Purification and Properties of Human Serum Cholinesterase

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Cholinesterase was purified from human serum by a three-stage procedure involving chromatography on DEAE-cellulose at pH 4.0, an electrofocusing technique and gel filtration on Sephadex G-200. The final product was purified 13 000-fold with a yield of 54%, and only one protein and one cholinesterase band could be demonstrated by polyacrylamide-disc electrophoresis. The catalytic properties appeared to be unchanged by the purification procedure. The molecular weight was determined by both ultracentrifugation in a density gradient and gel filtration, and values close to 366 000 were obtained. The isoelectric point of cholinesterase was estimated to be pH 3.99. The method appears suitable for the preliminary purification of the rare genetic variants of human cholinesterase.

A number of isoenzymes of cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) have been demonstrated in normal human serum by gel electrophoresis. Harris, Hopkinson & Robson (1962) found four bands (C_1 - C_4) by using two-dimensional electrophoresis on paper or starch gel with naphthyl acetate as substrate. The slowest (C_4) band contained about 90% of the esterase activity. Juul (1968) detected 12 isoenzymes by using polyacrylamide-disc electrophoresis and butyrylthiocholine as substrate.

In addition to this normal heterogeneity there are a number of genetic variants that appear to be determined by two independent autosomal genes (see Lehmann & Liddell, 1966). An unusual variant at one genetic locus (Ch_2) produces an extra slow-moving band (C_5) on gel electrophoresis (Harris, Hopkinson, Robson & Whittaker, 1963). Three unusual variants have been described at the other (Ch_1) locus. (1) The variant determined by the Ch_1^D gene has a higher K_m with all substrates and is less strongly affected by many but not all inhibitors. Dibucaine [2-butoxy-*N*-(2-diethylaminoethyl)cinchoninamide] is the most commonly used inhibitor to differentiate this variant from the normal form (Kalow & Genest, 1957; Kalow & Davies, 1958; Davies, Marton & Kalow, 1960). (2) The Ch_1^F gene determines a variant that is less strongly inhibited by sodium fluoride (Harris & Whittaker, 1961). (3) The Ch_1^S gene determines a variant that has very little or no enzyme activity (Liddell, Lehmann & Silk, 1962). These three unusual variants are of medical importance, as the affected homozygote shows a marked sensitivity to the muscle relaxant suxamethonium [bis-(2-dimethylaminoethyl) succinate].

The purification of the cholinesterase variants

from the serum of both normal and unusual homozygotes is an essential preliminary step before the difference in molecular structure can be investigated. Some of the cholinesterase variants are extremely rare. The purification procedure must therefore involve only small losses if useful amounts of enzyme protein are to be obtained. This paper describes such a procedure, developed on plasma from normal individuals.

METHODS

Cholinesterase assay with benzoylcholine. The cholinesterase concentration was measured by the method of Kalow & Lindsay (1955). Measurements were made with a Unicam SP.800 recording spectrophotometer fitted with a constant-temperature cell housing maintained at 25°C. One unit was defined as the amount of enzyme that hydrolysed 1 μ mol of benzoylcholine chloride/min.

Cholinesterase assay with acetylthiocholine. The method of Webber (1966) was used during the second stage of the purification procedure. The method was modified by using 0.067 M- H_3PO_4 adjusted to pH 7.4 with NaOH instead of tris-HCl, and reading extinctions at 408 nm instead of 412 nm. A correction was made for the spontaneous hydrolysis of the substrate.

Dibucaine number. This was measured by the technique of Kalow & Genest (1957).

Fluoride number. This was measured by the method of Harris & Whittaker (1961).

Percentage inhibition by RO2-0683. The dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide (RO2-0683, Hoffman-La Roche, Basle, Switzerland) is another differential inhibitor of the cholinesterase variants. The percentage inhibition produced by this compound was measured by repeating the assay procedure after the cholinesterase preparation had been preincubated for 2 h at room temperature with 0.067 M Sorensen's buffer, pH 7.4, containing 0.01 μ M-RO2-0683.

Measurement of protein concentration. For accurate

work the method of Waddell (1956) was used. For the monitoring of column eluates, the E_{280} was used as an estimate. Both techniques were calibrated with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.).

Electrophoresis techniques. Cellulose acetate electrophoresis was performed on strips (25 cm $\times$ 5 cm) manufactured by Oxo Ltd. (London S.E.1, U.K.) with a current of 2 mA/cm applied across the strips. Barbitone buffer, pH 8.6, was used: the concentration in the electrode compartment was 0.05 M and a 0.07 M solution was used to impregnate the cellulose acetate.

Agar-gel electrophoresis was performed on glass plates (7.6 cm $\times$ 2.5 cm) coated with 3 ml of Ionagar no. 2 (Oxo Ltd.) in 0.07 M-barbitone buffer, pH 8.6. A potential of 200 V was applied for 2½ h.

Horizontal starch-gel electrophoresis was performed by the method of Smithies (1959), with Connaught hydrolysed starch; Connaught Medical [Research Laboratories, Toronto, Ont., Canada (lot no. 132), 0.033 M-sodium borate, pH 8.68, as electrode buffer and 0.032 M-tris-citrate, pH 8.68, in the gel.

Polyacrylamide-disc electrophoresis was performed by a modification of the method of Ornstein (1964) and Davies (1964). A sample gel was not used and the samples were applied on top of the running gel in a mixture containing 5% (w/v) sucrose. The gel consisted of 7.5% (w/v) acrylamide, 0.06% (v/v) NNN'N'-tetramethylenediamine (TEMED) and 0.035% ammonium persulphate in 0.024 M-tris, adjusted to pH 8.7 with M-HCl. The buffer in the electrode compartments was 0.005 M-tris-0.038 M-glycine, pH 8.4. A current of 5 mA/tube was applied for 2-3 h.

Cholinesterase activity was identified in all of the above electrophoretic techniques by the method of Pinter (1957). Protein bands in the cellulose acetate and agar-gel techniques were stained with 0.2% Ponceau S in 3% trichloroacetic acid followed by immersion in several changes of 5% (v/v) acetic acid (A.R.). In the starch-gel and polyacrylamide-gel methods protein was stained by immersion of the gel in 1% Amidoshwartz 10B in acetic acid-water-methanol (2:4:5, by vol.). The gels were then immersed in the same mixture until the background was unstained.

Moving-boundary electrophoresis was performed in a Perkin-Elmer model 38 Tiselius apparatus. The buffer was 0.1 M-barbitone, pH 9.0, and a current of 10.5 mA was used. Schlieren optics were used to locate the boundaries. Serial pictures were taken up to about 90 min.

Immunoelectrophoresis was performed by the method of Scheidegger (1955) in agar gel mounted on glass plates (8 cm $\times$ 8 cm). The gel was made by the method described above. After electrophoresis antisera were placed in slots in the gel and the plates were incubated in a moist atmosphere for 19 h at room temperature.

Immunodiffusion. This was performed in 1% agar plates, 3 mm thick. Samples (0.2 ml) of the cholinesterase preparation were placed in the peripheral wells and antiserum (0.5 ml) in the central well. The plate was incubated for about 72 h at 4°C.

RESULTS

Purification methods

Human plasma was obtained from blood that had been stored for more than 3 weeks at 4°C by the

Blood Transfusion Service. A mixture (120 ml) containing 2 g of sodium citrate and 3 g of anhydrous dextrose had been added to each 540 ml bottle as an anticoagulant. Only plasma samples that appeared to contain the usual cholinesterase variant were used. The criteria were dibucaine and fluoride numbers greater than 80 and 57 respectively, over 90% inhibition by RO2-0683 and the absence of an extra (C_2) cholinesterase band on polyacrylamide-gel electrophoresis. Fibrinogen was removed by adding 1 ml of M-calcium chloride to each 100 ml of plasma. The mixture was left at 37°C until a fibrin clot formed (usually about 2 h). The clot was removed with a glass rod and the serum was centrifuged to remove residual precipitate. This procedure removed about 9% of the total protein without detectable loss of cholinesterase activity.

First stage: chromatography on DEAE-cellulose at pH 4.0. DEAE-cellulose (DE52; Whatman) (100-125 g) was precycled by stirring for 30 min in 2 l of 0.5 M-hydrochloric acid and washing with water on a Buchner funnel until the pH was about 6.5. The procedure was repeated with 0.5 M-sodium hydroxide. The DEAE-cellulose was then equilibrated by repeated dispersion in 2 l of 0.02 M-sodium acetate buffer, pH 4.0, with intervening filtration. The equilibrated cellulose was then resuspended in 2 l of this buffer and allowed to settle for 30 min. The supernatant and suspended fines were removed, fresh buffer was added and the whole was transferred to a filtering flask. Negative pressure was applied to remove dissolved gases.

The cellulose was packed in a column (diam. 2.8 cm). The column was packed in stages containing about 10% of the total cellulose. Between each stage the majority of the buffer was drained from the column. The next portion of cellulose suspension was stirred with a glass rod to mix it with the top of the previous portion. The acetate buffer was then run through the column from a reservoir positioned so that the hydrostatic pressure at the top of the column was approximately equal to the column length. This was continued until the column length became constant, at about 46 cm.

Serum (200 ml) was dialysed repeatedly against the same buffer until the pH reached 4.0, and the precipitate was removed by centrifuging. The dialysis residue was applied to the column at 20 ml/h. The column was washed with 0.02 M-acetate buffer until the E_{280} of the eluate fell below 0.005.

The eluate contained 95% of the total protein and no detectable cholinesterase activity. Constant-volume gradient elution was performed with increasing proportions of 0.2 M-sodium chloride in 0.02 M-acetate buffer. The flow rate was 10 ml/h and fractions (10 ml) were collected. Fig. 1 shows that

three protein peaks were obtained, with the cholinesterase activity in the first and major fraction.

Of the original activity, 90–120% was recovered, with a purification of 300- to 400-fold. The cholinesterase-containing fractions were concentrated by vacuum dialysis against 0.02M-acetate buffer, pH 4.0. Polyacrylamide-gel electrophoresis of the concentrate showed 14 protein bands and all four cholinesterase isoenzymes. Electrophoresis with both cellulose acetate and agar-gel showed only one

protein band, whereas with starch gel four faint bands were obtained. Three protein peaks were obtained with moving-boundary electrophoresis.

Second stage: electrofocusing in a pH gradient. Electrofocusing was performed at 4°C with an LKB (Stockholm, Sweden) column of 450ml capacity (LKB 8102) and Ampholines with a pH range between 3 and 5 (LKB 8151). The column was prepared with a density gradient of sucrose according to the manufacturer's instructions. The concentrated preparation from the first stage (37mg of protein, 200 units of cholinesterase) was dialysed against 0.5% Ampholine solution and added to one of the fractions when filling the column. The voltage applied to the column was gradually raised during the first 6h from 100 to between 300 and 400V. This voltage was continued for a further 72h. The energy did not exceed 3W. The column was emptied in 2ml fractions. The results are shown in Fig. 2. Eight protein and two cholinesterase peaks were found. Polyacrylamide-gel electrophoresis showed that the first cholinesterase peak contained five protein bands and all four cholinesterase isoenzymes, and the second contained eleven protein bands and only one (C_4) cholinesterase fraction. The first cholinesterase peak occurred at a pH of 3.99. This stage purified cholinesterase by a further three- to four-fold with a yield of 80% of the original cholinesterase activity in the first peak.

The fractions containing the first cholinesterase peak were concentrated by vacuum dialysis against 0.02M Sorensen's buffer, pH 7.4. When this concentrate was again subjected to electrofocusing only one protein peak with a pronounced tail was formed.

Fig. 1. Chromatography of human serum on DEAE-cellulose at pH 4.0. A gradient of 0.2M-NaCl in 0.02M-sodium acetate buffer was employed. The flow rate was 10ml/h and fractions (10ml) were collected. ●, E_{280} ; ▲, cholinesterase activity (with benzoycholine).

Fig. 2. Fractions (2ml) obtained by electrofocusing of a partially purified cholinesterase preparation in a pH gradient between pH 3 and 5 at 4°C. A potential of about 350V was applied for 72h, and the column was stabilized with a sucrose density gradient. ●, E_{280} ; ▲, cholinesterase activity (with acetylthiocholine).

This peak coincided with the enzyme activity. Polyacrylamide-gel electrophoresis showed the same five protein and four enzyme bands.

Third stage: gel filtration. The concentrated preparation obtained from the second stage was further purified by gel filtration on a column of Sephadex G-200 [Pharmacia (G.B.) Ltd., London W.13, U.K.]. Of the second stage preparation, 3.0ml containing 117 units of cholinesterase and 6.48mg of protein was applied to the Sephadex column (20 cm × 2 cm). The column was eluted with 0.02M Sorensen's buffer, pH 7.4, in 5ml fractions. The results are shown in Fig. 3. The enzyme-containing fractions were again concentrated by vacuum dialysis against 0.02M Sorensen's buffer, pH 7.4. Cholinesterase was further purified by a factor of 8.7, with a recovery of 64%. The poor recovery was thought to be due to the marked

instability of cholinesterase activity in the purified preparation. On polyacrylamide-gel electrophoresis only one protein band was detected with one zone of cholinesterase activity in the same position. This position corresponded to that of the C₄ fraction.

The results obtained with all three purification procedures are summarized in Table 1.

Properties of the purified preparations

Stability. Cholinesterase activity in untreated serum is very stable (Keilin & Wang, 1947). During purification the activity became increasingly unstable. The stability was unaffected by pH in the range 4.0–7.4 and was identical at 4°C and at room temperature. Storage at –20°C produced an initial loss of 20% of the activity.

The stability was improved by (1) increasing the concentration of the preparation and (2) the presence of 10% (w/v) sucrose, albumin (0.63g/100ml) or gum acacia (5g/100ml). The preparation was most stable on DEAE-cellulose in 0.02M-acetate buffer, pH 4.0, at 4°C. Of the activity, 90% was then recovered after storage for 2 months by elution with 0.02M-acetate buffer, pH 4.0, containing 0.1M-sodium chloride.

Purity. The results of polyacrylamide-gel electrophoresis are shown in Fig. 4. Only one faint protein band was detected in the final preparation. The presence of other proteins in much lower concentrations cannot be excluded. Cellulose acetate, agar-gel and starch-gel techniques detected a smaller number of bands in the first-stage preparation and were thought to be less sensitive as a test for purity.

Immunoelectrophoresis and immunodiffusion were performed on all the preparations with an anti-serum prepared against whole human serum (Baxter Hyland Laboratories Ltd., Thetford, Norfolk, U.K.). Many precipitin arcs were found with untreated serum, three arcs with the first-stage and none with the second- and third-stage preparations. Immunodiffusion gave similar results.

Fig. 3. Purification of the second-stage preparation by chromatography on a Sephadex G-200 column (20 cm × 2 cm). Fractions (5 ml) were eluted with 0.02M Sorensen's buffer, pH 7.4. ●, E_{280} ; ▲, cholinesterase activity (with acetylthiocholine).

Table 1. *Typical purification of cholinesterase from 200 ml of defibrinated human plasma*

The table includes the number of protein and cholinesterase bands detected by polyacrylamide-gel disc electrophoresis.

Purification stage	Total protein (mg)	Total cholinesterase (units)	Specific activity (units/mg)	Purification	Yield (%)	No. of protein bands	No. of cholinesterase bands
Starting material	14000	180	0.013			17	4
First stage	37	211	5.7	439	117	14	4
Second stage	8.6	160	18.6	1430	89	5	4
Third stage (final preparation)	0.42	73*	174	13400	54*	1	1

* Only 73% of the second-stage preparation was further purified.

Fig. 4. Polyacrylamide disc electrophoresis of the cholinesterase preparations. A, B, C and D are the protein fractions obtained from defibrinated plasma, first-stage, second-stage and final-stage preparations respectively. E, F, G and H are the cholinesterase isoenzymes of the same four preparations. The shading indicates the relative intensity of staining.

Similar experiments were performed with anti-serum prepared by immunizing rabbits against the final preparation. The antiserum completely inhibited the benzoylcholine-hydrolysing activity of human serum when equal volumes were incubated at 37°C for 24h. Only one precipitin arc was seen against all preparations with immunodiffusion, and with immunoelectrophoresis of the first- and second-stage preparations. Immunoelectrophoresis of serum gave a diffuse precipitin zone around the point of application whereas no arc was seen with the final preparation. In all preparations an arc staining for cholinesterase activity was seen.

Catalytic properties. The Michaelis constants of the cholinesterase in the third-stage preparation were determined with benzoylcholine, acetylthiocholine and butyrylthiocholine and compared with the values found in untreated plasma. In each case the reactions were performed in 0.067M Sorensen's buffer, pH 7.4, at 25°C. There was no significant difference between the values for the untreated plasma and those for the final preparation.

In a similar manner the inhibitory effect of various concentrations of dibucaine hydrochloride, sodium fluoride and RO2-0683 were measured with 50 μ M-benzoylcholine, 5 mM-acetylthiocholine and 7.5 mM-butyrylthiocholine as substrates. Identical results were obtained with both normal human plasma and the final-stage preparation.

The specific activity of one final-stage preparation,

purified 13000-fold, was measured. In each case the measurements were performed in 0.067M Sorensen's buffer, pH 7.4, at 25°C.

The following specific activities (in μ mol/min per mg of protein) were obtained: with 50 μ M-benzoylcholine, 175; with 5 mM-acetylthiocholine, 818; and with 7.5 mM-butyrylthiocholine, 1145.

Physical properties. The isoelectric point of cholinesterase was at pH 3.99, as determined by the electrofocusing technique.

The $s_{20,w}$ of the cholinesterase activity in the final preparation was found to be 12.44. The technique used involved centrifuging for 13h at 37000 rev/min at 3°C in the 5 $\times$ 3ml swing-out rotor of the Christ Omega II ultracentrifuge. A linear 5–20% (w/v) sucrose gradient in 0.067M Sorensen's buffer, pH 7.4, was employed. The machine had previously been calibrated with preparations of marker proteins of known $s_{20,w}$.

Both the first-stage and final preparations were chromatographed on a column (37 cm $\times$ 1.8 cm) of Sagavac 8 (Seravac Laboratories Ltd., Maidenhead, Berks., U.K.). The column had been precalibrated with commercially obtained marker proteins of known Stokes radius and molecular weight (myoglobin, horse-radish peroxidase, yeast alcohol dehydrogenase, fibrinogen and albumin dimer). The distribution coefficient of cholinesterase was calculated from the formula of Laurent & Killander (1964) and a value of 0.430 was obtained. Stokes radius

Table 2. *Purification of cholinesterase from human serum by various workers*

Authors	Technique	Yield (%)	Purification factor
Surgenor & Ellis (1954)	Ethanol precipitation	7	3400
Malström, Levin & Bowman (1956)	Purification of commercial Harvard fraction IV 6-3 by chromatography on calcium phosphate gel and Dowex 2	Not known	70-fold of fraction IV 6-3
Connell & Shaw (1961)	Chromatography on Selectacel-DEAE-cellulose at pH 4.2	53	45
Svensmark & Kristensen (1963)	Ammonium sulphate precipitation; electrophoresis on cellulose	8	5000
Goedde, Fuss, Ritter & Baitsch (1965)	Ammonium sulphate precipitation; absorption on alumina C _γ gel I and II; chromatography on calcium phosphate gel followed by gel filtration on Sephadex G-200	Not stated precisely; <20	1400
Haupt, Heide, Zwisler & Schwick (1966)	Rivanol-ammonium sulphate precipitation; absorption on aluminium hydroxide gel; zone electrophoresis on polyvinylchloride; gel filtration on Sephadex G-200	11	10000

and molecular weight were both determined graphically by the method of Siegel & Monty (1965). The value for Stokes radius lay between 77 and 91 and the molecular weight appeared to be 365 000. The latter figure compared with a value of 367 000 calculated from the $s_{20,w}$ by the formula of R. L. Watts (unpublished work) derived from the data of Virden, Watts, Watts, Gammack & Raper (1966).

DISCUSSION

The purification technique described was the best sequence discovered after investigation of several possible methods. Table 2 shows the previous work on cholinesterase purification. The main advantage of the present method is the greatly improved yield. All the previous work, except that of Connell & Shaw (1961), employed fractional precipitation with either ammonium sulphate or ethanol. These methods involve much higher losses than the chromatographic and electrophoretic techniques used in the present work. High yield is particularly important for purification of the cholinesterase variants, were the available material is limited. This technique has been used to purify cholinesterase from both $Ch_1^DCh_1^D$ and $Ch_1^FCh_1^F$ homozygotes (P. K. Das & J. Liddell, unpublished work).

Cholinesterase was purified 13 000-fold. No other proteins were detected in the final product, although their presence could not be excluded. Similar degrees of purification were obtained by Haupt *et al.* (1966) with human serum and by Jansz & Cohen (1962), who purified horse serum cholinesterase by between 11 000- and 14 000-fold with a yield of 5-

11%. Both groups of workers could not detect contaminating proteins in their final preparation.

Only four cholinesterase isoenzymes were detected in untreated serum by polyacrylamide-disc electrophoresis and staining with α -naphthyl acetate. After the present work was complete, Juul (1968) detected 12 bands of cholinesterase activity after the same electrophoretic technique and staining with butyrylthiocholine and copper. The differences in results are probably due to the different staining procedures.

The catalytic properties of cholinesterase did not appear to be affected by the purification procedure. The value of 12.44 for $s_{20,w}$ compares with that of 11.9 obtained by Surgenor & Ellis (1954) and 10.7 by Haupt *et al.* (1966).

The estimate of pH 3.99 for the isoelectric point compares with a value of pH 2.9-3.0 by Svensmark & Kristensen (1963), who used a paper-electrophoresis method.

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