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A covalent affinity column for the purification of acetylcholinesterase

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SUMMARY

2-Aminoethyl *p*-nitrophenyl methylphosphonate, a potential inhibitor of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), was coupled to extended sepharose, thus forming covalent affinity support for purification of acetylcholinesterase. Cholinesterase from electric eel was trapped on the column by forming a covalent bond with the insoluble matrix. The enzyme was recovered from the column in very good quality by using the reactivator 2-(hydroximinomethyl)-1-methylpyridinium iodide (2PAM) or 1,1′-trimethylene bis(4-hydroximinomethyl pyridinium) dibromide (TMB_4). The column functioned as a covalent affinity column for eel cholinesterase and α-chymotrypsin (EC 3.4.4.5).

The purification of biological active macromolecules using selective water-insoluble adsorbents ('affinity chromatography') is based on reversible inhibitors that are attached by a covalent bond to an insoluble matrix[1].

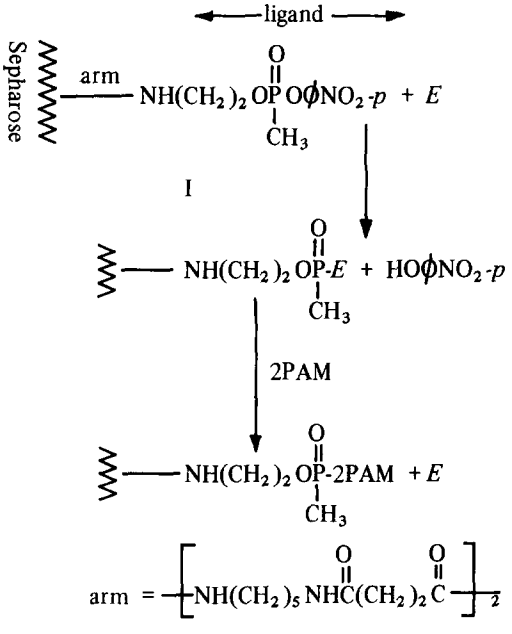
Acetylcholinesterase (acetycholine hydrolase, EC 3.1.1.7) from eel has been purified by ammonium sulfate fractionation and ultracentrifugation[2,3] and also by column chromatography[4].

Affinity chromatography has been applied successfully to the purification of acetylcholinesterase[5–7]. In these cases the specific ligand was based upon phenyltrimethylammonium ion, a competitive inhibitor of this enzyme. Kalderon *et al.*[5] concluded that this affinity column is also capable of binding negatively charged proteins, nonspecifically. As a result, purification was not complete. On the other hand, starting with partially purified eel enzyme Berman and Young[6] claimed complete purification of

Abbreviations: 2PAM, 2-(hydroximinomethyl)-1-methylpyridinium iodide; TMB_4, 1,1′-trimethylene bis(4-hydroximinomethyl pyridinium) dibromide.

acetylcholinesterase by using the same ligand with a different extension arm. Red cell enzyme required a different ligand[6]. Recently, Rosenberry *et al.*[7] have obtained very good purifications based on the affinity gel of Kalderon *et al.*[5] but with a doubly extended arm as emphasized by Cuatrecases and Anfinsen[1].

Acetylcholinesterase (and other esterases) is very readily inhibited 'irreversibly' by a wide variety of organophosphate triesters containing a good leaving group. A covalent phosphoryl enzyme derivative is formed. Nevertheless, the activity of inhibited acetylcholinesterase, depending on the nature of the phosphoryl moiety and the enzyme source, can be restored quite rapidly by specific nucleophiles such as 2-(hydroximinomethyl)-1-methylpyridinium iodide (2PAM)[8,9], 1,1′-trimethylene bis(4-hydroximinomethyl pyridinium)dibromide (TMB_4)[10,11], or by less specific nucleophiles such as fluoride ion[12] or hydroxylamine[13]. Thus, an affinity column (I) might be made by attaching a suitable organophosphate inhibitor to an insoluble matrix. In this way the enzyme would be covalently trapped by the column but could be released by suitable nucleophiles:



Scheme 1

The ligand we have used in this work is 2-aminoethyl *p*-nitrophenyl methylphosphonate hydrochloride (II) prepared according to the scheme:

$$CH_3P(O)(OCH_3)_2 \xrightarrow{PCl_5} CH_3P(O)Cl_2 \xrightarrow{p\text{-nitrophenol}}$$

$$CH_3P(O)(O\text{Ø}NO_2\text{-}p)Cl \xrightarrow{2\text{-Aminoethanol}} CH_3P(O)(OCH_2CH_2NH_2\cdot HCl)(O\text{Ø}NO_2\text{-}p)$$

II, m.p. 148–150°C

Scheme 2

The ligand is unstable in aqueous solutions and is only a poor inhibitor, but *N*-acyl derivatives of the ligand are very potent inhibitors, and more stable. Supports with relatively high specific activities of 0.2–1 μmole/ml packed gel (measured as *p*-nitrophenol that is released by 0.1 M NaOH or 0.1 M NaF) were obtained.

Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated by CNBr[14] and extended by conventional methods[15], using 1,5-diaminopentane, succinic anhydride and the coupling agent *N*-ethyl-*N'*-dimethylaminopropyl carbodiimide hydrochloride The ligand (II) was attached to the extended sepharose to give the active support I.

A short column (0.8 cm × 1.5 cm) was packed with I (ligand concentration 0.2 μmole/ml) and washed free from *p*-nitrophenol with phosphate buffer at pH 7.0 and room temperature (0.05 M phosphate adjusted to *I* 0.5 with NaCl, 0.02% gelatine). The flow rate was 1 ml/min.

Even after washing with 100 ml of buffer the effluent contains about $1 \cdot 10^{-7}$ M, bound *p*-nitrophenol and readily inhibits eel cholinesterase. Enzyme so inhibited is readily reactivated with 2PAM. Thus, a mobile phase inhibitor that is very potent is present, but it does not interfere with trapping the enzyme on the column. More than 90% of eel enzyme applied to the column in volumes of 0.05–2.0 ml and run at 1 ml/min is trapped (Fig. 1a).

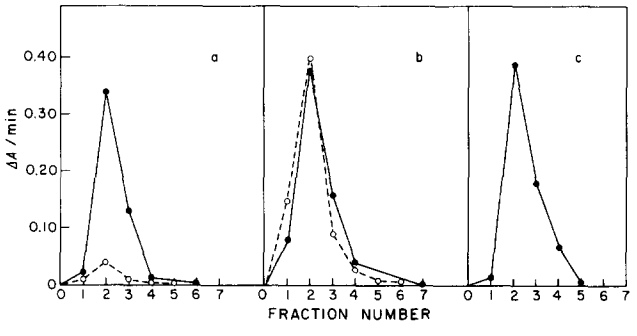


Fig. 1. Elution profile of eel cholinesterase on various derivatives of sepharose. For further details see text. (a) •——•, diethyl phosphoryl cholinesterase (eel) eluted on I. ○- - -○, eel cholinesterase eluted on I. (b) •——•, pretreatment of I with NaF. ○- - -○, pretreatment of I with 2PAM. (c) Extended sepharose without ligand. Enzyme activity assayed at pH 7.0 according to Ellman procedure[16].

The role of the ligand in trapping cholinesterase is well demonstrated in Fig. 1a where 96% of eel cholinesterase previously inhibited with diethyl phosphorofluoridate ($1 \cdot 10^{-5}$ M) was recovered in the effluent. Further studies showed that the gel loses its ability to bind eel cholinesterase by pretreatment with $1 \cdot 10^{-2}$ M 2PAM at pH 8.0, or $1 \cdot 10^{-1}$ M NaF at pH 8.0 (Fig. 1b). Fig. 1c demonstrates that the extended gel without the ligand did not retard eel cholinesterase to any extent.

Even after extensive washings with high salt buffer the trapped enzyme remains bound to the gel. There is enzymatic activity in the column which can be eliminated by diethyl phosphorofluoridate ($1 \cdot 10^{-5}$ M) and restored easily with 2PAM ($1 \cdot 10^{-4}$ M). To remove the enzyme from the gel, 2 ml of TMB_4 or 2PAM solution ($1 \cdot 10^{-2}$ M, at pH 8.0, I 0.5) was passed through the column and the gel left with the oxime solution (not flowing) for 16 h at room temperature (25–27°C). When small amounts of enzyme were applied to a column containing $2 \cdot 10^{-4}$ M bound ligand and enzyme reactivated with $1 \cdot 10^{-2}$ M TMB_4, 35–40% of the enzyme was recovered. An additional period of 48 h released another 45–50% of the enzyme, thus giving a total recovery of 80–90% of the enzyme.

The enzyme was separated from the mixture of oxime (2PAM or TMB_4) and *p*-nitrophenol using Sephadex G-50. For studies of purification larger amounts of enzyme were used so that the recovered enzyme would have a sufficiently large optical absorbance. In these cases 2PAM was used as the reactivator and the ligand concentration was $6 \cdot 10^{-4}$ M. Lower recovery rates were obtained.

The degree of purification of eel cholinesterase was estimated by measuring the specific activity (mmoles/h per mg) of the enzyme preparation before and after the purification procedure. Enzyme activity was assayed at pH 7.0 (0.02 M phosphate, 0.02 M $MgCl_2$ and 0.1 M NaCl) spectrophotometrically using acetylthiocholine as substrate ($2.75 \cdot 10^{-3}$ M) at 25°C[16]. (The molar absorbance index for the thiophenol was $1.40 \cdot 10^4$.) Protein concentrations of the crude preparations were estimated by using $E^{1\%}_{280} = 10$ which is the median value for proteins. For the final preparations the value $E^{1\%}_{280} = 18.0$ (1-cm cells) was used. The experimental value of the absorbance index for pure enzyme depends upon the method of protein determination[7]. We have selected 18.0 to be in keeping with the value used by Rosenberry *et al.*[7] so that we can readily compare specific activity values. We have also assayed one preparation under the conditions used by Rosenberry *et al.*[7], automatic titration at pH 7.4, 25°C, 0.1 M NaCl, 0.02 M $MgCl_2$, 0.005% gelatin, and 2.7 mM acetylcholine bromide. The specific activity of this preparation so evaluated was 525. This value is in the range reported by Rosenberry *et al.* for repeated purifications but less than their highest value of 610. We have no thoughts that our enzyme is pure because no special efforts were made to secure specificity. The column is potentially capable of trapping all serine esterases, and all such covalently bound esterases are potentially capable of being released from the column. For example, α-chymotrypsin (EC 3.4.4.5) is also trapped by this column, and in this case we could ascertain that an equivalent of *p*-nitrophenol was released. On the other hand no human serum albumin was trapped by the column.

The emphasis of this note is not on the purity of the product but rather on the method. As far as we can ascertain this is the first report of a covalent affinity column★. We believe this type of purification method has much potential value. Specificity can be achieved in principle in a number of ways. We find that when enzyme in the presence of $1 \cdot 10^{-3}$ M 3-hydroxy phenyltrimethylammonium ion, which is a very good reversible inhibitor, is applied to the column all the enzyme comes through. Presumably other serine esterases would be trapped. Specificity might also be achieved by changing the leaving group of the ligand. The column might be improved by using a longer arm length.

We have some evidence that removal of the covalently bound enzyme from the column is not as straightforward as envisaged. It appears that enzyme may be released from the column in an inhibited state spontaneously and very slowly. An ester linkage (as well as carbamate bond) between the arm and the matrix might be involved in such preparation.

This question is being investigated, and we are also pursuing precise methods for securing completely pure enzyme. What we have described in this note is a covalent affinity column that yields enzyme of very good quality in one step starting with a very crude enzyme preparation.

Table I indicates clearly that the described gel (I) is capable of purifying eel cholinesterase starting with a partially purified preparation (Worthingon S.A. 1079 μM unit/mg) or a very crude preparation (ammonium sulphate fractionation of eel tissue).

TABLE I

THE PURIFICATION OF EEL CHOLINESTERASE BY I (LIGAND CONCENTRATION $6 \cdot 10^{-4}$ M) AT ROOM TEMPERATURE (25–27°C)

For further details see text.

Specific activity of starting enzyme	*Protein recovered[a] in effluent (%)*	*Enzyme activity[a] recovered in effluent (%)*	*Time of recovery ($1 \cdot 10^{-2}$ M 2PAM, pH 8.0)*	*Yield (%)*	*Specific acitvity of purified enzyme (mmoles/mg per h)*
9	98	3	16 h	22 (48)[b]	480[e]
77	79	10	40 h	38 (57)[c]	450[e]
9	98	10	6 days[d]	55	525[f]

[a] Protein and enzyme activity not trapped by the column during the enzyme charging step.
[b] Total enzyme recovered after 64 h.
[c] Total enzyme recovered after 112 h.
[d] 5°C.
[e] Acetylthiocholine hydrolysis as described in text.
[f] pH 7.4 automatic titrator acetylcholine substrate. $E^{1\%}_{280} = 18.0$ used for estimation of protein; conditions comparable to Rosenberry *et al.*[7].

★ A gel containing a carbamate ligand used by Berman and Young[6] is potentially capable of functioning as a covalent affinity support.

It is also apparent from Table I that longer periods of incubation will release more enzyme from the column.

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REFERENCES

1 For extensive review and references see: P. Cuatrecases and C.B. Anfinsen, *Annu. Rev. Biochem.*, 40 (1971) 259.
2 M.A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, 168 (1947) 223.
3 H.C. Lawler, *J. Biol. Chem.*, 234 (1959) 799.
4 L.T. Kremzner and I.B. Wilson, *J. Biol. Chem.*, 238 (1963) 1714.
5 N. Kalderon, I. Silman, S. Blumberg and Y. Dudai, *Biochim. Biophys. Acta*, 207 (1970) 560.
6 J.D. Berman and M. Young, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 395.
7 T.L. Rosenberry, H.W. Chang and Y.T. Chen, *J. Biol. Chem.*, 247 (1972) 1555.
8 I.B. Wilson and S. Ginsburg, *Biochim. Biophys. Acta*, 18 (1955) 168.
9 D.R. Davies and A.L. Green, *Disc. Faraday Soc.*, 20 (1955) 269.
10 F. Hobbiger, D.G. O'Sullivan and P.W. Sadler, *Nature*, 182 (1958) 1498.
11 E.J. Poziomek, B.F. Hackley and G.M. Steinberg, *J. Org. Chem.*, 23 (1958) 714.
12 E. Heilbronn, *Acta Chem. Scand.*, 18 (1964) 2410.
13 I.B. Wilson, *J. Biol. Chem.*, 190 (1951) 111.
14 R. Axén, J. Porath and S. Ernback, *Nature*, 214 (1967) 1302.
15 P. Cuatrecases, *J. Biol. Chem.*, 245 (1970) 3059.
16 G.L. Ellman, K.D. Courtney, V. Andres, Jr and R.M. Featherstone, *Biochem. Pharmacol.*, 7 (1961) 88.