PSEUDOCHOLINESTERASE FROM HORSE SERUM

I. PURIFICATION AND PROPERTIES OF THE ENZYME

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SUMMARY

The enzyme pseudocholinesterase has been purified from horse serum to the extent of about 14000 times the starting material in a 10% yield. The enzyme behaves as a single component on ultracentrifugal and electrophoretic analysis, indicating a high degree of homogeneity. The purification procedure and some properties of the purified enzyme are described.

INTRODUCTION

During the course of our work on the active site of esterases[1] it became desirable to obtain large quantities of highly purified pseudocholinesterase from serum. The purification of this enzyme from horse and human serum has been attempted by a number of investigators[2–8]. The greatest purity was achieved by STRELITZ[5] who obtained enzyme preparations from horse serum of a purity about 5000 times greater than the starting material. For our purposes the method was unsatisfactory in view of the rather low final yield of enzyme (about 5%). Because of the extremely small quantities of the enzyme in serum the necessity exists to work up vast amounts of serum if large quantities of the enzyme are required. It is the purpose of this paper to describe an improved method for the purification of pseudocholinesterase from horse serum by which enzyme preparations are obtained in a 10% yield. Moreover the present procedure yields enzyme preparations which are purer than any of those earlier described in the literature and which are homogeneous on ultracentrifugal and electrophoretic analysis.

Also some properties of the purified enzyme are described in this communication.

METHODS AND MATERIALS

Enzyme assay[9]

The titration method was used to determine the enzymic activity of pseudocholinesterase. An appropriately diluted enzyme solution was mixed with 1 ml substrate (freshly prepared solution of 200 mg acetylcholine chloride per 5.5 ml water), 0.5 ml 0.01 *M* phosphate buffer, pH 7.5, and water to a final volume of 10.0 ml.

The reaction was carried out at 25° and the pH was maintained at 7.5 by the

continuous addition of 0.01 *N* NaOH by means of a Radiometer auto-titrator. The amount of NaOH *versus* time was recorded for 10 min and found to be strictly linear provided that the amount of enzyme was such that approx. 0.2 ml of NaOH was used/min. The contribution due to autolysis of substrate was negligible under these conditions.

Unit enzymic activity

A unit of enzymic activity was defined as the amount of enzyme which under these conditions requires the addition of 4 μl 0.01 *N* NaOH/h. Manometric determination of enzymic activity was carried out as described by STRELITZ[5]. It was found that in order to convert STRELITZ' units to our titrimetric units it is necessary to multiply the former by a factor of 1000.

Protein nitrogen

Routinely the amount of protein N in the enzyme preparations was determined in a Beckman model DU spectrophotometer. An absorbancy of 1.0 at 280 mμ corresponds to 1 mg protein/ml as shown by dry weight determinations for the enzyme preparations up to the 4th stage in the purification procedure. It was shown using the Kjeldahl method that these preparations contain 15% protein N. For the final enzyme preparation an absorbancy of 2.5 at 280 mμ corresponds to 1 mg protein/ml. The latter relation was estimated from an amino acid analysis of the protein using a Beckman-Spinco Amino Acid Analyzer and assuming that the total weight of the protein is determined by the sum of the weights of the constituent amino acid residues.

Specific activity

The specific activity of the enzyme preparations is expressed as units enzymic activity/mg protein N.

Electrophoresis

Electrophoresis was carried out on cellulose columns as described by PORATH[10]. Cellulose powder from Munktell, Sweden, was washed with water to remove small particles and suspended in the buffer to a thick slurry which was freed of air under reduced pressure and poured into the column. The column was provided with a cooling jacket for the circulation of icewater during electrophoresis. The buffer[10] contained 60 ml triethylamine/10 l of water which was brought to the desired pH of 8.5 by CO_2.

RESULTS

Purification of pseudocholinesterase

The enzyme was purified from horse blood. Horse blood was collected in large containers and whipped in order to prevent coagulation. The blood was stored overnight in the cold room and the serum was collected the next day. The results of the purification of three preparations are summarized in Table I.

First $(NH_4)_2SO_4$ precipitation: The first stage in the purification procedure was similar to that of STRELITZ[5]. 27-l portions were worked up at a time, first at neutral and then at acid pH to obtain 21 l of a clear fluid. Spinning of these large quantities in 15 and 12-l batches was carried out in a Heine centrifuge. Foaming was prevented by adding occasionally a few drops of octanol.

TABLE I

PURIFICATION PROCEDURE OF PSEUDOCHOLINESTERASE FROM HORSE SERUM

	Preparation No.	*Volume (ml)*	*Units × 10⁻⁷ (total)*	*mg N (total)*	*Units/mg N*	*Yield (%)*	*Purification factor*
Serum	1	27 000	16.2	225 000	720	—	1
	2	27 000	17.0	290 000	590	—	1
	3	27 000	15.4	270 000	570	—	1
1st $(NH_4)_2SO_4$ precipitation	1	21 200	12.7	19 000	6700	78	9
	2	20 000	9.0	12 400	7300	53	12
	3	21 000	8.5	14 700	5800	55	10
2nd $(NH_4)_2SO_4$ precipitation	1	125	3.8	540	70 400	23	98
	2	95	3.4	410	83 000	20	140
	3	210	4.7	1400	33 500	31	60
3rd $(NH_4)_2SO_4$ precipitation	1	40	2.9	104	280 000	18	390
	2	44	3.0	88	340 000	18	580
	3	32	3.4	96	350 000	22	620
Ultracentrifuge	1	10	2.3	37	620 000	14	860
	2	8	2.1	17	1 240 000	12	2100
	3*	5	2.3	19.2	1 200 000	15	2100
Electrophoresis	1	5	1.8	2.3	7 800 000	11	10 800
	2	4	0.9	1.1	8 200 000	5	14 000
	3	8	3.4	4.3	8 000 000	10	14 000

* In this stage of this experiment we added $3.0 \cdot 10^7$ units (volume 11 ml) corresponding to 28 mg N, 1 100000 units/mg N prior to electrophoresis.

Second $(NH_4)_2SO_4$ precipitation: 150 g solid ammoniumsulphate were dissolved for each 1000 ml of the fluid of the preceding stage. The suspension which is still at the low pH owing to the acid treatment of the preceding stage, was left overnight in a round bottom flask, the incubation being an essential step in the subsequent purification procedure. The next day most of the supernatant could be siphoned off and the precipitate was collected on large Büchner funnels (Whatman No. 5 paper, 24 cm in diameter), suction being continued until the filter cake was almost dry. The filter cake could be stored at —20° without loss of enzymic activity.

The filter cake was taken up in 1 l of water. 200 g of solid ammoniumsulphate were dissolved by stirring and the precipitate was removed in the centrifuge (Servall Angle Type centrifuge, model SS-22, 12000 rev./min for a 5 min at room temperature). For each litre of the supernatant fluid were added 235 g solid ammoniumsulphate. The precipitate was collected in the Servall centrifuge as above. The precipitate was dissolved in 50 ml of distilled water and dialyzed against distilled water at 4° (several changes) until free of ammoniumsulphate. At the end of the dialysis the solution was spun in the centrifuge in order to remove the precipitate which formed during dialysis.

Third $(NH_4)_2SO_4$ precipitation: The volume of the solution was adjusted to bring the amount of protein N to 4 mg/ml. An amount of saturated ammoniumsulphate corresponding to approx. 0.9 of the volume of the enzyme solution was slowly added with stirring at room temperature.

To obtain optimum purification this step is usually tried out on a small scale. From these small scale preliminary experiments it was found that optimum purifi-

cation could be expected for the preparations 1, 2 and 3 in Table I when the volumes of saturated ammoniumsulphate were respectively 0.76, 0.90 and 0.88 of the volumes of the enzyme solutions. The bulk of these preparations were fractionated accordingly. The precipitate was removed in the Servall centrifuge. The supernatant solution was brought to 0.7 saturation with solid ammoniumsulphate and the precipitate collected in the Servall centrifuge. The precipitate was dissolved in 25 ml of 0.01 *M* phosphate buffer, pH 7.0, and dialyzed against this buffer at 4° until free of ammonium sulphate.

Ultracentrifugal purification: Further purification was achieved in the Spinco model L centrifuge. The solution was spun for 16 h at 40000 rev./min (No. 40 rotor) at 5°. The supernatant fluid was carefully siphoned off and the pellet dissolved in approx. 10 ml 0.01 *M* phosphate buffer, pH 7.0. This solution was subjected to another ultracentrifugal run as above. The final pellet was dissolved in 3 ml of 0.01 *M* phosphate buffer, pH 7.0.

Column electrophoresis: The final step in the purification procedure was performed by column electrophoresis. The enzyme solution was equilibrated with triethylamine–CO_2 buffer, pH 8.5, by dialysis for 16 h at 4°, and applied to the cellulose column (62 cm in length, 2.7 cm in diameter). Electrophoresis was carried out in the cold room at 4°. A voltage of 760 V (current 25 mA) was applied for 72 h, using ice water as the coolant. At the end of this time the column was eluted with the buffer collecting 3.0-ml fractions. Protein and enzymic activity were determined in the fractions. In Fig. 1 the result of a representative experiment is presented.

Fractions with enzymic activity were pooled and the enzyme was precipitated by the addition of solid ammoniumsulphate to saturation. The enzyme was collected

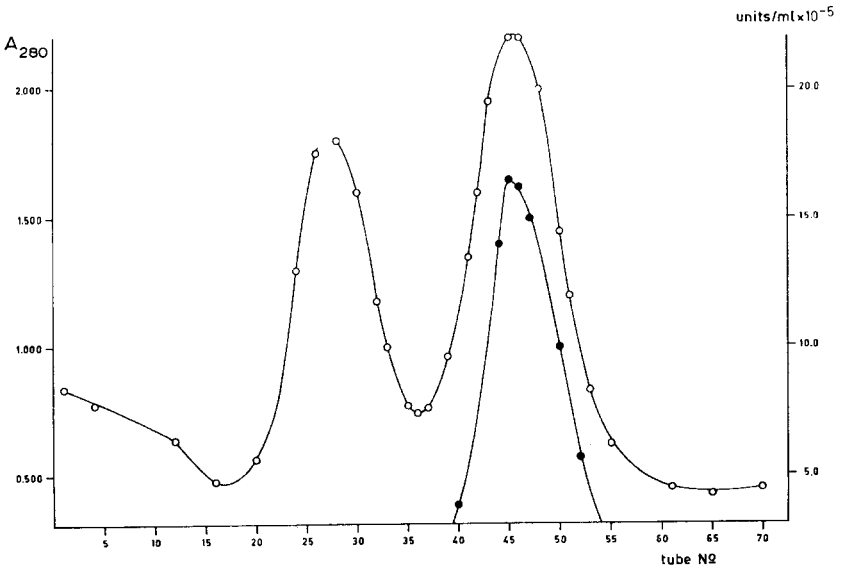

Fig. 1. Purification of pseudocholinesterase by column electrophoresis. Preparation 3 from Table I. Cellulose column (62 cm in length; 2.7 cm in diameter). 760 V (25 mA) for 72 h. Trietylamine–CO_2, pH 8.5, was used as the buffer. After electrophoresis the column was eluted with the buffer, 3.0-ml fractions being collected at a rate of approx. 1.0 ml/min. The absorbancy at 280 mμ, ○—○, and enzymic activity, ●—●, in the fractions were determined. Fractions 40–52, inclusive, were pooled. The properties of the final enzyme preparation are presented in Table I (preparation 3).

in the Servall centrifuge (model SS-1A) in the cold and dissolved in 4 ml 0.01 *M* phosphate buffer, pH 7.0. The enzyme solution was freed of ammoniumsulphate by dialysis against phosphate buffer. The enzyme solution was stored at —20°.

Properties of the purified enzyme

Stability: The enzyme is stable in concentrated solutions (approx. 3 mg protein/ml) in the cold as well as at room temperature. The enzyme activity does not fall off appreciably on storage in 0.01 *M* phosphate buffer, pH 7.0 at —20°. In dilute solutions the enzyme gradually loses activity although it was not necessary to add stabilizing agents such as gum acacia in the titrimetric test for enzymic activity at 25° as suggested by STRELITZ[5] for the manometric assay at 37°. However, the yields which we obtained on column electrophoresis in the last stage of the purification procedure were greatly increased by using concentrated solutions of enzyme, indicating that the enzyme loses activity even in the cold in dilute solutions.

Substrates: It may be calculated that 1 mg of our purest enzyme preparation (10^7 units/mg protein N) is able to hydrolyze about 60 mmoles of acetylcholine chloride/h at 25°. It is well known that pseudocholinesterase does not hydrolyze acetylcholine esters exclusively but hydrolyzes a number of other compounds as well. The hydrolytic activity of the purified enzyme towards a number of compounds is shown in Table II. The compounds were either dissolved in a 1% solution in water or homogenized with water in this concentration. Five millilitres of these solutions were assayed. The values obtained were corrected for spontaneous hydrolysis of the esters. The results are presented in Table II. The figures refer to the percentage of the effect caused by the same amount of enzyme on acetylcholine chloride. Acetylcholine hydrolysis as well as hydrolysis of phenyl acetate and tributyrine by the enzyme is completely inhibited by 10^{-5} *M* eserine.

TABLE II

HYDROLYSIS OF ESTERS BY PURIFIED PSEUDOCHOLINESTERASE

The figures refer to hydrolysis rate taking acetylcholine hydrolysis as 100. The concentrations of the esters were 0.5% (v/v) for the liquids and 0.5% (w/v) for the solids.

Acetylcholine chloride	100
Butyrylcholine chloride	175
Phenyl acetate	46
Tributyrin	14
Methyl butyrate	< 2
Triacetin	< 2
Amyl acetate	< 2
Ethyl butyrate	< 1
Ethyl acetate	< 1
Ethyl formate	< 1
Acetyl-β-methylcholine	< 1

pH optimum: The pH optimum of the purified enzyme is at 8.0 as determined with acetylcholine as substrate. Autolysis of substrate is appreciable at pH 8.0. At pH 7.5 where autolysis of substrate is negligible under the conditions of the assay the enzyme displays 95% of its maximum activity.

Ultracentrifugal analysis: On ultracentrifugal analysis (Spinco, Model E) of the purified enzyme one component was observed with $s_{20} = 9.9$ as may be seen from

Fig 3. For the human serum cholinesterase an $s_{20,w}$ of 12 has been tentatively concluded by SURGENOR AND ELLIS[7]. In the earlier stages of the purification procedure the enzyme is contaminated with slower moving material ($s_{20} = 3.5$) some of which still appears as a minor peak in Fig. 3. It has been shown by sampling and testing both fractions after an ultracentrifuge run that only the component with $s_{20} = 9.9$ is endowed with enzymic activity.

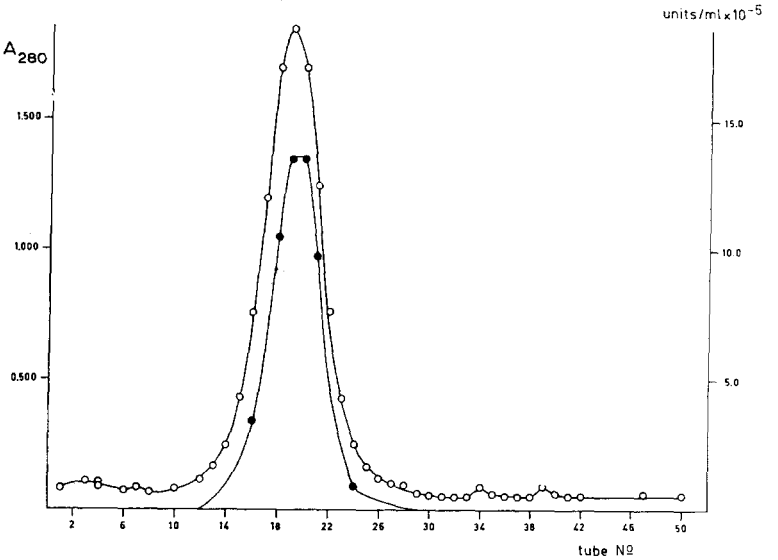

Fig. 2. Reelectrophoresis on a cellulose column (40 cm in length, 2.7 cm in diameter) of pseudocholinesterase preparation of Fig. 1. A total of $3.0 \cdot 10^7$ units enzymic activity, containing 4 mg protein N, volume 3.5 ml was subjected to electrophoresis for 40 h (760 V, 30 mA) as described under Fig. 1. Fractions 15–23, inclusive, were pooled and the enzyme collected. A total of $1.8 \cdot 10^7$ units enzyme activity was recovered. A total of 1.9 mg N was obtained corresponding to a specific activity of 10^7 units/mg N.

Electrophoretic analysis: An amount of the purified enzyme corresponding to $3.0 \cdot 10^7$ units enzymic activity and 4 mg protein N was again subjected to electrophoresis on a cellulose column. The result of this experiment is presented in Fig. 2. It may be seen that enzymic activity and protein content of the fractions run perfectly parallel. Moreover both protein and enzymic activity peaks show a great degree of symmetry. This indicates a high degree of homogeneity of the enzyme preparation.

Inhibitors: The enzyme is inhibited by diisopropyl phosphorofluoridate[11,12] and isopropyl methylphosphoryl fluoridate (sarin) in low concentrations. The purified enzyme was completely inhibited by [^{32}P]sarin by incubation of the enzyme (10^7 units/mg protein N) in a concentration of 0.37 mg protein/ml with sarin in a concentration of $7.7 \cdot 10^{-6}$ *M*. The inhibited enzyme was thoroughly dialyzed in the cold to remove the excess of sarin. It was found that $43.7 \cdot 10^{-10}$ gram atom of P was bound to 0.37 mg protein as shown by radioactivity determinations. This corresponds to 1 gram atom of P bound per 84000 g protein. There is firm evidence that the organophosphate inhibitors react specifically at the active site(s) of this enzyme[13]. This knowledge allows a calculation of the turnover number of pseudocholinesterase of 84000 molecules acetylcholine hydrolyzed/min at 25° per active center of the enzyme. EASSON AND STEDMAN[14] have estimated a turnover number of 89400/min.

If it is assumed that per molecule of enzyme one molecule of inhibitor is bound after complete inhibition as shown for chymotrypsin[15], the number of 84000 corresponds to a maximum molecular weight of the pseudocholinesterase. However, a much higher molecular weight would be expected on account of a sedimentation coefficient of 9.9. Further work on this problem is now in progress.

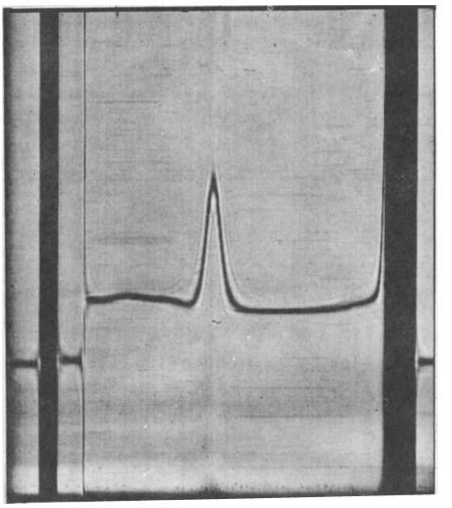

Fig. 3. Sedimentation pattern of pseudocholinesterase in 0.01 *M* phosphate buffer, pH 7.0, 50 min after reaching 50740 rev./min. The enzyme concentration was 4 mg/ml and the temperature 20°. The sedimentation coefficient (not corrected for the buffer and concentration) was 9.9 Svedberg units.

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