

REVERSIBLY BOUND LIGANDS FOR HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY: APPLICATION TO SERUM CHOLINESTERASE

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Introduction

In this paper we present a new method for immobilizing ligands to solid supports which can be used in the development of high performance affinity chromatography (HPAC) columns for protein purification. A surfactant consisting of a hydrophobic tail and a long polar head group is derivatized by the attachment, to the end of the polar group, of a ligand that will bind specifically to the desired protein. In aqueous solutions the ligand-modified surfactant binds very strongly to hydrophobic surfaces, such as octadecyl-bonded silica packing materials that are commonly used for reverse-phase chromatography. The solid support with the adsorbed surfactant can be used to carry out the purification of the desired protein by affinity chromatography. However, when desired, the ligand can be removed from the column with organic solvents. This makes the packing reusable for other types of applications, and it allows removal of the ligand prior to sterilization of the support.

The technique can result in very large ligand loadings per volume of packing when compared to covalent solid phase derivatization methods. Since the surfactant-ligand is synthesized in solution the chemistry of derivatizing the surfactant with affinity ligand is easier to control than the solid-ligand reaction, the resulting yield and purity of the final product is higher. By mixing the surfactant that has been modified by ligand attachment with other surfactants prior to adsorption it is also possible to control the ligand loading as well as the nature of the interaction of the proteins in solution to the solid matrix.

As an example of this method, we present results on the attachment of pyridine to a non-ionic ethoxylated alcohol, octaethylene glycol n-hexadecyl ether ($C_{16}E_8$), via activation with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride). The resultant ligand-modified surfactant ($C_{16}E_8$ -Pyridinium) was found to be a reversible competitive inhibitor of serum cholinesterase (E.C. 3.1.1.8). This affinity surfactant was adsorbed to octadecyl-bonded porous silica particles. The support was tested for use in an affinity column in the purification of human and horse cholinesterase from the respective raw sera.

Experimental Procedures

Synthesis of $C_{16}E_8$ -Pyridinium. $C_{16}E_8$ was derivatized using a procedure based on the method described by Nilsson and Mosbach (1). Pyridine was attached to $C_{16}E_8$ by tresylation of the

surfactant followed by nucleophilic substitution with the inhibitor. Initially, 600 mg of $C_{16}E_8$ (1.0 mmole) was dissolved in 10 ml of dichloromethane and the solution was cooled to 4°C. Then 400 μ l of pyridine and 400 μ l of tresyl chloride were added to the surfactant solution. The tresylation reaction proceeded for 1.5 hours with stirring, whereupon an additional 400 μ l of pyridine was added. The solution was stirred at ambient temperature for 16 hours. The reaction mixture was then dried under vacuum in a rotary evaporator and redissolved in 10 ml of distilled water.

Purification of $C_{16}E_8$ Pyridinium. An aqueous solution containing the product (10 ml) was applied to a stainless steel column (2.5 x 25 cm) packed with Davisil™ octadecyl-bonded silica (30-40 μ m particle size, 300Å pore size). The column had been washed with 300 ml of 0.1% trifluoroacetic acid (TFA) in water (solvent A) at the rate of 2.0 ml/min, prior to sample application. The surfactant was completely retained by the column, since the column eluate had no detectable surface activity (as gauged by its inability to foam in an aqueous solution). The surfactant was eluted with a 120 minute linear gradient from solvent A to 100% solvent B (methanol: iso-propanol: acetonitrile: TFA, 6:3:1:0.1 by volume). The flowrate during elution was maintained at 3.0 ml/min and 10 ml fractions were collected and redissolved in water to gauge surface activity. Pyridine has an absorbance maximum at 256 nm in water ($E_m=2826 M^{-1}cm^{-1}$). Therefore, the absorbance of the column effluent was monitored at a similar wavelength, namely 259 nm.

Enzyme Kinetic Assays and Inhibition Studies. The activity of cholinesterase solutions was determined by the method of Ellman et al. (2) using butyrylthiocholine as substrate. One unit of enzyme activity corresponds to the hydrolysis of one μ mole/min of a one mM butyrylthiocholine solution at 25°C in 0.05 M Tris-HCl buffer at pH 8.0. The enzyme-catalyzed reaction product, thiocholine, is bound to dithiobisnitrobenzoate (10 mM in 0.1 M phosphate buffer at pH 7.0) and the absorbance of the complex followed at 412 nm. The Michaelis-Menten parameters, K_m and V_{max} , for the cholinesterase-catalyzed cleavage of butyrylthiocholine in these buffer conditions are 1.29 μ M and 103 mM/min, respectively. The inhibition of cholinesterase by $C_{16}E_8$ -Pyridinium and by pure surfactant $C_{16}E_8$ was measured by following the rate of butyrylthiocholine cleavage in the presence of the two surfactants. The corresponding K_i values were determined by the method of Dixon (3) using different substrate concentrations.

Chromatographic Studies. A test HPAC column was prepared from an Upchurch Scientific HPLC pre-column (2 cm length, 2 mm inside diameter) packed with 21.3 mg of Davisil™ octadecyl-bonded silica. The column was washed with 20 ml of methanol followed by 20 ml of deionized water. The column was then treated with a 10^{-4} M solution of $C_{16}E_8$ -Pyridinium (50 ml), continuously applied until the absorbance at 259 nm was constant. The decrease in the solution's absorbance at 259 nm was used to determine the extent of surfactant adsorption to the column. The column was rinsed with 100 ml of 0.05 M Tris-HCl buffer, pH 8.0 prior to application of enzyme solutions.

Protein Determination. A reference protein solution was prepared by dissolving 0.75 mg of bovine serum albumin (determined by dry weight) in 10 ml of 0.05 M Tris-HCl buffer, pH 8.0. Aliquots of this solution were analyzed for absorbance at 214 nm using an HPLC system. The injection valve was connected to the detector inlet with a 40-cm piece of stainless steel tubing (0.17 mm internal

diameter). A standard curve was generated by plotting the integrated peak areas versus the amounts of BSA injected.

Results and Discussion

Synthesis and Purification of C₁₆E₈-Pyridinium. The only surface active peak that absorbed at 259 nm emerging from the reverse phase chromatographic packing described in the section above showed significant inhibition of the activity of cholinesterase and was therefore judged to be the desired product. The product yield was 71.9%. The structure of the ligand-modified surfactant is shown in Figure 1.

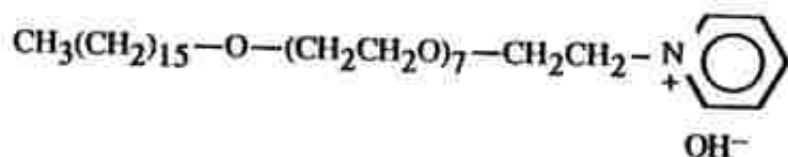


Figure 1: Structure of C₁₆E₈-Pyridinium

Binding of C₁₆E₈-Pyridinium to Cholinesterase in Bulk Solution. Cholinesterase is a large (MW=348,000) hydrophobic enzyme which is inhibited competitively by quaternary pyridinium salts, as well as by other quaternary ammonium aromatic compounds, including phenyltrimethylammonium (PTA) which has a K_i of 220 μM (4). The ratio of enzymatic reaction rate V_L/V_O in the presence of C₁₆E₈ and C₁₆E₈-Pyridinium is shown in Figure 2. Here V_L is the reaction velocity in the presence of the ligand (either modified or unmodified surfactant) and V_O is the reaction velocity in the absence of ligand at the same concentration of enzyme and substrate. The pure surfactant C₁₆E₈ has no effect on the enzymatic reaction rate up to concentrations of 0.1 mM. The surfactant-inhibitor reduces the enzymatic activity much more powerfully than the analogous quaternary ammonium inhibitor PTA. The data for the C₁₆E₈-Pyridinium are well fit by the equation for Michaelis-Menten kinetics for reversible competitive inhibition with a K_i of 0.514 μM up to concentrations of about 5 μM. Beyond this concentration, the enzymatic reaction rate remains constant at about 10% of the uninhibited rate. It is clear from the very small value of K_i measured for C₁₆E₈-Pyridinium when compared to the pure PTA that there is strong secondary binding of the surfactant-inhibitor to the enzyme through hydrophobic interactions.

Immobilization of C₁₆E₈-Pyridinium on Octadecyl-bonded Silica. The test HPAC column was saturated with C₁₆E₈-Pyridinium as described in *Methods*. Leakage of the surfactant-inhibitor from the packing was minimal during elution with aqueous buffers, even in the presence of 3 M guanidine hydrochloride. Washing with 2 liters of water (3.33 x 10⁴ column volumes) caused leakage of only 5% of the surfactant, as gauged by monitoring the column effluent at 259 nm. On the other hand, the surfactant could be readily removed with the use of a solvent mixture of methanol and isopropanol (6:4 V/V).

Using the molar extinction coefficient of the C₁₆E₈-Pyridinium (3118 M⁻¹ cm⁻¹) the specific adsorption of the surfactant-inhibitor to the reverse phase material was determined to be 0.37

$\mu\text{moles/mg}$ of packing. Since the density of silica particles is about 1.5 grams/ml, the average ligand loading is roughly 550 $\mu\text{moles/ml}$ of packing. This is a very high ligand capacity compared to typical values for agarose affinity columns of 1-2 $\mu\text{moles/ml}$ of packing and also when compared to chemically-activated silica HPAC columns. For example, the ligand density for N^6 -(6-aminohexyl)-5'-AMP coupled to tressyl-activated silica was reported to be 0.028 $\mu\text{moles/mg}$ of packing (5), from which a value of about 42 $\mu\text{moles/ml}$ of packing can be calculated.

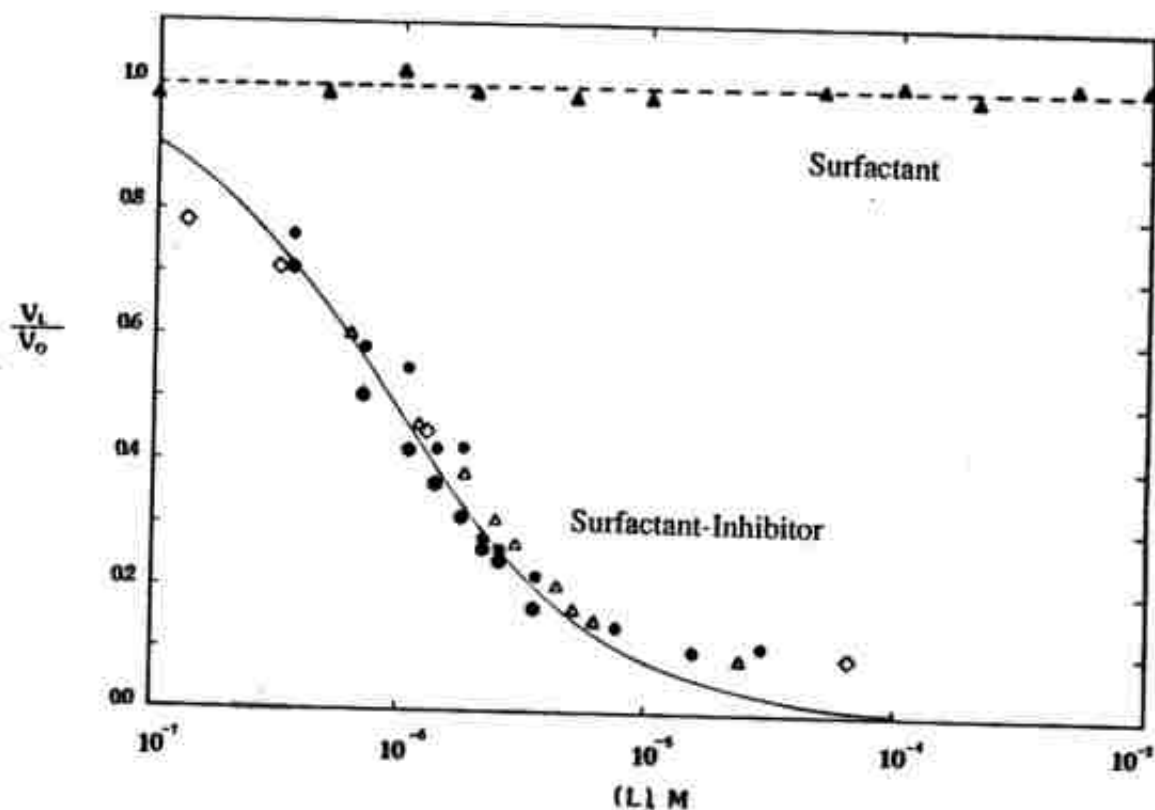


Figure 2: Dependence of cholinesterase-catalyzed reaction velocity ratio on pure surfactant C_{16}E_8 (solid triangles) and surfactant-inhibitor C_{16}E_8 -Pyridinium (open diamonds, closed circles, open triangles, and closed hexagons). The solid line nearest the C_{16}E_8 -Pyridinium data is the prediction of the equation for Michaelis Menten kinetics for competitive reversible inhibition with a K_i of 0.514 μM . The closed circles correspond to a cholinesterase concentration of 2.86 μM and a substrate (butyrylthiocholine) concentration of 1.0mM, the open triangles to a cholinesterase concentration of 2.46 μM and a substrate concentration of 1.0mM, the open diamonds to a cholinesterase concentration of 0.286 μM and a substrate concentration of 1.0mM, and the closed hexagons to a cholinesterase concentration of 2.46 μM and a substrate concentration of 0.5mM.

To determine the effect of unmodified surfactant on protein retention, a test column was saturated with unmodified C_{16}E_8 . This column was tested with horse cholinesterase and bovine serum albumin and in neither case were the proteins retained. These results indicate that the column surface was effectively covered with surfactant molecules. This is consistent with the high ligand loadings

observed, since these surfactant concentrations can be explained by a membrane-like configuration in which the hydrophobic tails of the surfactant molecules are aligned side by side, anchored to the octadecyl groups of the matrix. Since the ethoxy chains of the surfactant molecules are non-ionic and hydrophilic, the surface of the reverse phase column can be changed from hydrophobic and strongly protein-sorptive to hydrophilic and non-protein-sorptive.

High Performance Affinity Chromatography. The Pyridinium-HPAC column was tested for its potential use in the purification of horse and human serum cholinesterase. The specific activity of cholinesterase in the samples of horse and human serum were 0.054 and 0.066 units/mg protein, respectively (estimated using absorbance at 214 nm). Application of 200 μ l aliquots (3.33 column volumes) of both horse and human serum to the column resulted, in each case, in the elution of a broad and large unretained protein peak. In both cases, application of the same 2-minute gradient to 1.0 M NaCl resulted in elution of a single peak containing cholinesterase activity.

In the case of horse serum, the amount of cholinesterase recovered corresponded to 84.2% of the activity applied and with the human serum, the activity yield as 80.7%. The specific activities of the purified cholinesterase from horse and human serum were 15.1 and 5.18 units/mg of protein, respectively. Protein amounts were determined from the integrated peak areas and a BSA standard curve. These values correspond to 280 and 79-fold enrichments of cholinesterase. Figure 3 shows the HPAC elution profile for the purification of the human serum cholinesterase.

An approximate purity can be calculated for the serum cholinesterase solutions obtained using the specific activity values of 684 and 546 units/mg of protein, reported for electrophoretically pure horse and human serum cholinesterase (6). The purity of the HPAC-purified horse and human serum cholinesterase preparations would then be 2.2 and 0.95%, respectively. These low purities were largely the result of the low resolving capability of the small test column used. Significantly greater purification could be obtained with the use of a longer and more efficient column, optimized chromatographic conditions, and the use of a specific eluting agent such as choline chloride or procainamide. Increased purifications could also be obtained by adjusting the ligand density so that the column capacity is closer to the concentration of the enzyme solutions applied.

Conclusion

In this study we have shown that non-ionic ethoxylated alcohols can be modified by the attachment of ligands which can bind specifically to a desired biomolecule. The modified surfactant can be adsorbed essentially irreversibly from aqueous solution to a conventional octadecyl reverse phase silica packing. The resulting solid is a high-capacity affinity sorbent. When desired, the modified surfactant can be eluted with organic solvents, and the packing can be sterilized and reused. Using this ligand-immobilization technique we were able to achieve high yields and significant purification of cholinesterase from horse and human serum in a single step. Additional details of some of these experiments will soon appear in an article by Torres et al. (7). We believe that this method will have wide applicability to many other biospecific separations.

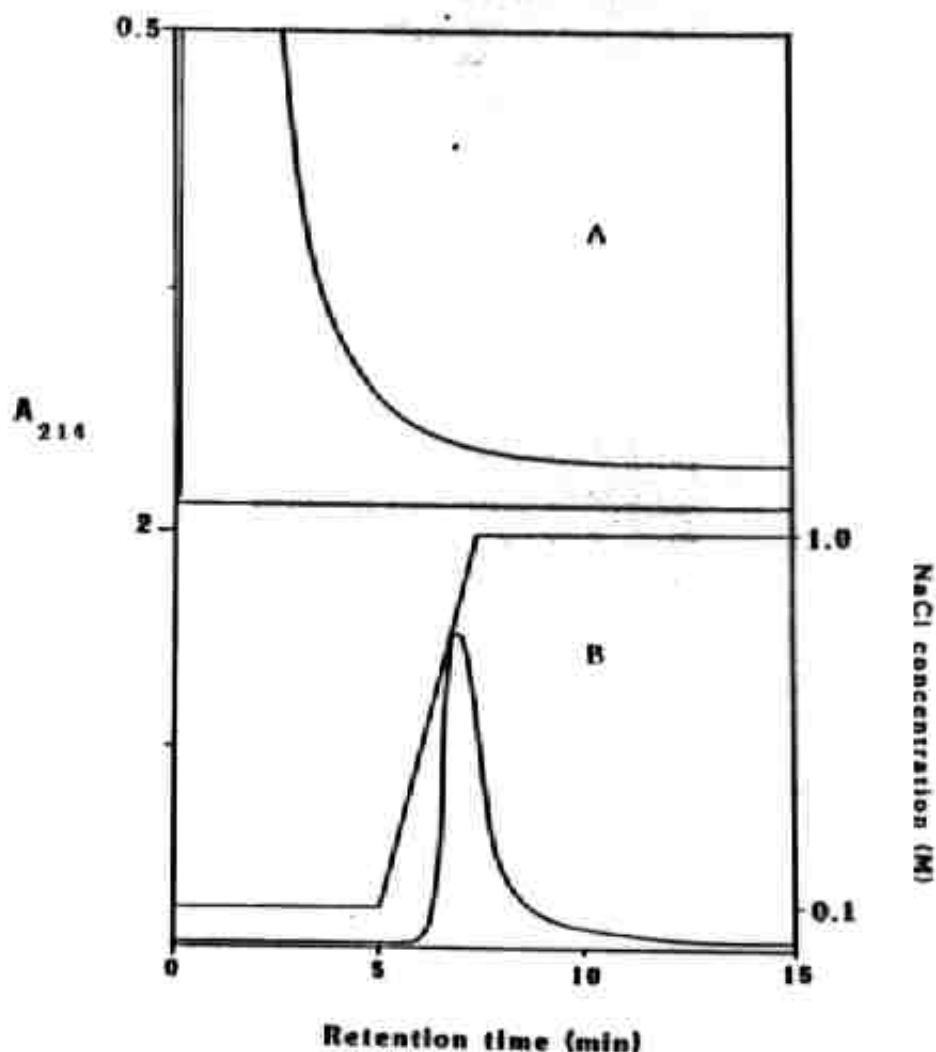


Figure 3. Elution profile of the purification by HPAC of human serum cholinesterase. A 200- μ l aliquot of human serum (56.8 mg/ml by absorption at 280 nm) was applied to the HPAC column. The specific activity of cholinesterase in the serum was 0.066 units/mg. Panel A shows the elution of unretained protein. Panel B shows the elution of a peak containing cholinesterase activity upon application of a 2-min linear gradient to 0.05 M Tris-HCl, pH 8.0, 1.0 M NaCl. The specific activity of the purified cholinesterase was 5.18 units/mg, corresponding to a 79-fold purification.

REFERENCES

1. Nilsson, K., Mosbach K.: Immobilization of Ligands With Organic Sulfonyl Chlorides, *Methods in Enzymology*, 104: 56-69, 1984.
2. Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.H.: A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity, *Biochem. Pharm.*, 7: 88-95, 1961.

3. Dixon, M.: The Determination of Enzyme Inhibitor Constants, *Biochem. J.*, 55: 170-171, 1953.
4. Kilpatrick, B.F.: Purification of Horse and Human Butyrylcholinesterases Purified on a Large Scale by Procainamide Affinity Chromatography, , Ph.D. Thesis, Department of Biochemistry, North Carolina State University, 1980.
5. Nilsson, K., Mosbach, K.: Immobilization of Enzymes and Affinity Ligands to Various Hydroxyl Group Carrying Supports Using Highly Reactive Sulfonyl Chlorides, *Biochem. Biophys. Res. Comm.*, 102: 449-457, 1981.
6. Ralston, J.S., Main, A.R., Kilpatrick, B.F., Chasson, A.L.: Use of Procainamide Gels in the Purification of Human and Horse Serum Cholinesterase", *Biochem. J.*, 221: 243-250, 1983.
7. Torres, J.L., Guzman, R., Carbonell, R.G., Kilpatrick, P.K.: Affinity Surfactants as Reversibly Immobilized Ligands for High Pressure Affinity Chromatography, *Anal. Biochem.* , In Press, 1988.

SUMMARY

Pyridine was coupled covalently to a non-ionic ethoxylated alcohol: octaethyleneglycol n-hexadecyl ether. This modified surfactant was found to be a reversible, competitive inhibitor of horse serum cholinesterase. The surfactant bound irreversibly, in aqueous media, to octadecyl-bonded reverse phase silica particles commonly used for High Performance Liquid Chromatography. The amount of ligand bound was found to be 500 μ moles/ml of packing, a concentration that is over 100 times higher than what can be normally bound to agarose affinity chromatography supports. With this packing, a 280-fold purification of cholinesterase from horse serum and a 79-fold purification of human serum cholinesterase were accomplished, with yields greater than 80%, using a 2 cm long column and a 7 minute elution time. The affinity surfactant could be eluted from a column using a 6:4 V/V mixture of methanol and isopropanol. This technique should be generally applicable in the development of biospecific supports for high performance affinity chromatography.