AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINE ACETYL-HYDROLASE. EC 3.1.1.7
A new straight chain aliphatic inhibitor

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1. Introduction

Affinity chromatography has proven to be a very useful method for purification of acetylcholinesterases. Berman and Young [1] first applied Cuatrecasas' method [2] to purify acetylcholinesterase from the electric organ of the electric eel (*Electrophorus electicus*) and from bovine erythrocyte membranes.

Agarose serving as a solid support was fitted with a 'spacer' of considerable length, at the end of which a specific enzyme-inhibitor was attached. As inhibitors Berman and Young [1] used trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride and similar derivatives. The active site of the enzyme interacts with the solid-bound inhibitor and thus the enzyme cannot be washed out with conventional buffers. The bound enzyme was removed with a soluble inhibitor (edrophonium, tensilon^{R}).

In the course of our studies to purify acetylcholinesterase we found that a 'simple' inhibitor: 1-(*N, N, N*-trimethylammonium)-6-hexylamine bromide hydrobromide which serves as the bound inhibitor, gives even better results. This parallels the results of Schwyzer and Frank [3], who demonstrated, that the use of tetramethylammonium chloride, instead of edrophonium for elution of the affinity bound enzyme, gives almost the same results and is more easily removed from the recovered enzyme by dialysis.

2. Materials and methods

2.1. *Preparation of 1-(N, N, N-trimethylammonium)-6-hexyl-amine bromide hydrobromide*

The substance was first synthesized by Lott and Krapko [4] by acetylation of *N, N*-dimethylhexamethylenediamine followed by methylation with methyl bromide and hydrolysis. We used the methods of Elslager et al. [5] and Ledochowski et al. [6] to synthesize 1-bromohexylphthalimide-6, starting from 1,6-dibromohexane. The dibromide is treated with potassium phthalimide and trimethylamine to yield 1-(*N, N, N*-trimethylammonium)-6-phthaliminohexane bromide, which after hydrolysis gives the desired 1-(*N, N, N*-trimethylammonium)-6-hexylamine bromide hydrobromide.

2.2. *Preparation of affinity gel*

Sepharose 2B* was treated according to Berman and Young [1], but instead of coupling 3,3′-diaminodipropylamine, hexamethylendiamine was used. Succinic anhydride was coupled as earlier suggested by Cuatrecasas [2]. Another molecule of hexamethylendiamine was added by the aid of carbodiimide**, followed by a second coupling of succinic anhydride to form an adequate 'spacer'. At the end of this spacer with the aid of carbodiimide** the inhibitor was at-

* Pharmacia Fine Chemicals, Uppsala: from Instrumenten-Gesellschaft, CH 8049 Zürich, Switzerland.

** *N*-Cyclohexyl-*N*′-[β-(*N*-methylmorpholino)-ethyl]-carbodiimide-*p*-toluensulfonate, 'MERCK': from Bender und Hobein AG, Riedlistr. 15a, CH 8042 Zürich, Switzerland.

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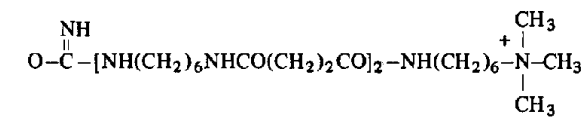
tached to form the following structure:



Agarose

2.3. *Preparation of crude enzyme*

We prepared crude enzyme solutions according to Nachmansohn et al. [7–9]. For our purpose the most convenient methods were step 1 and 2 after Leuzinger et al. [10, 11] who used as starting material the electric organ of the eel (*Electrophorus electricus*). We used deep frozen electric organs of *Torpedo marmorata.* The crude enzyme solution was chromatographed on Sephadex G-100* with 0.5 M NaCl–phosphate buffer at pH 7. The active fraction served as our starting material for affinity chromatography. This material was centrifuged at 30 000 *g* to give a clear solution.

2.4. *Affinity chromatography*

Five ml of clear enzyme solution (total activity 50 000 units) were made up in an equal amount of 0.02 M phosphate buffer pH 7, and applied to a K 26/70 column*, filled with 150 ml of prepared Sepharose. Fractions were monitored continuously by UV spectroscopy for protein content and enzyme activity was measured sample by sample with acetylcholine (4×10^{-3} m) as substrate with automatic titration***

3. Results

With 0.02 M phosphate buffer a protein peak was eluted without enzyme activity. We further eluted with a 0.5 M NaCl–0.02 M phosphate buffer another protein peak with no enzyme activity. Final elution was done with 0.5 M NaCl–0.02 M phosphate–0.2 M tetramethylammonium bromide at pH 7. The third peak, small in amount of protein compared to the two preceding peaks, contained highly active enzyme fractions. The inhibitor trimethylammonium bromide was removed by washing in an AMICON cell † with 0.001 M phosphate buffer pH 7. The total yield was 46 480 units (93%).

4. Discussion

For theoretical reasons acetylcholinesterase is an ideal enzyme for affinity chromatography. The anionic centre of the active site carries a negative charge, which *in vivo* attracts under physiological conditions the positive charged nitrogen of acetylcholine. *In vitro* it attracts any positively charged nitrogen, surrounded by methyl groups and one substituent which is not too bulky. Berman and Young [1] used a phenyl-trimethylammonium group, placed at the end of a spacer which was firmly bound to the supporting agarose. Thus only the anionic centre of the active site is involved in binding the enzyme. Lately Schmidt and Raftery [12] used compounds of much simpler type, [*N*-(ε-aminohexanoyl)-3-aminopropyl]-trimethylammonium bromide, to trap cholinergic receptor proteins by affinity chromatography. It is very likely that cholinergic receptor proteins and acetylcholinesterases have a similar anionic centre. The simplest fixed compound to fit the anionic centre would be a long, straight, aliphatic chain fitted at a solid support and carrying a positively charged nitrogen at the free end. For this reason we used 1-(*N, N, N*-trimethylammonium)-6-hexylamine bromide as described above. Our spacer carries polar groups since they were needed for its synthesis only, and thus approaches the ideal model. The spacer has to have a length of about thirty C-atoms. When it is shorter the enzyme does not bind in an adequate manner and may be washed off the column with conventional buffers of sufficient ionic strength [Dr. U. Brodbeck, Med. Chem. Institut., University of Bern, personal communication according to own experiment]. Similar considerations apply for recovering the enzyme. As Schwyzer and Frank [3] already have demonstrated the smallest

* See page 1.

*** Metrohm Autotitrator, Metrohm, CH 9100 Herisau, Switzerland.

† Diaflo^{R} ultrafiltration membrane XM-100 A, 43 mm: from W. Meyer, Zihlmattweg 1, CH 6000 Lucerne 13, Switzerland.

molecule to fit the anionic centre: tetramethylammonium ion serves best for this purpose.

The typical elution profile for affinity chromatography of acetylcholinesterase shows three different protein peaks. The first peak is eluted with relatively weak buffer and shows almost no enzyme activity. The second peak, which is eluted with buffer of higher ionic strength shows low enzyme activity 'bleeding' at a low level until the enzyme is eluted under the third peak with a specific inhibitor. The third peak shows low protein content and high enzyme activity. With the new aliphatic inhibitor: 1-(*N, N, N*-trimethylammonium)-6-hexylamine, adequately bound to agarose and eluted under proper conditions, peak one and two do not show any significant enzyme activity. The enzyme is obtained in the fractions of the third peak in good yield (93%).

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