Studies on Cholinesterase

4. PURIFICATION OF PSEUDO-CHOLINESTERASE FROM HORSE SERUM

By FRIEDA STRELITZ, *Banting and Best Department of Medical Research, Banting Institute, University of Toronto, Toronto, Canada*

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The purification of cholinesterase from horse serum was first attempted by Stedman & Stedman (1935), who obtained a product 50–100 times more active than serum. McMeekin (1939) raised this to 150 times. By retaining the principle of Stedman's method, based mainly on fractionation with ammonium sulphate, we have obtained highly purified enzyme preparations with a hydrolyzing power about 5000 times greater than the source from which they were derived, 1 mg. of the purified preparation being able to hydrolyze approximately 2 g. of acetylcholine per hour. The following communication deals with the description of the purification and with some properties of the purified enzyme.

METHODS

Test of enzymic activity. The manometric method of Warburg was used to determine the activity of the enzyme. The main compartment of the Warburg vessel contained the enzyme together with $NaHCO_3$ and gum acacia; the acetylcholine chloride (Hoffmann-La Roche) or other substrate was added from the side-bulb. The final concentrations were: $NaHCO_3$ 0·025 M, acetylcholine 0·03 M, gum acacia 0·25%; volume 2 ml., temp. of water-bath 37·5°. The solutions were in equilibrium with 5% CO_2 in N_2 and the pH of 7·4 produced by the $NaHCO_3/CO_2$ buffer remained substantially constant during the test period. After the contents were mixed, manometric readings were taken at equal time intervals for 15 min.

The amount of enzyme should be so chosen that 100–150 μl. of CO_2 are evolved during the reaction time. Under these conditions the velocity of the reaction is directly proportional to the enzyme concentration. The spontaneous hydrolysis of acetylcholine accounts for less than 5% of the total gas pressures and can be neglected.

Activity units. The arbitrary unit to designate the activity of the enzyme is defined as the *amount of esterase* which, under the testing conditions described, causes the liberation of CO_2 at the rate of 1 ml./hr. from the bicarbonate, indicating the hydrolysis of 7·3 mg. of acetylcholine into acetic acid and choline. The degree of purity reached is expressed by the symbol Q_{ACh}, which represents the *amount of* CO_2 (in μl.) liberated in 1 hr. by 1 mg. of a dried preparation.

PREPARATION

The starting material was horse serum. Horse blood was collected in large containers and allowed to stand several days in the refrigerator. The serum was siphoned off and, if it was not used immediately, a few drops of toluene were added. When stored cold it could be kept for several months without loss of enzyme activity.

1st Stage. 2 l. of serum, containing 12,000 units of cholinesterase (Q_{ACh} 50) were mixed with 400 g. of solid ammonium sulphate and kept at room temperature for at least 1 hr., with occasional stirring. The heavy precipitate was then removed by centrifuging in a Sharples centrifuge. The supernatant fluid was brought to pH 2·8 (yellow to bromophenol blue) by addition of 5 N-H_2SO_4. The amount of acid required (about 2·5 ml./100 ml. of fluid) was first determined in a small sample, and added to the whole solution in two portions ($\frac{3}{4}+\frac{1}{4}$), the second portion being added at least 2 hr. after the first. The resulting precipitate was again removed by centrifugation in a Sharples centrifuge. The clear, almost colourless supernatant fluid contained 7000 units cholinesterase of Q_{ACh} 2000 (40-fold purification).

2nd Stage. 15 g. of solid ammonium sulphate were added to each 100 ml. of fluid and the precipitate was collected on a large Büchner funnel (Whatman filter no. 50, diameter 24 cm.), filtration being continued until the filter cake was almost dry.

When 10 l. of serum had been worked up to this stage, the combined filter cakes, which had been kept in the refrigerator, were dissolved in 1200 ml. of water, and 800 ml. of saturated ammonium sulphate were added to bring the ammonium sulphate concentration to 0·4 saturation. The suspension was filtered on a large Büchner funnel; the precipitate was washed with 500 ml. of 0·4 saturated ammonium sulphate and discarded. The combined filtrates contained 20,000 units of cholinesterase, Q_{ACh} 4000 (80-fold purification).

3rd Stage. The filtrate was mixed with 20 g. of solid ammonium sulphate for each 100 ml. of fluid and filtered thoroughly on a Büchner funnel (Whatman no. 50, 18 cm.). The filter cake was dissolved in 100 ml. 0·1 N-acetate buffer pH 3·5, and saturated ammonium sulphate was added to 0·45 saturation. The precipitate was centrifuged off, washed twice with 50 ml. 0·45 saturated ammonium sulphate in 0·05 N-acetate buffer pH 3·5

and discarded. The combined supernatant fluids contained 16,000 units of cholinesterase, Q_{ACh} 15,000 (300-fold purification).

4th Stage. Saturated ammonium sulphate was added to the solution to bring it to 0·66 saturation and the precipitate was collected on a Büchner funnel (Whatman no. 50, 11 cm.). The filter cake was dissolved in 50 ml. 0·1 N-acetate buffer pH 3·5, and saturated ammonium sulphate was added to 0·5 saturation. The precipitate was centrifuged off, washed twice with 25 ml. 0·5 saturated ammonium sulphate in 0·05 N-acetate buffer pH 3·5 and discarded. The combined supernatant fluids contained 12,000 units of cholinesterase, Q_{ACh} 25,000 (500-fold purification).

5th Stage. Saturated ammonium sulphate was added to the supernatant fluid to bring it to 0·66 saturation and the precipitate was collected on a Büchner funnel (Whatman no. 50, 11 cm.). The filter cake was dissolved in 40 ml. 0·1 N-acetate buffer pH 3·5, and saturated ammonium sulphate was added to 0·54 saturation. The precipitate was centrifuged off, washed twice with 20 ml. 0·54 saturated ammonium sulphate in 0·05 N-acetate buffer pH 3·5 and discarded. To the combined supernatant fluids saturated ammonium sulphate was added to 0·66 saturation. The precipitate was collected by centrifugation, dissolved in a small amount of water (about 10 ml.) and dialyzed in a cellophan bag against running tap water. By 16–20 hr. a fine precipitate had formed, which was removed by centrifugation. The yellow, slightly turbid solution contained 7000 units of cholinesterase, Q_{ACh} 50,000 (1000-fold purification).

6th Stage. The solution was mixed with 1 g. of Lloyd's reagent, centrifuged and the precipitate washed twice with 3 ml. of water. To the combined supernatant fluids 2 vol. of saturated ammonium sulphate were added. The precipitate was collected by centrifugation, dissolved in a small amount of water, dialyzed against running tap water for at least 48 hr. and dried *in vacuo* over $CaCl_2$. Yield 6000 units of cholinesterase, Q_{ACh} 120,000 (2400-fold purification).

7th Stage. The dried preparation, 50 mg., was dissolved in 4 ml. of water, centrifuged if not clear, and 0·6 ml. of N-acetate buffer pH 5·2 were added. After being cooled to just above its freezing-point (with solid CO_2) the solution was mixed with ½ vol. of cold acetone (about −10°), kept in the refrigerator for 15 min., and centrifuged cold. The precipitate was dissolved immediately in 4 ml. of water and the procedure just described was repeated. The resulting precipitate was dissolved in a small amount of water, giving a clear, slightly yellow solution. It was dialyzed against frequently changed distilled water for at least 24 hr. and finally dried *in vacuo* over $CaCl_2$. Yield 3000 units of cholinesterase, or 5% of the amount of enzyme present in the starting material, Q_{ACh} 250,000 (5000-fold purification).

Notes on the preparation. The figures mentioned represent averages and the yield and the degree of purification may vary considerably. Occasionally preparations were obtained with a Q value as high as 400,000. The best yield was found in autumn and early winter, when the cholinesterase activity of horse serum was from 20 to 50% higher than in summer.

Although evidently the enzyme has not yet been obtained pure, the small amounts of material available prohibited further purification. Larger quantities of the enzyme in its present state of purity would provide a suitable starting material with which to attempt crystallization.

PROPERTIES

Stability. The purified enzyme preparations from horse serum are much more stable than preparations from dog pancreas (Mendel & Mundell, 1943). Dialysis and drying does not result in any loss of activity. The dried preparation, which may be kept indefinitely, is readily soluble in water, and the solution, when stored cold, retains its activity for weeks. Very high dilution, however, which is rendered necessary for testing the activity of the purified preparation, results in destruction of the enzyme, unless a stabilizing agent, such as gum acacia, is added (Table 1). Similar observations have been made for other highly purified enzymes. Sumner (1927) lists a number of substances which prevent the destruction of crystalline urease by dilution with water, and Scott & Mendive (1941) found that addition of peptone and various other agents preserves the activity of highly diluted solutions of carbonic anhydrase.

Table 1. *Degree of enzyme destruction at various dilutions with water*

Amount of enzyme in 2 ml. of solution μg.	Activity remaining (% of full* activity)
12	55
6	37
3	22

* Full activity obtained by diluting with and testing in the presence of 0·25% gum acacia.

The purified enzyme is stable in 0·1 N-acetic acid and 0·01 N-NaOH, but is destroyed by 0·1 N-H_2SO_4 and 0·04 N-NaOH (Table 2).

Under the conditions defined earlier, the initial velocity of the hydrolysis of acetylcholine is directly proportional to the enzyme concentration and is independent of the substrate concentration, provided that an excess of substrate is present. As

Table 2. *Resistance of enzyme to acid and alkali*

Treatment*	Activity remaining (% of initial activity)
0·1 N-acetic acid	96
0·2 N-acetic acid	67
0·4 N-acetic acid	0
0·01 N-H_2SO_4	41
0·1 N-H_2SO_4	0
0·01 N-NaOH	97
0·02 N-NaOH	33
0·04 N-NaOH	0

* 1% enzyme solution kept for 1 hr. at room temperature with acid or alkali, then neutralized. Destruction expressed as percentage of activity of untreated control.

the substrate concentration is reduced the rate of hydrolysis decreases; with 0·001 M-acetylcholine the velocity of the reaction is only one-quarter of the maximum rate (Table 3).

Table 3. *Enzymic hydrolysis at various concentrations of acetylcholine*

Molarity of acetylcholine	µl. CO_2 evolved in 10 min.
0·056	162
0·042	155
0·028	158
0·016	142
0·008	131
0·004	107
0·002	69
0·001	42

Specificity. The purified enzyme does not act exclusively on acetylcholine but to a lesser degree hydrolyzes non-choline esters as well. A common esterase, present in horse serum and mainly responsible for the hydrolysis of tributyrin and methyl butyrate, is removed in the early stages of purification. However, as the purification proceeds, the rate of hydrolysis of tributyrin, as compared with that of acetylcholine, remains constant. As the purest preparations so far obtained still retain the power to hydrolyze tributyrin at high velocity (30% of that for acetylcholine) it would seem justified to assume that the same enzyme catalyzes both reactions (Table 4).

Table 4. *Hydrolysis of tributyrin by enzyme preparations at various stages of purification*

Enzyme preparation		Hydrolytic activity towards tributyrin* (% of activity towards acetylcholine)
Q_{ACh}	Degree of purification	
50	—	139
1,600	32-fold	31
10,000	200-fold	30
300,000	6000-fold	30

* Substrate concentration 0·03 M. Because of the low solubility of the substrate it was placed in the main compartment of the Warburg vessel, while the side-bulb contained the enzyme.

From the facts discussed above it is evident that the enzyme should be classified as a pseudo-cholinesterase (Mendel & Rudney, 1943), since it displays its maximum activity at high substrate concentrations and does not hydrolyze exclusively esters of choline.

SUMMARY

A method for the purification of pseudo-cholinesterase from horse serum is described and some of the properties of the purified preparation are discussed.

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The Analysis of Hair Keratin

2. THE DICARBOXYLIC AND BASIC AMINO-ACIDS OF HUMAN HAIR

BY J. M. R. BEVERIDGE AND C. C. LUCAS, *Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada*

(*Received* 3 *January* 1944)

Data concerning the composition of many keratins is still very incomplete, so a study of human-hair hydrolysates was undertaken. A more thorough examination of such hydrolysates would give information of theoretical importance to those interested in protein constitution and might possibly be of practical value in revealing new sources for some of the amino-acids. The fractionations described