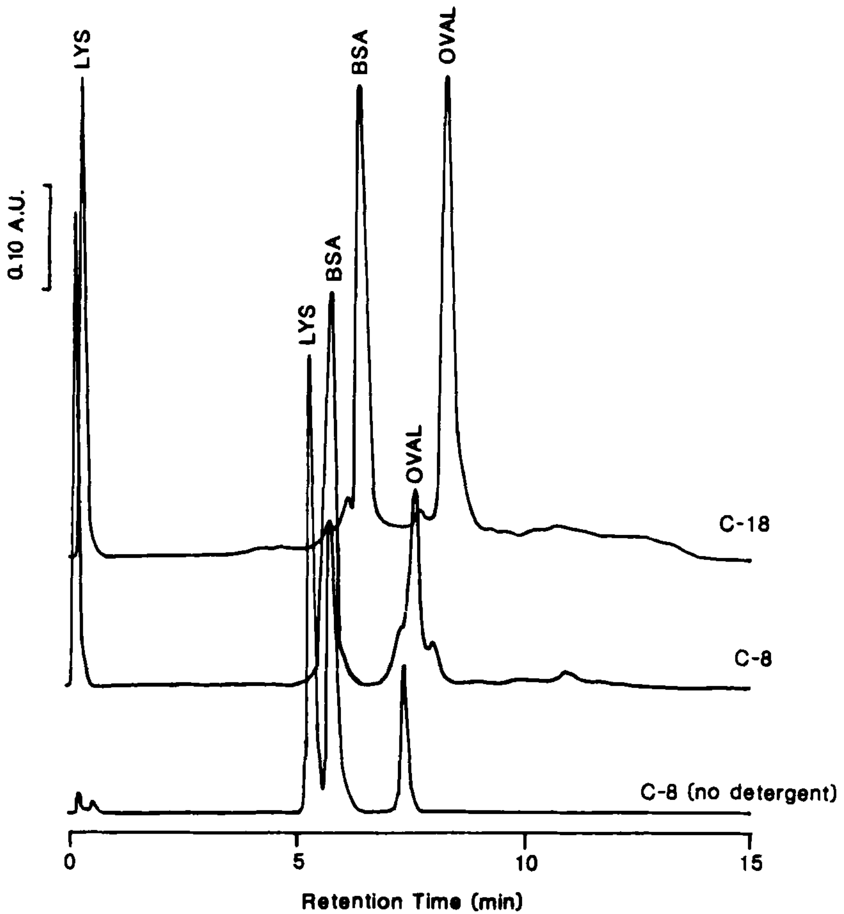

Figure 2. Comparison of protein retention on wide-pore packing materials in the presence of 0.2% -D-octylglucoside (C-18: BakerBond wide-pore C-18, C-8: Altex experimental packing, 300 A pore size, 5 u particle size).

TABLE 1.

Column performance in VSC-HPLC before and after exposure to detergents.

	no detergent		Brij-35		Brij-99		
Parameter	BSA	LYS	LYS	HLFA	LYS	BSA	HLFA
t_o (min)	7.63	8.85	8.03	9.63	7.76	8.46	8.36
t_g (min)	0.42	0.42	0.43	0.42	0.35	0.35	0.35
peak							
area	5330	2415	4261	11832	4155	3075	13776
height	287	90	234	484	278	77	324
width	37.1	53.7	36.4	48.9	29.9	79.9	85.0
$w_{1/2}$ (min)	0.18	0.27	0.18	0.24	0.15	0.40	0.41
N'	8888	5400	9876	8158	13519	2277	2114

(BSA = bovine serum albumin; LYS = lysozyme; HLFA= human lymphocyte function antigen.)

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elute from the "detergent modified" column. The retention time of bovine serum albumin showed a slight increase (8.85 to 9.17 min.), but this was well outside the observed variation in retention time from run-to-run (8.83 to 8.87 min.). Additional washing did not change these results.

Chromatograms of the membrane protein, HLFA, showed good peak symmetry, but 'ghost' peaks appeared in subsequent blank injections.

Large Pore Silicas

A comparison of results obtained on columns packed with BakerBond wide-pore C-18 to those from columns packed with Altex C-8 did not reveal any

significant differences. In general, retention times were shorter on the C-8 column, as would be predicted based on the hydrophobicity of the two columns. Unlike the small pore Nucleosil material, there was a large shift in retention time after the large-pore materials were exposed to detergent (Figure 2). The retention time of lysozyme shifted from approximately 6 min to about 0.7 min on both the C-8 and C-18 colums.

The large shift in retention time observed for lysozyme when large pore columns were exposed to detergent was used to determine how much detergent was neccessary to saturate the chromatographic column. This was accomplished by pumping detergent containing solvents through the column as described in the methods section and periodically injecting lysozyme and running a short gradient to determine the retention time. Approximately 110 mg of detergent was passed through the column before the retention time stabilized (Figure 3).

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Membrane Proteins

Results of the chromatography of membrane proteins are shown in Figures 4 and 5. Human lymphocyte function-antigen (HLFA) chromatographed as a single peak (Figure 4). Multiple injections were made of this protein to determine both reproducibility and protein recovery (Table 2). The results of this experiment were then compared to the results obtained with the soluble protein, lysozyme (Tables 2 and 3). Retention time for both HLFA and lysozyme showed little variation, and the ratio of peak area to protein

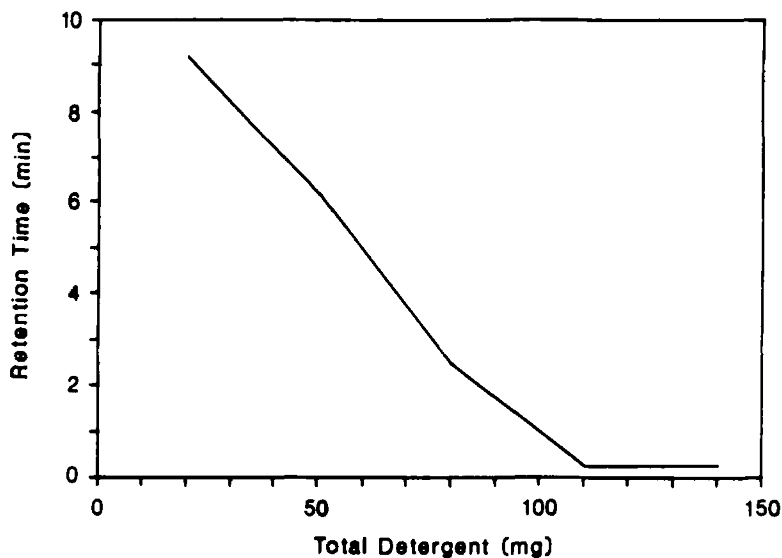

Figure 3. Column equilibration with β-D-octylglucoside as monitored by the change in retention time of lysozyme.

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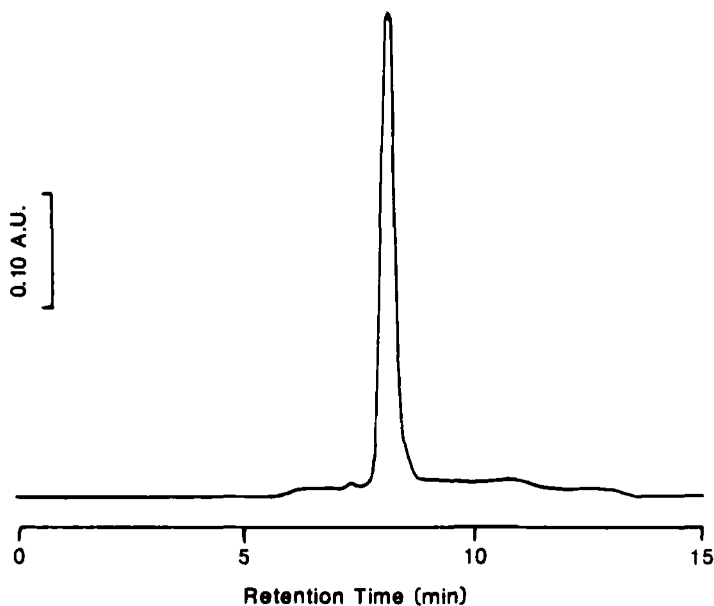

Figure 4. Reversed-phase chromatography of human lymphocyte function-antigen in the presence of 0.2% β-D-octylglucoside.

TABLE 2.

Run-to-Run Variability of Retention Time and the Ratio of Peak Area and Protein Load.

Protein Injected (μg)	Retention Time (min)	Peak Area	Area/μg
lysozyme			
144.0	8.01	16537	115
36.0	8.04	5096	141
14.4	7.97	2265	157
7.2	8.00	970	135
2.9	8.04	435	150
1.4	8.03	201	143
HLFA			
20.2	8.08	5041	250
20.2	8.09	4564	226
20.2	8.12	5350	265
10.1	8.10	2800	277
5.0	8.09	1511	302

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TABLE 3.

Protein recovery in the the presence of 0.2% β-D-octylglucoside.

PROTEIN	COLUMN LOAD (μgram)	FRACTION VOLUME (μl)	ABS 280 nm	PERCENT RECOVERY
lysozyme	36.0	490	0.182	95.3
	14.4	540	0.066	95.1
	144.0	470	0.622	77.8*
HLFA	20.2	1000	0.095	94.0
	20.2	1000	0.088	87.1
	20.2	1000	0.099	98.0
	10.1	1000	0.050	99.0
	5.0	1000	0.026	103.0

* A large peak at the void volume indicated that the column was overloaded in this trial.

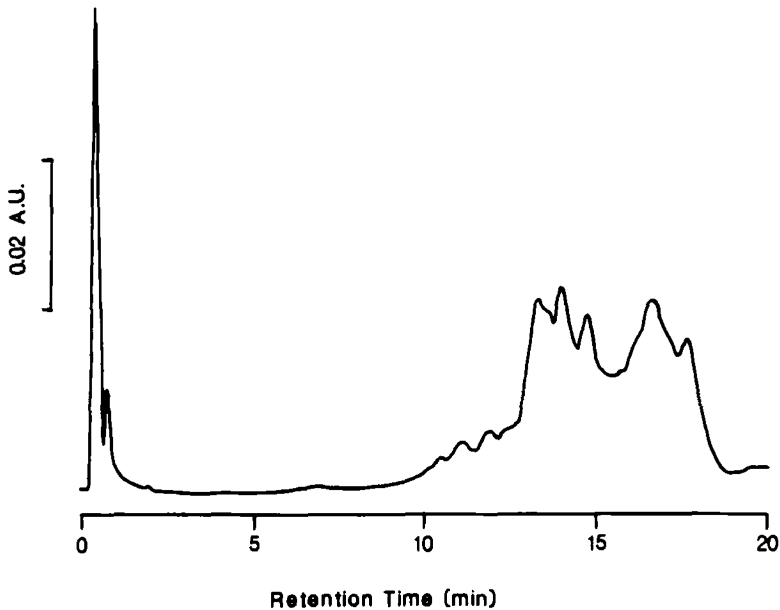

Figure 5. Reversed-phase chromatography of human erythrocyte acetylcholinesterase in the presence of 0.2% β-D-octylglucoside (peaks marked with an asterisk contain acetylcholinesterase).

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load varied about 10% for both proteins. This variation may be due to errors in integration, but did not show a strong correlation to protein load. Both lysozyme and HLFA were recovered in over a 95% yield.

Acetylcholinesterase chromatographed as a complex of fused peaks (Figure 5) two of which contained acetylcholinesterase as determined by SDS-PAGE and screening for esteractic activity (B.J. Earls, personal communication). Acetylcholinesterase produced 'ghost' peaks in subsequent blank injections with about 66% of the protein eluting in the original chromatogam.

DISCUSSION

The ability of short columns to resolve proteins has previously been noted (24). Additionally, it was found that the improved resolution could not be explained by a change in peak width alone. The enhanced efficency noted in this study exhibits the same behavior as both peak width and retention time are changing (see Table 1), although the changes in peak width appear to have a greater effect on improved efficiency. The reduced peak width can be caused by either a reduction in multi-site interactions, which are often associated with the chromatography of proteins, or by lessening the extent of protein denaturation and unfolding that occurs during the chromatography. Only lysozyme showed any significant change in retention time, and then only when chomatographed on a large pore silica. If the enhancement of efficency were due to decreased denaturation then the result should have yielded either a slight increase in retention time, as the less denatured proteins would be more nearly globular and could exchange with the interstitial volume, or no change in retention time, but peak width would decrease as the mass transfer between the bulk solvent and the interstitial volume is also more rapid. This is consistent with the behavior of BSA and ovalbumin, but not that of lysozyme. If a decrease in the multisite interactions were responsible for the reduced peak width then the retention time should also have decreased. It is therefore proposed that the increase in efficiency is due to a combination of reduced multi-site interactions and reduced denaturation.

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