Partial Purification and Some Properties of a Cholinesterase from Bush Bean (*Phaseolus vulgaris* L.) Roots

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A cholinesterase was partially purified from bush bean (*Phaseolus vulgaris* L.) roots by using acridinium-based ligand affinity chromatography. The procedure gave a 78-fold increase in specific activity, although at least three inactive contaminants remained. The enzyme activity was maximal against acetyl esters of choline and was inhibited by neostigmine. Di-isopropyl phosphorofluoridate completely inhibited activity at concentrations greater than 0.1 mM. The catalytic centre activity was 2×10^{-4} times that of electric eel acetylcholinesterase. Cholinesterase activity appeared as a peak ($s = 4.2 \pm 0.1$ S) after isokinetic sedimentation. The Stokes radius was 4.00 nm and the apparent molecular weight was $72\,700 \pm 1900$. The smallest active and native form of the enzyme appeared to be a monomer. This contrasts with animal acetylcholinesterases, in which the smallest active and native forms are multimeric.

Acetylcholine concentrations are regulated *in vivo* by several factors including the activity of acetylcholinesterase (EC 3. 1. 1. 7) (Nachmansohn, 1959; Ruch & Patton, 1965). It has been proposed (Jaffe, 1972; Fluck & Jaffe, 1974a) that acetylcholine mediates the effect of light on bioelectric and subsequent physiological responses in plant tissues through the involvement of phytochrome. Interest in the regulation of acetylcholine concentration in plants has increased with the accumulation of evidence supporting an endogenous role for this substance (Fluck & Jaffe, 1974a).

Acetylcholine may be hydrolysed by a variety of plant esterases including citrus acylesterase (Jansen *et al.*, 1947; Schwartz *et al.*, 1964), wheat-germ esterase (Jansen *et al.*, 1948; Mounter & Mounter, 1962), cucurbitacin esterase (Schwartz *et al.*, 1964), sinapine esterase (Tzagoloff, 1963) and a cholinesterase (Riov & Jaffe, 1973; Kasturi & Vasantharajan, 1976). The citrus acylesterase and wheat-germ esterase have K_m values of 1.6 and 1.0 M respectively for acetylcholine, but have not been tested for inhibition by Dip-F or carbamate inhibition. The cucurbitacin esterase activity is unaffected by 10 μ M-Dip-F and is slightly stimulated by 10 μ M-eserine. The sinapine esterase has a lower K_m (660 μ M) for acetylcholine, but is only marginally inhibited by 10 mM-eserine.

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Abbreviations used: Dip-F, di-isopropyl phosphorofluoridate; Dip, di-isopropyl; SDS, sodium dodecyl sulphate; MAC-Sepharose, 1-methyl-9-[N- β -(ϵ -amino-hexanoyl)- β -aminopropylamino]acridinium bromide-Sepharose

The cholinesterase has been partially purified from mung bean roots and subsequently from other plant tissues (Riov & Jaffe, 1973; Fluck & Jaffe, 1974c; Kasturi & Vasantharajan, 1976). It resembles animal acetylcholinesterase in that it is inhibited by neostigmine ($I_{50} = 0.6 \mu$ M) and to a lesser extent by eserine ($I_{50} = 0.9$ mM) (I_{50} is the inhibitor concentration at which enzyme velocity is decreased to 50% of that observed in absence of inhibitor). It also shows maximal activity against acetyl esters, it has a K_m of less than 200 μ M for acetylcholine or acetylthiocholine and it is prone to substrate inhibition.

Purification of acetylcholinesterase to homogeneity from various animal tissues involved the use of either the combination of $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration and ion-exchange chromatography (Kremzner & Wilson, 1963; Leuzinger & Baker, 1967) or affinity chromatography (Kalderon *et al.*, 1970; Berman & Young, 1971; Dudai *et al.*, 1972; Rosenberry *et al.*, 1972; Rosenberry & Richardson, 1977). A variety of affinity chromatography matrices have been used to purify the enzyme from electric eel electro-plaque tissue. The most suitable ligands for affinity purification of the native asymmetric molecular forms of the eel enzyme are derivatives of acridine (Dudai *et al.*, 1972; Rosenberry & Richardson, 1977).

This study was undertaken to identify cholinesterase activity in bush bean (*Phaseolus vulgaris* L.) roots, to purify the enzyme by using acridinium-based ligand affinity chromatography so that physical studies could be undertaken and to compare its physical properties with those of the acetylcholinesterases from animal sources.

Experimental

Materials

[³H]Dip-F and NCS tissue solubilizer were obtained from Amersham/Searle, Arlington Heights, IL, U.S.A. Dip-F was from Aldrich, Milwaukee, WI, U.S.A. pHisolytes were obtained from Brinkman Instruments, Wesbury, NY, U.S.A. Acetylcholine chloride, acetylthiocholine iodide, butyrylcholine chloride, butyrylthiocholine iodide, catalase, cytochrome *c*, decamethonium bromide [decamethylene-bis(trimethylammonium bromide)], 5,5'-dithiobis-(2-nitrobenzoic acid), β -galactosidase, neostigmine [(*m*-hydroxyphenyl)trimethylammonium bromide dimethylcarbamate], myoglobin, ovalbumin and propionylthiocholine were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Sepharose and Sephadex gels were from Pharmacia, Uppsala, Sweden. All other chemicals were obtained locally and were of A.R. quality when available.

Bush bean (*Phaseolus vulgaris* L. var. Top Crop Green Pod) seeds were surface sterilized with 0.5% (w/v) NaOCl for 15 min, rinsed four times with water, and grown in vermiculite for 9 days in a dark cabinet at room temperature ($24 \pm 2^\circ\text{C}$). Roots were separated and washed with cold tap water to remove vermiculite.

Methods

Cholinesterase activity assay. The activity of cholinesterase was determined by the spectrophotometric method of Ellman *et al.* (1961) as modified by Fluck & Jaffe (1974b). The volumes of all reactant solutions were tripled and the neostigmine (25 μM) preincubation and assay times were 15 min each. Except for column or gradient effluent fractions, all enzyme assays were performed in duplicate or triplicate. Replicates agreed within 2%. In experiments designed to examine the effects of a given substance on enzyme activity, 30–60 μl of stock solution of the substance was prepared in water (or propan-2-ol for Dip-F) and this replaced the same volume of buffer in the assay. A unit of activity is defined as the amount of enzyme that converts 1 μmol of substrate/s under the assay conditions used.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951) as modified by Eggstein & Kreutz (1955), with bovine serum albumin as standard. The protein content of the chromatography column eluate was monitored at A_{280} .

Preparation of MAC-Sepharose 2B. 1-Methyl-9-[*N*- β -(ϵ -aminohexanoyl)- β -aminopropylamino]-acridinium bromide was synthesized by the method of Dudai & Silman (1975) as modified by Webb & Clark (1978). MAC-Sepharose 2B was prepared from CNBr-activated Sepharose 2B (March *et al.*, 1974). An affinity matrix containing 2 μmol of 1-

methyl-9-[*N*- β -(ϵ -aminohexanoyl)- β -propylamino]-acridinium bromide/ml of Sepharose-2B was found to be best for the experiments described here.

Extraction and partial purification of cholinesterase Roots (344 g) were added to 788 ml of 20 mM-potassium phosphate buffer, pH 7.0, and homogenized in a Waring Blendor for 3 min. The homogenate was centrifuged at 4800g for 20 min at 4°C . The supernatant was filtered through Whatman no. 1 filter paper and the residue was resuspended in 788 ml of the extraction buffer containing 5% (w/v) $(\text{NH}_4)_2\text{SO}_4$. The slurry was stirred for 30 min at 4°C and centrifuged at 4800g for 20 min. The supernatant was filtered through two layers of Whatman no. 1 filter paper, adjusted to 80% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ at 4°C and centrifuged at 4800g for 20 min. The pellet was resuspended in 30–100 ml of 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl and dialysed overnight against the same buffer at 4°C . The non-diffusible material was clarified by centrifugation at 15000g for 10 min. The supernatant could be used immediately or stored at 0°C for up to 1 month without loss of activity.

Samples (5–20 ml) were applied to a column (3.5 cm $\times$ 95 cm) of Sepharose 6B equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl. Gel filtration was performed at a flow rate of 50–60 ml/h. Fractions were collected and assayed for cholinesterase activity and protein.

The pooled cholinesterase fraction (87 ml) from gel filtration was applied to a column (1.5 cm $\times$ 2.5 cm) of MAC-Sepharose 2B (2 μmol /ml of gel) equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl at a flow rate of 6–8 ml/h. After the entire sample had entered, the column was washed with 2 column volumes of the equilibration buffer and was then eluted with approximately 3 column volumes of equilibration buffer containing 1 M-NaCl. Fractions were collected throughout the loading, washing and elution procedures, and assayed for cholinesterase activity and protein. The total activity eluted was compared with that of the loaded sample to determine the recovery of cholinesterase. The 1 M-NaCl eluate (approx. 30 ml) was concentrated to 2–3 ml by ultrafiltration through an Amicon XM50 membrane filter (Amicon Corp., Lexington, MA, U.S.A.) and stored at 0°C for no longer than 12 days. This solution was used in the following experiments unless indicated otherwise. The column was regenerated by washing with approximately 4 bed volumes of equilibration buffer containing 5 M-guanidinium chloride, followed by at least 5 bed volumes of equilibration buffer.

Isoelectric focusing. Thin-layer isoelectric focusing was performed in 2 mm Sephadex G-75 layers (Radola, 1973). Samples (100–200 μg of protein) were

dialysed against 10mM-potassium phosphate buffer, pH 7.0, before their application 7–10cm from the cathode end of the plate. Focusing was carried out at 4°C at 200V for 8h and 500V for an additional 10h, at which time the standard proteins were focused. Measurements of the pH were made directly with a flat membrane glass electrode (Desaga, Heidelberg, West Germany). Protein was detected by the paper print method (Delincée & Radola, 1972) using Coomassie Brilliant Blue R-250. Cholinesterase was detected by assaying samples (approx. 100 μ l) from the dextran layer. All experiments were run in triplicate.

Labelling with Dip-F. A portion (56 μ l) of [3 H]Dip-F (0.26mg/ml in propylene glycol, 3.4Ci/mmol) was added to enzyme solutions (final volume 0.81 ml) and the tubes were incubated for 20min at 37°C. (Skin contact was avoided and operations were performed in a fume hood.) Reaction mixtures were dialysed against 4 $\times$ 3 litres of 20mM-phosphate buffer, pH 7.0, containing 1.0M-NaCl for 18h at 4°C and then assayed for cholinesterase activity. Catalytic centre activity was determined by a modification of the method of Cohen *et al.* (1967) in which the butyrylcholine concentration was lowered to 20mM. All procedures were performed in triplicate on duplicate preparations. For radioactivity counting, 10ml of dioxan-based scintillation fluid (Bray, 1960) was added to 50 μ l samples. Samples were counted for radioactivity at 45% efficiency in an Isocap 300 liquid-scintillation spectrometer (Searle Analytic Inc., Des Plaines, IL, U.S.A.) for 10min or terminated at 800000c.p.m. All activities were below the coincidence counting range of the spectrometer. Background radioactivity was counted in duplicate before each set of samples and subtracted from c.p.m. values. A quench curve was prepared for efficiency determinations by the channels-ratio method (Wang & Willis, 1965).

Disc gel electrophoresis. Mixtures containing 25–100 μ l samples of enzyme (30–150 μ g of protein), 5 μ l of 0.05% (w/v) Bromophenol Blue in water and solid sucrose to 5% (w/v) were applied to 7% (w/v) acrylamide gels and electrophoresis was performed at pH 8.3 (Davis, 1964) in duplicate on at least duplicate preparations. Gels were stained with 1% (w/v) Amido Schwartz in 7% (w/v) acetic acid and destained in 7% acetic acid. The gels were sliced immediately after electrophoresis and individual 2mm slices from duplicate gels were assayed for cholinesterase activity in the presence or absence of neostigmine.

SDS/polyacrylamide-gel electrophoresis. [3 H]Dip-cholinesterase and standard proteins (bovine serum albumin, β -galactosidase, catalase, glyceraldehyde 3-phosphate dehydrogenase, myoglobin and ovalbumin) were dialysed against 100mM-sodium phosphate buffer, pH 7.2, containing 0.1% (w/v) SDS for

18h at room temperature. Samples (75 μ l) were mixed with 75 μ l of either 100mM-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS, 20% (w/v) sucrose, 0.002% (w/v) Pyronin Y and 40mM-dithioerythritol, or the same solution without dithioerythritol. Mixtures were incubated at 100°C for 5min, cooled and 20–100 μ l samples were applied to 5% (w/v) acrylamide gels (0.4cm $\times$ 10cm) containing 0.1% (w/v) SDS (Weber & Osborn, 1969). Electrophoresis was performed at 2mA/gel for 1h and 5mA/gel for an additional 5.5h. The tracking dye front was marked with India ink. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in methanol/water/acetic acid (5:5:1, by vol.) overnight at 60°C and destained with 5% (v/v) methanol in 7.5% (v/v) acetic acid at 60°C for 24h. Gels were scanned at 550nm in a Gilford 240 spectrophotometer (Gilford Instruments, Mississauga, Ontario, Canada). Gels were placed on solid CO₂ for 15min and sliced in 2mm sections. Slices were placed in scintillation vials containing 0.6ml of NCS tissue solubilizer/water (9:1, v/v) and kept for 20h at 20°C followed by 2h at 50°C. Then 8ml of scintillation fluid (Bray, 1960) was added, and samples were counted for radioactivity at 35% efficiency for 20min.

Sedimentation in isokinetic sucrose gradients. Isokinetic sucrose gradients (10–29.3%, w/v) were prepared by the theoretical formulation of Noll (1967) as applied by Morrod *et al.* (1975). Cholinesterase or cholinesterase labelled with [3 H]Dip-F, and a mixture of standard proteins (myoglobin, catalase and β -galactosidase), were dialysed against 20mM-phosphate buffer, pH 7.0, containing 1M-NaCl. Mixtures containing 175 μ l of the active enzyme or 125 μ l of the [3 H]Dip-enzyme plus 50 μ l of the standard protein mixture, and solid sucrose to 5% (w/v) were applied to the top of the gradient, overlaid with buffer, and centrifuged at 40000rev./min in a Beckman SW41 rotor for 18h at 5°C in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). Gradients were eluted at a flow rate of approx. 12ml/h and fractions (0.5ml) were collected and assayed for enzyme activity or radioactivity. β -Galactosidase was assayed as described by Massoulié & Rieger (1969), and myoglobin and catalase were monitored by their A_{405} . A calibration curve was prepared for each gradient by using the standard proteins. All sedimentation experiments were run in duplicate.

Determination of Stokes radius and molecular weight. A Sepharose 6B column (0.8cm $\times$ 70cm) was equilibrated with 20mM-potassium phosphate buffer, pH 7.0, containing 1M-NaCl. The standard proteins, Blue Dextran 2000 and K₃Fe(CN)₆ (2mg/ml of each) were applied at a flow rate of 5ml/h. The partition coefficient (K_D) was determined (Massoulié & Rieger, 1969) and a calibration curve was prepared

by plotting the Stokes radius (R_s) of standard proteins versus $(-\log K_D)^{\dagger}$. The K_D of cholinesterase was determined in a separate run and the Stokes radius obtained by interpolation on the standard curve. The molecular weight of cholinesterase was determined by the graphical method of Bon *et al.* (1976), in which the product of sedimentation coefficient and Stokes radius was plotted against molecular weight for standard proteins and the molecular weight for cholinesterase was obtained by interpolation on this graph.

Results and Discussion

Identification of cholinesterase

Criteria for the identification of acetylcholinesterase activity in plants include inhibition by neostigmine and maximal activity against acetyl esters of choline (Fluck & Jaffe, 1974a). Enzyme activity that satisfied these criteria was identified in extracts of *P. vulgaris*. All esterase activity against acetylthiocholine in preparations purified beyond $(\text{NH}_4)_2\text{SO}_4$ precipitation was inhibited by concentrations of neostigmine that were effective against acetylcholinesterase activity in animals (Augustinson, 1960, 1963) and plants (Riov & Jaffe, 1973; Kasturi & Vasantharajan, 1976). This property was exploited in activity assays to correct for spontaneous or non-specific hydrolysis of acetylthiocholine.

Partial purification of cholinesterase

A small-scale purification procedure has been developed to give a 78-fold increase in specific activity (Table 1). The yield was 14% of the activity extracted with 5% $(\text{NH}_4)_2\text{SO}_4$. The purification obtained after $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration was similar to that reported from the mung-

bean enzyme (Riov & Jaffe, 1973). The use of MAC-Sepharose 2B yielded enzyme of 2–3-fold greater specific activity than that recovered from mung bean roots and 5–6-fold greater than that from pea roots (Kasturi & Vasantharajan, 1976).

Substantial purification was achieved by passage of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme through Sepharose 6B, and subsequent chromatography on MAC-Sepharose 2B resulted in further purification, although the yield after this step was low (Fig. 1). NaCl (1.0M) in buffer gave the most satisfactory recovery from MAC-Sepharose 2B. Decamethonium (60mM) totally inhibited bean root cholinesterase and no more enzyme was eluted by 50mM-decamethonium after salt elution. Use of a 0–40mM-decamethonium gradient for salt elution gave lower recoveries at comparable specific activities. Acetylcholine (5mM) was ineffective in eluting the bean enzyme from MAC-Sepharose 2B. Attempts at further purification involving ion-exchange chromatography, affinity elution chromatography (Scopes, 1977) and preparative isoelectric focusing were not successful.

Purity of cholinesterase

The results of electrophoresis in 7% acrylamide gels showed that, despite the very high specific activity achieved by these isolation procedures, the cholinesterase was not purified to homogeneity (Fig. 2). There were two densely stained bands (mobilities 0.07 ± 0.3 and 0.69 ± 0.03 with respect to Bromophenol Blue) and a lightly stained region (mobility 0.39–0.49), but only one band (mobility 0.07) contained cholinesterase activity. The other major band could have been a non-active component of the enzyme. This interpretation is consistent with a report (Das *et al.*, 1977) of active and inactive components of erythrocyte acetylcholinesterase.

Table 1. *Partial purification of cholinesterase from P. vulgaris roots*

Methods are detailed in the text. The extent of purification is based on the specific activity of dialysed root homogenates. Values presented are from one preparation and represent at least two others.

Fraction	Volume (ml)	Protein (mg)	Total units	Specific activity (units/mg of protein)	Recovery [% of 5%-(NH_4) ₂ SO ₄ extract]	Purification (-fold)
Crude extract (in buffer)	848	N.D.	500	N.D.	—	—
5% (w/v) $(\text{NH}_4)_2\text{SO}_4$ extract of residue	695	662.3	4239	6.4	100	2.3
80% satd. $(\text{NH}_4)_2\text{SO}_4$ ppt. (resuspended)	30	149.2	1775	11.9	42	4.2
Sepharose 6B	87	74.2	1722	23.2	41	8.2
MAC-Sepharose 2B (after ultrafiltration)	2.9	2.6	589	222.9	14	78.8

Fig. 1. Elution profile of *P. vulgaris* root cholinesterase after chromatography on MAC-Sepharose 2B containing $2.0 \mu\text{mol}$ of ligand/ml

The sample obtained after gel filtration was applied in 20mM-potassium phosphate buffer, pH 7.0, containing 0.2M-NaCl. The column was washed with the buffer, then eluted with the buffer containing 1M-NaCl. See under 'Methods' for details. ●, Cholinesterase activity; ○, A_{280} (protein).

Fig. 2. Polyacrylamide-gel electrophoresis of cholinesterase from *P. vulgaris* roots

Cholinesterase prepared as described under 'Methods' was electrophoresed in 7% (w/v) polyacrylamide gels at pH 8.3. Gels were stained with 1% (w/v) Amido Schwartz (b). The mobilities shown in the protein electrophoretogram (a) are average values obtained from duplicate gels of three different preparations. The enzyme activity profile shows values from one of two duplicate experiments. ●, Cholinesterase activity.

1973; Kasturi & Vasantharajan, 1976). Maximal activity to acetylthiocholine was observed between 0.5mM and 0.7mM, and a Lineweaver-Burk regression line (slope = $0.014 \pm 0.003 \text{ s.d.}$) for points obtained at acetylthiocholine concentrations between 0.02mM and 0.5mM gave a calculated K_m of $56 \mu\text{M}$. In the range 0.5–0.7mM, cholinesterase activity towards acetylthiocholine was three times that observed for either propionyl- or butyryl-thiocholine. This agrees with reports for mung bean root enzyme (Riov & Jaffe, 1973).

pi

The pI of cholinesterase was 5.3 ± 0.1 , and the enzyme precipitated at this pH with a loss of more than 90% of its activity. The autolysed 11S globular form of the eel enzyme has a pI of 5.3 (Leuzinger *et al.*, 1968) or 4.5 (Chen *et al.*, 1974). Morrod (1975) reported variable values (pI = 3.6–4.9) for asymmetric forms of that enzyme. The pI values of the erythrocyte acetylcholinesterases range between 3.0 and 5.3 (Das *et al.*, 1977). The variability among these reports may reflect instability of acetylcholinesterases in this pH range.

Estimated catalytic centre activity

Dip-F completely inhibited cholinesterase at concentrations greater than 0.1mM. The inhibition of enzyme activity by 0.1mM-Dip-F was decreased to 29% in the presence of 20mM-butyrylcholine in 20mM-potassium phosphate buffer, pH 7.0, for 15min before the addition of Dip-F. The cholinesterase catalytic centre activity, calculated after reaction of the enzyme with $[^3\text{H}]$ Dip-F, was $197 \pm 5 \text{ mol}$ of acetylthiocholine/min per mol of Dip-F reaction site. The value represents a minimum and is approx. 2×10^{-4} times analogous values of eel acetylcholinesterase (Rosenberry, 1975). This result, together with the established importance of acetylcholine in the eel electric organ (Florey, 1966) and the low quantities observed in plants (Fluck & Jaffe, 1974a; Hartmann & Kilbinger, 1974; White & Pike, 1974), supports the view that the considerable difference in catalytic centre activity between the eel enzyme and that from beans reflects the different requirements for rapid hydrolysis of acetylcholine in the two organisms.

Isokinetic sedimentation

The results of isokinetic sedimentation of partially purified cholinesterase are shown in Fig. 3. Cholinesterase activity appeared as one major peak with a sedimentation coefficient of $4.0 \pm 0.1 \text{ S}$. Sedimentation of $[^3\text{H}]$ Dip-cholinesterase resulted in a single

Substrate affinity

The hydrolytic activity of purified cholinesterase on acetylthiocholine showed substrate inhibition characteristic of acetylcholinesterases from plant and animal sources (Augustinsson & Nachmansohn, 1949; Wilson & Bergman, 1950; Riov & Jaffe,

Fig. 3. Isokinetic sedimentation of cholinesterase from *P. vulgaris* roots and of standard proteins

Samples contained β -galactosidase ($s=15.9S$), catalase ($s=11.4S$), myoglobin ($s=2.0S$) and either 54 units of cholinesterase activity (a) or 54 units of activity quantitatively inhibited by 0.1 mM-Dip-F (b). Sucrose gradients were calculated by using an average $\bar{v}$ of 0.745 (Smith, 1970) for standard proteins. The basis for this procedure is the report (Martin & Ames, 1961) that only small errors in determined sedimentation coefficients arise from using average values for $\bar{v}$. The s values were not corrected for the variations in actual $\bar{v}$ values of the individual proteins. The samples were sedimented in the sucrose gradients as described under 'Methods'. Fractions (0.5 ml) were assayed for cholinesterase activity (a) or radioactivity (b). β -Galactosidase activity was measured by A_{420} after assay by the method of Massoulie & Rieger (1969) and myoglobin and catalase were monitored by A_{405} . ●, Cholinesterase; ○, radioactivity; ▲, β -galactosidase; △, catalase; □, myoglobin; ■, s .

radioactive peak ($s=5.0\pm0.4S$). We conclude that all of the radioactivity recovered from sedimentation of 3H -labelled preparations existed as [3H]Dip-cholinesterase.

Determination of the Stokes radius and molecular weight

The Stokes radius of cholinesterase ($K_D=0.647$) determined from the regression line of standard proteins was 4.00 nm. This value and the sedimenta-

tion coefficient of $4.2\pm0.1S$ were used to obtain the product $R_e \cdot s = 16.8\pm0.4$. Interpolation of this value on the graph of $R_e \cdot s$ versus molecular weight for standard proteins [myoglobin, 16 890; catalase, 24 750; β -galactosidase, 51 530 (Smith, 1970)] gave an apparent molecular weight for cholinesterase of $72\,700\pm1900$.

Cholinesterase extracted from mung-bean (Riov & Jaffe, 1973) and pea (Kasturi & Vasantharajan, 1976) roots by the same procedure as used in this study was eluted in the void volume of Sephadex G-200

(mol. wt. above 200000) by using 20mM-phosphate buffer. An approx. 80000-mol. wt. species was observed in elution profiles of cholinesterase extracted from mung bean roots with 5% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (Riov & Jaffe, 1973). It is likely that this 80000-mol.wt. species represents the smallest active and native form of root cholinesterase. This is in contrast with animal acetylcholinesterases, in which observed active and native forms are always multimeric (Millar & Grafius, 1970; Dudai & Silman, 1971; Chen *et al.*, 1974; Taylor *et al.*, 1974; Morrod, 1975).

SDS/polyacrylamide-gel electrophoresis

A [^3H]Dip-F-labelling strategy (Bellhorn *et al.*, 1970; Berman, 1973) was used to detect catalytic subunits of bean root cholinesterase. The observations that (i) [^3H]Dip-cholinesterase sedimented as a single particle corresponding to a slightly more globular form of cholinesterase, (ii) no other [^3H]H-labelled particles were observed in sedimentation gradients (Fig. 3*b*) and (iii) the preparations were free from contaminating esterase activity led to the conclusion that Dip-cholinesterase was a suitable form of the enzyme in which to detect catalytic subunits in SDS/polyacrylamide gels. The major Dip-F-labelled peak in the SDS/polyacrylamide gels of reduced and non-reduced cholinesterase corresponded to a mol.wt. of 61000 ± 2000 , with minor

bands at 77000 ± 2000 , 26000 ± 1000 and 17500 ± 500 (Fig. 4). The 77000- and 61000-mol.wt. peaks of material corresponded to distinctly stained protein peaks in the gel scans.

The presence of active-site serine (as indicated by [^3H] labelling) in protein fragments of different molecular weight, which was observed after SDS/polyacrylamide-gel electrophoresis, contrasts with the apparently homogeneous placement of label obtained after sedimentation analysis in both the bean cholinesterase and eel acetylcholinesterase. The mol. wt. value of 73000 calculated for the intact enzyme from sedimentation and gel-filtration results corresponds quite well to 77000 calculated for the highest-molecular-weight fragment observed in SDS/polyacrylamide gels. We conclude that this component corresponds to the intact monomeric form of bush bean root cholinesterase and that the lower-molecular-weight fragments are derived by proteolysis of the monomer. The differences observed between band patterns for non-reduced and reduced [^3H]Dip-cholinesterase on SDS/polyacrylamide gels suggest the presence of intrachain disulphide bonds in the monomer, which are also effective in stabilizing the degraded fragment (mol. wt. 61000). Intra- and inter-chain disulphide-bond cleavage has been proposed to explain the SDS/polyacrylamide-gel electrophoresis migration pattern for eel acetylcholinesterase under reducing and non-reducing conditions after either tryptic degradation or

Fig. 4. SDS/polyacrylamide-gel electrophoresis of non-reduced and reduced cholinesterase from *P. vulgaris* roots. Cholinesterase was denatured without reducing agent (a, b) and in the presence of 40mM-dithioerythritol (c, d). Gels were stained with Coomassie Blue, scanned at A_{550} (a, c), sliced into sections and assayed for radioactivity (b, d). Molecular weights were derived from a calibration curve (see under 'Methods') and are average values from duplicate gels. K=1000 daltons; precision = $\pm 10\%$.

endogenous proteolysis (Dudai & Silman, 1974; Morrod *et al.*, 1975). No active-site labelled fragments of less than 55000–60000 mol.wt. have been reported for either tryptically or endogenously degraded eel enzyme, indicating an apparent resistance to continued proteolytic cleavage (Dudai & Silman, 1974; Morrod *et al.*, 1975). It is not clear whether such cleavage does occur during the initial steps in purification of the bean root cholinesterase. Active proteinases are well known from plant tissues (Ryan, 1973) and rapid techniques aimed at decreasing proteolysis may increase the recovery of the intact monomer.

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