Biochimica et Biophysica Acta, 445 (1976) 147—157

BBA 67886

ACETYLCHOLINESTERASE OF THE HOUSE-FLY HEAD

AFFINITY PURIFICATION AND SUBUNIT COMPOSITION

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(Received January 16th, 1976)

Summary

1. Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) of house-fly head tissue was solubilized as a 7.4-S form by autolysis for 80—100 h at 25°C and pH 8.0.

2. The autolysed enzyme was purified by affinity chromatography, firstly on Con-A-Sepharose and subsequently on *m*-trimethylammoniumaniline-Affi-Gel® 202. This sequence permitted overall purification yields of approx. 50% of the solubilized enzyme.

3. The 7.4-S purified enzyme was essentially homogeneous on polyacrylamide gel electrophoresis, and its specific activity coincided with the highest previously reported for fly-head acetylcholinesterase.

4. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and β-mercaptoethanol revealed two major polypeptide components of molecular weight 82 000 and 59 000. Each of these polypeptides contained diisopropylphosphofluoridate-binding sites, as shown with [^{3}H]diisopropylphosphofluoridate.

5. The results suggest a strong structural similarity between fly-head acetylcholinesterase and the purified electric eel enzyme.

Introduction

Insect acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) has been isolated in multiple forms which vary with the mode of solubilization [1—4]. The aggregates present in salt extracts have been shown to be interconvertible and convert to a 7.4-S form of molecular weight approx. 160 000 following autolysis [3—5]. Krysan and Kruckeberg [4] proposed this form as the "funda-

Abbreviation: iPr_2P-F, diisopropylphosphofluoridate.

mental unit" of insect acetylcholinesterase, but recent studies [5,6] have characterized smaller active forms of the enzyme. Steele and Smallman [5] demonstrated that 7.4-S form of house-fly head acetylcholinesterase was cleaved by disulphide reduction into an active 5.3-S form of molecular weight about 80 000. The latter form was obtained as a 'native' component of fresh tissue extracts, and was suggested [5] as the best candidate for the fundamental unit of the house-fly enzyme.

A thorough characterization of the 5.3-S component appears essential for an understanding of the protomer structure of the fly-head enzyme, but such characterization is attendant on successful purification. Any approach to purification of the 'native' 5.3-S form appears subject, however, to major difficulties imposed by the low proportion of total activity residing in this form. Of the 30% of fly-head acetylcholinesterase that separates as 'natively' soluble after centrifugation at 100 000 × *g* for 1 hour [1,3], four higher aggregates have been shown to contribute activity in addition to the 5.3-S form [5].

Solubilization of the enzyme by autolysis at high salt concentrations and pH 8.0 [3,4], offers the advantage of releasing over 70% of the enzyme in a form that has been shown to be homogeneous by gel filtration and polyacrylamide gel electrophoresis [3,5]. Moreover, the enzyme solubilized by autolysis appears indistinguishable from the 'native' 7.4-S form and can be converted to the 5.3-S form by disulphide reduction [5].

The present report details the purification of autolysed fly-head acetylcholinesterase by affinity chromatography, and characterization of the subunit structure of this enzyme.

Materials and Methods

Materials

Heads were collected from 3—4 day old house-flies of the DDT/S strain [5] by the method of Moorefield [7], and stored at —20°C until further use.

Bovine γ-globulin, α-methyl-D-mannoside, sodium dodecyl sulphate, β-mercaptoethanol, Coomassie brillant blue R, and guanidine hydrochloride were purchased from Sigma Chemical Company, St. Louis, Missouri. Sephadex G-25, G-200, and Con-A-Sepharose were products of Pharmacia Fine Chemicals Inc., Dorval, Quebec. Affi-Gel® 202 was obtained from Bio-Rad Labs., Richmond, California. 1,3-[^{3}H]dissopropylphosphorofluoridate (specific activity 3.9 Ci/mmol) was purchased from Amersham/Searle, Don Mills, Ontario. *m*-Trimethylammoniumaniline was synthesized by the procedures of Chan et al. [8]. Edrophonium chloride was a gift of Hoffman La Roche, Basle, Switzerland. Other reagants were the same as used previously [5].

Assay methods

Acetylcholinesterase activity was determined by the method of Ellman et al. [9] at 25°C and pH 8.0, using acetylthiocholine iodide (0.75 mM) as substrate. The measurements were made on a Cary Model 14 spectrophotometer from changes in absorbance at 412 nm. On unit of activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of acetylthiocholine iodide

per min at 25°C, and the specific activity is expressed as μmol hydrolysed per mg of protein per min. Protein concentration was determined by the method of Lowry et al. [10], with bovine serum albumin as standard.

Enzyme purification

Frozen fly-heads were homogenized (10% w/v) in 1.0 M NaCl, 0.1 M potassium phosphate buffer (pH 8.0) as described previously [5]. Homogenates were filtered free of large debris, and autolysed after the method of Krysan and Kruckeberg [4] for 80—100 h at 25°C, with constant shaking and daily pH adjustments to pH 8.0. Solubilization of the enzyme was influenced by the rate of rotary shaking; our preparations were shaken on a New Brunswick Gryrotory Shaker Model G-10 at a speed setting of 2.0. Autolysed preparations were centrifuged at 40 000 × *g* for 1 h at 4°C (Sorvall RC2-B centrifuge, SS-34 rotor), and the enzyme was obtained in the resulting supernatants. Solid ammonium sulphate was added to these supernatants and the fractions which precipitated between 20—45% w/v (without pH adjustment at 4°C) were isolated by centrifugation at 40 000 × *g* for 30 min. After redissolving in autolysis buffer, these fractions were given a final centrifugation at 40 000 × *g* for 30 min, and the supernatants were run at 20 ml/h and 4°C over a 1.6 × 40 cm affinity column of Con-A-Sepharose equilibrated with the same buffer. Preliminary studies established that, under our conditions, fly-head acetylcholinesterase was quantitatively adsorbed to this column provided the sample preparations contained less than 6 units/ml bed volume. After washing the adsorbed protein with about 5 column volumes, elution was achieved by addition of α-methyl-D-mannoside (10% w/v) to the starting buffer. Acetylcholinesterase appeared within the first 150 ml of the elution front. Following buffer exchange to 0.1 M NaCl, 30 mM Tris · HCl (pH 8.0) on Sephadex G-25 (column 2.5 × 70 cm), the preparations were run at 20 ml/h and 4°C over the second affinity column (1.6 × 16 cm), equilibrated with the same buffer. This affinity resin was made by attaching *m*-trimethylammoniumaniline to the extended arms of Affi-Gel® 202, according to the procedures of Cuatrecasas [11]. After washing the adsorbed protein with 5—10 column volumes of buffer (when absorbance of the eluate at 280 nm returned to zero), elution was achieved by the addition of edrophonium chloride (10 mM) to the buffer. Acetylcholinesterase appeared within the first 20 ml of the elution front; over 50% of the released enzyme was free of detectable edrophonium inhibition. The remaining edrophonium was removed by gel filtration on Sephadex G-150, after the 20-ml affinity product had been concentrated to about 5 ml in a Minicon B-15 concentrator (Amicon). Chromatography on Sephadex G-150 was performed at 26—28 ml/h and 4°C, on a calibrated column (2.5 × 78 cm) equilibrated and developed with 0.1 M NaCl, 30 mM Tris · HCl (pH 8.0) as described earlier [5]. Fractions of high activity were pooled, concentrated to about 200 units/ml in the Minicon B-15, and stored at −20°C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in Tris/glycine buffer (pH 8.3) after Davis [12], and visualization of acetylcholinesterase activity were performed as previously described [5]. Protein bands were stained 4—16 h in a solution of

0.25% w/v Coomassie blue in methanol/water/acetic acid (5 : 5 : 1 by volume). Destaining was accomplished by exhaustive washing in an aqueous solution of 7.5% v/v acetic acid, 5% v/v methanol.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed after the discontinuous pH 8.3 system of Laemmli [13]. Separation gels (0.5 × 7 cm) contained 10% acrylamide, and spacer gels were made 2.5% (C = 20%) acrylamide. Both gel buffers and the reservoir buffer contained 0.1% w/v sodium dodecyl sulphate; the reservoir buffer also contained 0.1% v/v β-mercaptoethanol. Acetylcholinesterase samples and standard proteins (bovine serum albumin, bovine γ-globulin, ovalbumin, pepsin, and myoglobin) were denatured prior to sodium dodecyl sulphate electrophoresis by heating for 3 min at 100°C in a solution of 10% v/v glycerol, 60 mM Tris · HCl (pH 6.8) containing 2% w/v sodium dodecyl sulphate and 5% v/v β-mercaptoethanol. Electrophoresis was performed at 1.0 mA per tube until the marker dye bromophenol blue had migrated through the spacer gel (0.5 cm). The run was completed at 2.0 mA per tube, until the dye front had migrated 6.5 cm in the separation gel. Polypeptides were visualized by staining with Coomassie blue as above, and a molecular weight calibration curve was determined after the method of Shapiro et al. [14] from the relative mobilities of the standard proteins.

[^{3}H]iPr_2P-F labelled acetylcholinesterase was denatured and electrophoresed similarly. The labelled enzyme was prepared at 25°C by incubation with [^{3}H]-iPr_2P-F (final concentration approx. 50 μM) until complete inhibition of enzymic activity was obtained. Unbound label was removed from the phosphorylated enzyme by dialysis overnight against 4 changes of 60 mM Tris · HCl (pH 6.8). Following electrophoresis, the distribution of ^{3}H-labelled components was determined from gel slices (1.5 mm) prepared for scintillation counting by digestion for 2 h at 60°C in 0.5 ml hydrogen peroxide (30%). Counting was performed in Aquosol fluor (New England Nuclear) with a Nuclear Chicago Unilux III.

Results

Purification procedures

In preliminary studies it was found that autolysed fly-head acetylcholinesterase could be purified to >500 units/mg, by one-step affinity chromatography on Affi-Gel with substituted side arms having specific affinity for the anionic site in the active center of acetylcholinesterase [8]. However, with crude autolysed fly-head preparations the binding capacity of this affinity resin diminished drastically with use. This diminution was retarded by column regeneration after the method of Chen et al. [15], using 6 M guanidine hydrochloride and extensive washing with 1.0 M NaCl solutions. Notwithstanding these measures, visible contamination of the substituted Affi-Gel column by black-brown pigmented material remained, and the purification yields continued to decline. To overcome this problem, a partial purification of crude autolysed preparations was performed. Conventional procedures of ammonium sulphate fractionation (20—45% w/v), DEAE-cellulose and Sephadex G-200 chromatography, were examined. These steps achieved an essentially pigment-free product, but as ob-

served by Huang and Dauterman [16], low recoveries (≤55%) of enzyme activity were obtained.

These various difficulties were finally resolved by performing a two-step sequence of affinity purification. The first affinity step involved chromatography of the 20—45% w/v ammonium sulphate fraction on Con-A-Sepharose. Fig. 1 shows a typical elution pattern from this column. Enzyme recoveries exceeded 75% in all runs, and the product was essentially free of pigmented material. This binding of fly-head acetylcholinesterase convincingly demonstrates the glycoprotein nature of the enzyme. Moreover, this affinity step resulted in a 5—10-fold purification, a result similar to that obtained by Wiedmer et al. [17] with acetylcholinesterase from a variety of vertebrate scources.

Following buffer exchange on Sephadex G-25, the Con-A-Sepharose product was introduced to the substituted Affi-Gel column. Fig. 2 shows a typical pattern of enzyme elution from this affinity column. Less than 1% of the bound acetylcholinesterase was lost from the resin by exhaustive washing. Edrophonium chloride (10 mM) released the enzyme in recoveries that ranged from 62% to 94% with increasing amount of enzyme applied, and the binding efficiency of the column remained essentially unaltered with use. The fractions containing acetylcholinesterase activity (volume 20 ml) were concentrated and then subjected to gel filtration on Sephadex G-150. Fig. 3 shows the elution results of this final purification step.

An example of a purification run is presented in Table I. It can be seen that gel filtration on Sephadex G-150 failed to increase significantly the purification. However, this step removed traces of pigmented material of low molecular weight in addition to edrophonium chloride. Presumably, these pigmented contaminants are non-proteinaceous, and are absorbed non-specifically to the sub-

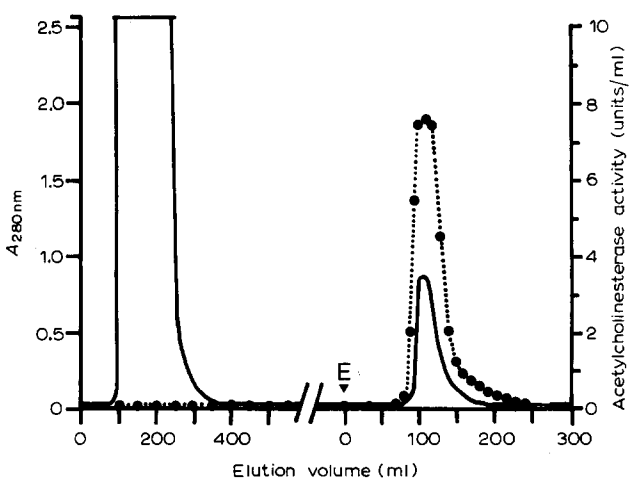

Fig. 1. Affinity chromatography of fly-head acetylcholinesterase on Con-A-Sepharose (column 1.6 X 40 cm). Elution of the enzyme sample (20—45% w/v ammonium sulphate fraction of an autolysed preparation) was performed at 20 ml/h and 4°C: at E, α-methyl-D-mannoside (10% w/v) was added to the elution buffer (1.0 M NaCl/0.1 M potassium phosphate (pH 8.0). The solid line is the absorbance at 280 nm; the dashed line refers to the acetylcholinesterase activity determined by the Ellman et al. [9] assay. Recovery of the applied activity (418 units) was 79.2%.

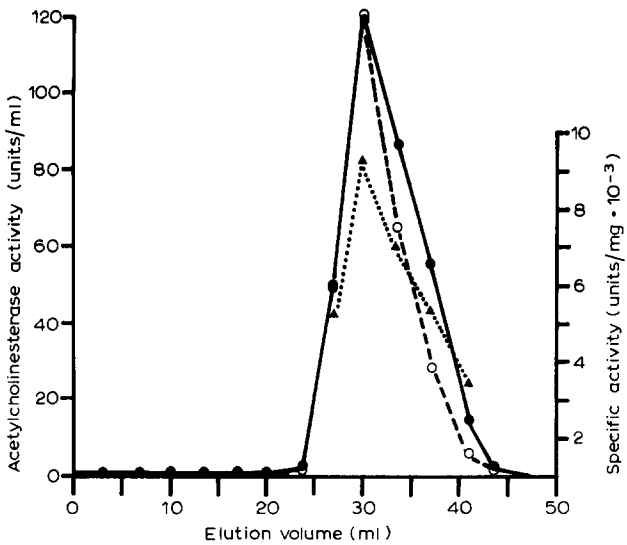

Fig. 2. Affinity chromatography of fly-head acetylcholinesterase on Affi-Gel® 202 with the ligand *m*-trimethylammoniumaniline attached to the side arms. The enzyme sample obtained from Con-A-Sepharose chromatography was applied to the column (1.6 × 16 cm) at 20 ml/h; after washing the column with 0.1 M NaCl, 30 mM Tris · HCl (pH 8.0) until the eluant was protein-free (approx. 5 column volumes), the adsorbed enzyme was eluted with edrophonium chloride (10 mM) in the same buffer. ○ is the acetylcholinesterase activity prior to removal of edrophonium chloride; ●, acetylcholinesterase activity after removal of edrophonium chloride by dialysis; ▲, specific activity (units/mg) of the recovered enzyme (82%).

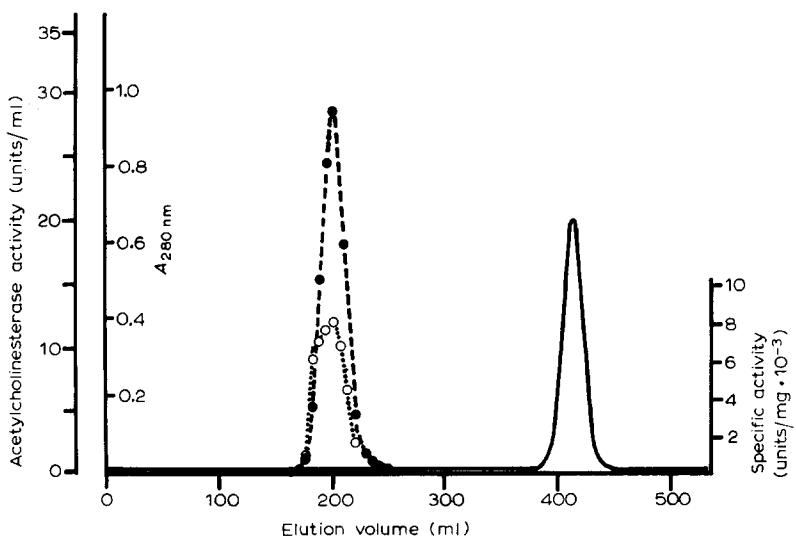

Fig. 3. Gel-filtration of fly-head acetylcholinesterase on Sephadex G-150 (column 2.5 × 78 cm). The enzyme obtained from substituted Affi-Gel chromatography was concentrated to 6.7 ml (containing 112 units/ml) and eluted at 26.8 ml/h and 4°C with 0.1 M NaCl, 30 mM Tris · HCl (pH 8.0). The solid line refers to the absorbance at 280 nm; the dashed lines refer to acetylcholinesterase activity; ●, units/ml; ○, units/mg. Recovery of the applied activity (752 units) was 94.3%. From the calibration curve [5], the peak at 205 ml corresponded to a molecular weight of 160 000 ± 10 000.

TABLE I

PURIFICATION OF HOUSE-FLY HEAD ACETYLCHOLINESTERASE

	Purification step	Total activity (units)	Specific activity (units/ml)	Specific activity (units/mg)	Purification factor	Yield (%)
1	Zero time, crude homogenate (50 g; 10% w/v)	1551	3.1	0.25	—	100
	Zero time, 40 000 × *g*, 1-h supernatant	236	0.47	0.09	—	15
2	Autolysed 74 h 40 000 × *g*, 1-h supernatant	1368	2.7	0.61	1	88
3	$(NH_4)_2SO_4$ fraction 20—45% (w/v)	1233	17	2.2	3.5	80
4	Con-A-Sepharose affinity chromatography *	962	2.2	13.3	21	62
5	Substituted Affi-Gel affinity chromatography	789	39	578	936	51
5	Sephadex G-150 gel-filtration	706	15	590	955	46

* This affinity step was carried out in 3 separate runs. After buffer exchange on Sephadex G-25, the fractions were recombined preparatory to affinity chromatography on substituted Affi-Gel.

stituted Affi-Gel resin. The specific activities of the purified enzyme averaged 585 and ranged from 383 to 762 units/mg. Individual fractions from each purification run had greater specific activities, and the highest observed was 939 units/mg. These values overlap with the specific activities of 469—952 units/mg obtained by Huang and Dauterman [16]. Large scale purification of fly-head acetylcholinesterase by this purification scheme should prove simple, convenient, and far more efficient than previous methods.

Characterization of purified fly-head acetylcholinesterase

When purified acetylcholinesterase was electrophoresed on 4—10% polyacrylamide gels, only one enzymatically active component was visualized (Fig. 4). This component appeared to be the only protein constituent as judged by staining with Coomassie blue. Our purified enzyme therefore differed significantly from that of Huang and Dauterman [16], which electrophoresed as two active and interconvertible forms, which they suggested were of the same or similar molecular weight but differed in total net charge.

No differences could be observed between the purified enzyme and that of the 7.4-S form of native or crude autolytic preparations. These coincidences pertained to electrophoretic characteristics, fractionation on Sephadex G-150, and cleavage by disulphide reducing agents [5]. Fig. 4 shows that cleavage with 1 mM β-mercaptoethanol produced a smaller active component, earlier established to be indistinguishable from the 5.3-S natively soluble form. On the basis

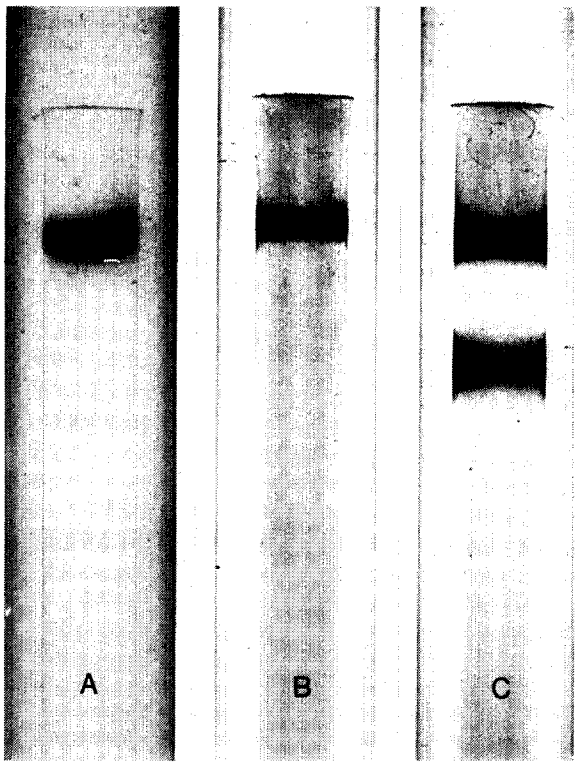

Fig. 4. Polyacrylamide gel electrophoresis in Tris/glycine buffer (pH 8.3) of purified 7.4-S fly-head acetylcholinesterase (specific activity 709 units/mg). 10% gels were stained for protein and acetylcholinesterase activity as described in Materials and Methods. (A) 20 μg sample stained for protein; (B) 2 μg sample (1.5 units) stained for acetylcholinesterase activity; (C) same sample as in B, but reduced by 1 mM β-mercaptoethanol (10 min exposure at 25°C), then run in parallel and stained for acetylcholinesterase activity.

of these observations, the enzyme was judged to have been purified in a form similar if not identical, in physicochemical properties, to the natively soluble 7.4-S form of molecular weight about 160 000 [3,5].

Subunit structure

The polypeptide components of the purified enzyme were determined by performing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and β-mercaptoethanol. The dominant polypeptide component revealed by this procedure (Fig. 5), was estimated [14] from the calibration curve to be 59 000 ± 2000 daltons. Fig. 5 shows polypeptides of 82 000 ± 4000 and 20 000 ± 2000 daltons, and a trace of (102 000 ± 6000)-dalton components were also found. The majority of [^{3}H]iPr_2*P*-F binding sites were contained in the 59 000-dalton component (Fig. 5). Some of the 82 000-dalton polypeptide also appeared to contain iPr_2*P*-F-binding sites, but no ^{3}H labelling was detected in the polypeptides of 20 000 daltons.

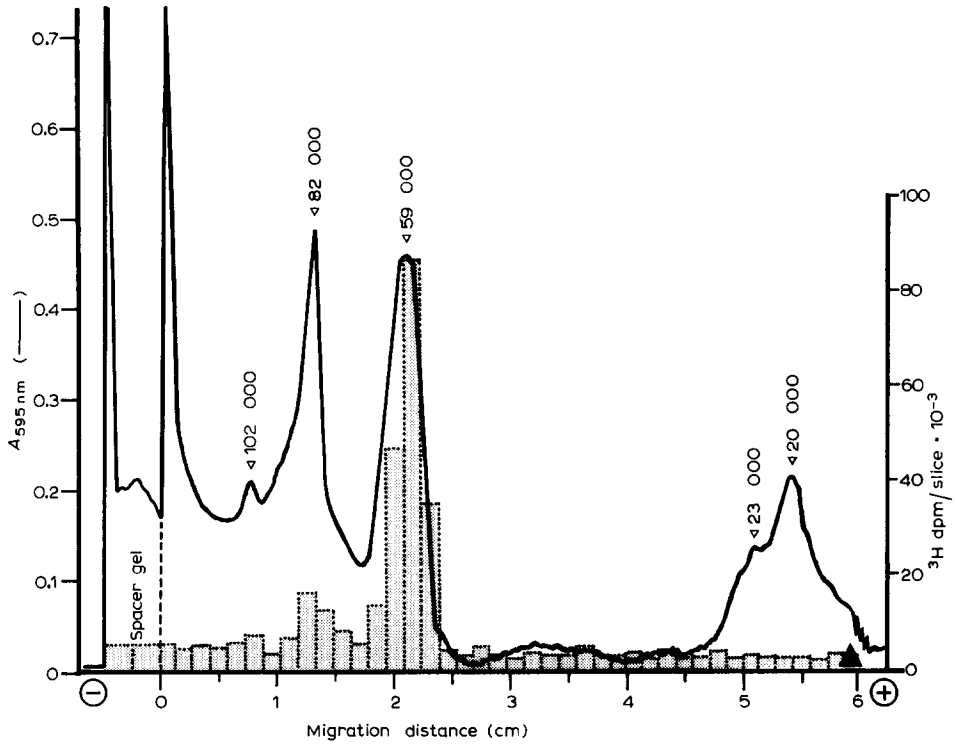

Fig. 5. Polypeptide components in purified fly-head acetylcholinesterase (specific activity 709 units/mg). Samples were denatured in sodium dodecyl sulphate and β-mercaptoethanol, then electrophoresed on 10% gels as described in Materials and Methods. The solid line is the absorbance of the stained gel at 595 nm (scanned on a Joyce-Loebl Chromoscan, slit 0.2 × 1.0 mm); the black triangle marks the migration peak of myoglobin when run as an internal standard. The symbols (e.g. 59 000) indicate the estimated molecular weights. The histogram refers to the ^{3}H dpm/1.5 mm gel slice obtained from a gel containing 10 μg of the same enzyme preparation which had been labelled with [^{3}H]iPr$_2$*P*-F prior to denaturation and electrophoresis.

General discussion

Our data on the polypeptide composition of purified 7.4-S fly-head acetylcholinesterase coincide well with recent data on the most thoroughly studied actylcholinesterase, that of the electric eel *Electrophorus electricus*. The coincidence with the results of Dudai and Silman [18] is particularly convincing. They observed that the 11-S forms of the eel enzyme consisted of two major polypeptides of 82 000 and 59 000 daltons, identical with our estimates for the major polypeptide components of the 7.4-S fly-head enzyme. This coincidence pertained also to the demonstration of polypeptide components containing iPr$_2$*P*-F-binding sites; [^{3}H]iPr$_2$*P*-F binding revealed these sites in both the 82 000- and 59 000-dalton polypeptides of each enzyme. Finally, Dudai and Silman [18] observed that the appearance of the 59 000-dalton component was accompanied by polypeptide fragments of about 25 000 daltons that contained no iPr$_2$*P*-F-binding sites. Similar polypeptides, also without iPr$_2$*P*-F-binding sites, were observed with the fly-head enzyme (Fig. 5).

These polypeptide consistencies indicate that, within our limits of observa-

tion, the fly-head and eel acetylcholinesterases have identical subunit structures and manifest similar fragmentation. The latter phenomenon has been established in the eel enzyme by the observation of relative polypeptide compositions of several 11-S enzyme preparations. In briefly autolysed eel tissue the principal polypeptide component was the intact 82 000 component, whereas after prolonged autolysis or storage the 59 000-dalton cleavage product predominated [15,18—21]. Presumably, fragmentation occurs without the release of cleavage products from the quarternary structure of the enzyme unless it is denatured. Our purified enzyme was obtained from tissue subsequent to extensive autolysis, and in apparent conformity the polypeptide pattern was similarly dominated by the 59 000-dalton component (Fig. 5). Possibly, this polypeptide arises from fragmentation of the 82 000-dalton polypeptide chain. This supposition is supported by the observation that disulphide reduction without denaturation (Fig. 4), cleaved the purified 7.4-S fly-head enzyme into an active 5.3-S form which has been shown [5] to have a molecular weight (about 80 000) consistent with that of an intact 82 000-dalton polypeptide chain. We believe the 102 000-dalton polypeptide component represents a trace impurity, and studies to resolve the fragmentation issue should establish this possibility.

Native fly-head extracts contain 7.8-, 8.1- and 11.8-S forms of acetylcholinesterase in addition to the 7.4- and 5.3-S species [5]. Preliminary studies (Steele, R.W. and Smallman, B.N., unpublished work) suggest these intermediate and higher aggregates have the same polypeptide composition as the purified 7.4-S enzyme. This result appears difficult to reconcile with the data indicating that these aggregates do not comprise simple oligomers [5] of the intact 82 000-dalton polypeptide chain. Similar findings, however, have been obtained for higher aggregates of the eel enzyme [19,20,22]. Our suggestion is that the 7.8-, 8.1- and 11.8-S fly-head aggregates may contain similar moieties to the 'tail components' which have been seen in the higher aggregates of the eel enzyme [23—25]. These moieties appear distinct from the catalytic subunits [19,20,22], and have proven refractory so far to characterization on sodium dodecyl sulphate gels.

Acknowledgements

We are grateful to Aspi Maneckjee for skilled technical assistance. This research was supported by National Research Council of Canada, Grant No. A2394 to B.N. Smallman.

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