CHARACTERISTICS OF THE CHOLINESTERASE OF THE VENOM OF THE CENTRAL ASIAN COBRA

V. M. Sorokin, Z. Nigmatov, and L. Ya. Yukel'son

UDC 591.105:577.15.598.126

Cholinesterase catalyzes the hydrolysis of choline esters to choline and the corresponding carboxylic acids. The presence of this enzyme in venoms of snakes of the Elapidae family is a characteristic feature of them; cholinesterase has not been found in venoms of snakes of the Viperinae and Crotalinae families [1-3]. In many properties, particularly in substrate specificity, the cholinesterase of venoms differs from enzymes with a similar action from other sources. Consequently, it cannot be included with the "true" cholinesterases (acetylcholine acetyl-hydrolase, E.C. 3.1.1.7) nor with the pseudocholinesterases (cholinesterases E.C. 3.1.1.8), which has been made a reason for separating it as an independent "ophiocholinesterase" [4].

A detailed investigation of the properties of cholinesterase and the determination of its biological value in snakes is impossible without the isolation of the enzyme in the purified state from the complex mixture of biologically active protein components forming snake venom, all the more since the latter also contains its inhibitor [5]. Several attempts at purifying venom cholinesterase are known. Thus, by precipitation with sodium sulfate and ammonium sulfate, preparations of cholinesterase purified by factors of 20 and 11, respectively, have been isolated from the venoms of Naja naja (cobra) and Bungarus fasciatus (bungarus). It was impossible to increase their specific activity by electrophoresis or by adsorption chromatography; i.e., they were practically free from contamination with other substances [6]. Eighteenfold purification of cholinesterase has been achieved by the passage of the venom of Naja oxiana Eichwald (Central Asian cobra) through Sephadex gel G-75. However, the enzyme preparation obtained, according to electrophoretic and enzymological analysis, included other biologically active factors of the venom – hyaluronidase, phosphatases, etc. [3, 7]. Using the method of ion-exchange chromatography on sulfoethyl-Sephadex C-50 followed by gel filtration on Sephadex G-25, we have increased the specific activity of the cholinesterase of the same venom by a factor of 46. Nevertheless, the results of an electrophoretic check have shown that the preparation contains two protein components [8]. The present paper discusses the process of the further purification of the cholinesterase and some of its properties.

EXPERIMENTAL

The enriched (by a factor of 46) preparation of cholinesterase from the venom of Naja oxiana Eichwald was rechromatographed on sulfoethyl-Sephadex C-50 in columns (15 × 300 mm). The sorption of the preparation on the cation-exchange resin was performed from 0.05 M ammonium acetate buffer, pH 5.5. Elution was performed with the same buffer solution with an increase in the concentration to 0.3 M and a change in the pH from 5.5 to 7.8. The eluent (volume 3-4 ml) was collected in a KhKOV-1 automatic fraction collector, the amounts of protein, from the absorption at 280 nm, and the cholinesterase activity were determined, the fractions were combined according to the protein "peaks," and they were freeze-dried, desalted on Sephadex G-25, again freeze-dried, and then investigated.

The cholinesterase activity was determined by Hestrin's method as modified by A. N. Panyukov [9]. The unit of activity of the enzyme corresponded to 1 μmole of substrate hydrolyzed in 60 min at 37°C.

Institute of Biochemistry, Academy of Sciences of the Uzbek SSR. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 783-789, November-December, 1972. Original article submitted March 10, 1972.

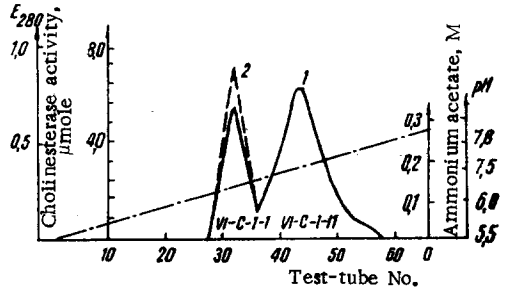

Fig. 1. Results of the rechromatography of the 46-times enriched preparation of cholinesterase [8] on sulfoethyl-Sephadex C-50: 1) concentration of protein (extinction at 280 nm); 2) cholinesterase activity. VI-C-1-1 and VI-C-1-2 are the fractions combined according to the protein "peaks."

TABLE 1. Cholinesterase Activity of the Venom and Its Fractions

Material	Specific Activity (μmole of hydrolyzed acetylcholine per 1 ml of protein)	Purification A^1/A	Yield, %
Cobra venom	0.9×10^3	–	100
Fraction VI (obtained by chromatographing the venom on sulfoethyl-Sephadex C-50 [8])	6.82×10^3	7.5	52
Fraction VI-C-1 (obtained after the passage of fraction (VI) through Sephadex G-25 [8])	41.57×10^3	46.0	51
Fraction VI-C-1-1 (obtained by rechromatographing fraction VI-C-1 Sephadex C-50)	3.68×10^3	4.0	13

Notes. A^1 Specific activity of the cholinesterase in the fractions; A in the cobra venom.

The disc electrophoresis of the protein preparations was performed by the method of Davis and Ornstein [10].

The toxicity of these preparations was determined by intraperitoneal injection into white mice weighing 18-20 g, and the results were treated statistically by the method of Litchfield and Wilcoxon [11].

Further Purification of the Cholinesterase. When the 46-times-enriched preparation of cholinesterase was separated on sulfoethyl-Sephadex C-50, we found in it (Fig. 1) two protein components, one of which was desorbed from the cation-exchange resin with a change in the concentration of the eluting ammonium-acetate buffer from 0.1 to 0.2 M and the second when the concentration was raised to 0.3 M. The cholinesterase appeared in the first fraction (test tubes Nos. 26-37), and its activity rose in parallel with the increase in the concentration of protein in the tubes (the maximum activity corresponded to the maximum absorption at 280 nm). Disc electrophoresis of this fraction showed that it was an individual substance (Fig. 2). On calculating the specific activity of the enzyme, we found that it had fallen by approximately 14 times, being $3.68 \cdot 10^3$ μmole instead of $41.57 \cdot 10^3$ μmole of the initial material. The yield of cholinesterase amounted to 13%; it was only four times more active than the whole cobra venom. The results of a determination of the cholinesterase activity in the first fraction, which we have denoted by the symbols VI-C-1-1, and of similar investigations of the whole cobra venom and also of the fractions obtained from it in various ways are given in Table 1.

The fall in the cholinesterase activity in the process of rechromatography on sulfoethyl-Sephadex can be explained by a separation of an aggregated cholinesterase enzyme system into two components. Changeux et al. [12], who have studied the acetylcholinesterase of the electric apparatus, assume that it consists of subunits with a catalytic activity and "regulator" subunits. The separation of them from one another may be accompanied by a fall in the activity of the enzyme as we have probably observed in the

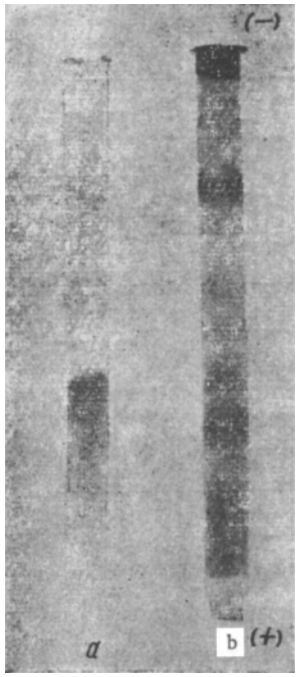

Fig. 2. Disc electrophoregram of cobra venom and of fraction VI-C-1-1 (polyacrylamide gel, glycine acetate buffer, pH 3;6, at a voltage of 200 V and a current strength of 2.5 mA per tube with a time of electrophoresis of 55 min). a) Fraction VI-C-1,1; b) cobra venom.

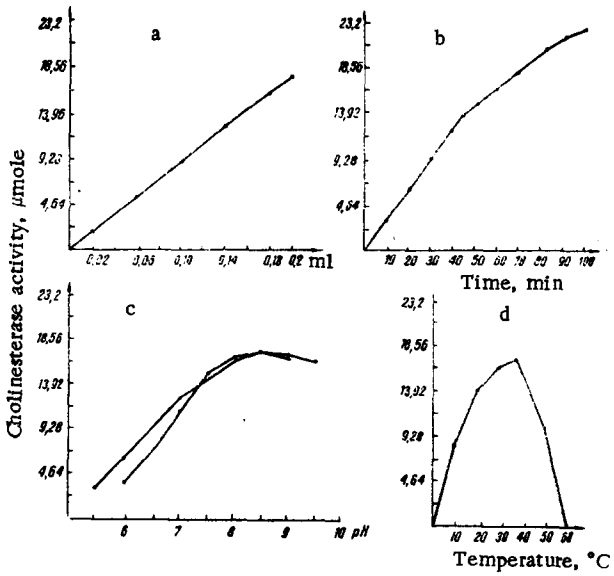

Fig. 3. Influence of the conditions of incubation on the cholinesterase activity of cobra venom (incubation medium 0.05 M tris–hydrochloric acid buffer or 0.033 M phosphate buffer), 0.2 ml (4 μg) of enzyme and 0.0193 M solution of the substrate – acetylcholine. a) Dependence of the activity on the concentration (0.1 ml of solution contains 2 μg of cholinesterase protein); b) dependence of the activity on the time of incubation; c) influence of the pH on the activity; 1) tris–hydrochloric acid buffer; 2) phosphate buffer (time of incubation 30 min); d) influence of the temperature on the activity (time of incubation 30 min).

investigation of the electrophoretically pure preparation of cholinesterase. Attempts to restore the initial activity of recombining fractions VI-C-1-1 and VI-C-1-2 proved unsuccessful. The restoration of activity on the recombination of subunits is apparently possible only when certain conditions unknown to us are observed. It is not excluded that in the process of separation conformational changes in the molecules of the subunits take place, as a consequence of which the cholinesterase activity is not restored when the two fractions are mixed. Nevertheless, in further experiments on the properties of the venom cholinesterase we used the VI-C-1-1 fraction, which was electrophoretically homogeneous.

Influence of the Conditions of Incubation on the Rate of Hydrolysis of Acetylcholine by Cobra Venom Cholinesterase. With an excess of substrate, the rate of an enzymatic reaction depends primarily on the concentration of the enzyme:

$$V = k\,(E),$$

where V is the rate of the reaction and (E) is the concentration of enzyme. This relationship is shown graphically in Fig. 3a. The activity of the venom cholinesterase rises linearly with an increase in the concentration of the enzyme in the samples. Such a linear relationship is characteristic for the majority of enzymes [13].

The dependence of the rate of hydrolysis of acetylcholine on the time of incubation is shown in Fig. 3b. In the time from 10 to 45 min, the cholinesterase activity rises linearly with the increase in the time of incubation. This shows that the hydrolysis of the acetylcholine takes place as a zero-order reaction. If the concentration of hydrolyzed substrate is denoted by X and the time by t, for the initial part of the curves in Fig. 3b it is possible to write the following equation:

$$k_0 = \frac{dX}{dt},$$

where k_0 is the rate constant of the zero-order reaction.

In our case, k_0 will be equal to 0.314; i.e., in 1 min 0.2 ml (4 μg) of cholinesterase venom hydrolyzes 0.314 μmole of acetylcholine. In the main, the curve of Fig. 3b corresponds to first-order reactions: after

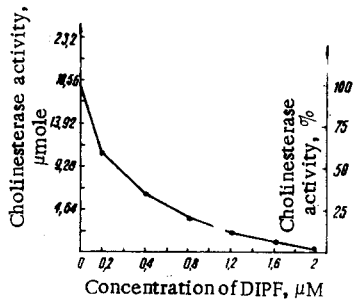

Fig. 4. Results of the inhibition of venom cholinesterase by diisopropyl phosphorofluoridate (DIPF). The incubation medium contained the same ingredients (see caption to Fig. 3) and various amounts on DIPF.

the lapse of 45 min, the rate of the reaction gradually falls and the kinetic curve approaches a line parallel to the axis of abscissas. The rate of such a reaction at each moment is determined by the equation

$$\frac{dX}{dt} = k(A - X),$$

where A is the initial concentration of the substrate and X is the concentration of the converted fraction of the substrate (the hydrolyzed acetylcholine).

Thus, like many other enzymatic processes, the hydrolysis of acetylcholine in the presence of venom cholinesterase, in the first period (up to 45 min) – when the degree of conversion is low, there is a considerable excess of substrate, and only a small amount of reaction products has been formed – is a zero-order reaction, and only when the exhaustion of a substrate begins and the reaction products accumulate does the rate fall and the process take place in the manner of first-order reactions. Consequently, it is desirable to estimate the activity of venom cholinesterase after incubation of the enzyme with the substrate for 20-30 min, and we took this into account.

We investigated the influence of the concentration of hydrogen ions in the incubation medium on the cholinesterase activity, and this both in 0.05 M tris–hydrochloric acid buffer and in 0.033 M phosphate buffer (Fig. 3c). In both cases, the graph of the dependence of the activity on the pH had a sigmoid nature. The optimum pH was found to be 8.0-8.5. The rate of hydrolysis of acetylcholine rose rapidly with an increase in the pH from 5.5-6.0 to 8.0-8.5, and, when the incubation medium was made more alkaline it fell, which is possibly due to the denaturation of the enzyme. Almost the same optimum pH (8.4-9.0) was found by Heilbron [14] and by Chowdhury [15], who performed experiments with whole venoms of the Elapidae family and cholinesterases purified from them. In both buffer systems (tris and phosphate) they observed a rapid increase in cholinesterase activity with a shift in the pH to 7.4, and then the activity changed little, becoming almost stable. The stability and marked action of cholinesterase at pH 7.4 has been reported by Chowdhury [15] and by Björk [16].

Figure 3d shows graphically how the temperature affects the cholinesterase activity. With a rise in the temperature the rate of hydrolysis of acetylcholine increases, as with any other enzymatic reaction. The maximum cleavage of acetylcholine takes place at 37-38°C. The same temperature optimum for cholinesterase was found in an investigation of the whole venom of the Central Asian cobra [3]. With a further rise in temperature the colinesterase activity falls; at 50°C it has fallen to somewhat less than half (41%), and at 60°C it is suppressed completely in the first few minutes. The cause of the sharp fall in the activity of the enzyme with a rise in the temperature is considered to be primarily its thermal denaturation [13]. The thermolability of venom cholinesterase has also been reported by other authors [17].

Influence of Diisopropyl Phosphorofluoridate (DIPF) on the Activity of Cobra Venom Cholinesterase. DIPF is a reagent which reacts specifically with serine. It is assumed that histidine participates in the reaction of DIPF with serine [18]. Consequently, the fall in enzymatic activity on treatment with DIPF may be considered an indication of the inclusion of serine and histidine in the "active center." Figure 4 shows the influence of DIPF on the activity of the cobra venom cholinesterase. With an increase in the concentration of DIPF to 2 μM the rate of hydrolysis of acetylcholine falls to zero. The results that we have obtained indicate that serine and histidine participate in the formation of the esterase center of the enzyme. The formation of the "active center" with the aid of these amino acids is common to all cholinesterases; the action of DIPF that has been shown is characteristic for many cholinesterases from other sources [19].

Substrate Specificity of Cobra Venom Cholinesterase. We have investigated the action of cobra venom cholinesterase on acetylcholine chloride, acetylcholine bromide, and butyrylthiocholine bromide. The results of the determination of the substrate specificity of the cholinesterase are given below.

Substrate	Specific activity of the cholinesterase, μmole/mg of protein
Acetylcholine chloride	$4.32 \cdot 10^3$
Acetylthiocholine bromide	$4.33 \cdot 10^3$
Butyrylthiocholine bromide	0

The venom cholinesterase hydrolyzes acetylcholine chloride and acetylthiocholine bromide equally actively, but has no effect on butyrylthiocholine bromide. It is known that the ester of choline and butyric acid is a reversible inhibitor of acetylcholine esterase [18]. It is possible just for this reason that the venom cholinesterase does not attack butyrylthiocholine bromide. In this respect it is similar to the "true" cholinesterases, which also act on acetylcholine chloride and acetylcholine bromide but do not hydrolyze butyrylthiocholine bromide [19]. The similarity of "ophiocholinesterase" to the true cholinesterases has also been reported by other authors [15].

Toxicity of Cobra Venom Cholinesterase. In experiments on mice we studied the biological action of the cholinesterase isolated, evaluating it according to its lethality for mice. On intraperitoneal administration of the preparation, no lethal outcome was observed even at doses of 8-10 mg/kg body weight. The lack of toxicity of the venom cholinesterase has been observed previously in experiments on the separation of the venom and the determination of the toxicity of the fractions [3, 20], and also in experiments on the lethal effect of venoms in which the cholinesterase was suppressed with the aid of specific inhibitors (proserine, DIPF) [3]. Nevertheless, the assumption has existed that venom enzymes, while themselves being nontoxic, may potentiate the action of the true toxins. In view of this, we studied the lethal effect of the neurotoxin isolated from cobra venom with LD_{50} 0.13 mg/kg body weight [21] in combination with the cholinesterase. The addition of the cholinesterase did not change the effectiveness of this neurotoxin; its LD_{50} value remained constant at 0.12-0.125 mg/kg body weight.

SUMMARY

1. By rechromatography on sulfoethyl-Sephadex C-50, an electrophoretically homogeneous preparation of cholinesterase has been obtained from the venom of Naja oxiana Eichwald.

2. The activity of the cholinesterase isolated depends on the concentration of the enzyme and the time and temperature of incubation, and also on the pH. The following must be considered the optimum conditions: time of incubation of the enzyme with the substrate 20-30 min, pH 8.0-8.5, temperature 37-38°C.

3. Diisopropyl phosphorofluoridate (DIPF) in a concentration of 2 μM completely suppresses the activity of the cobra venom cholinesterase.

4. The venom cholinesterase hydrolyzes acetylcholine chloride and acetylthiocholine bromide but has no effect on butyrylthiocholine bromide, in which respect it resembles the true cholinesterases.

5. Preparations of cobra venom cholinesterase do not possess a lethal action and do not potentiate the activity of the neurotoxins of the same venom.

LITERATURE CITED

1. N. K. Iyengar,, N. B. Sehra, B. Mukerji, and R. N. Chopra, Current Sci., 7, 51 (1938).
2. E. A. Zeller, Experientia, 3, 375 (1947).
3. L. Ya. Yukel'son and M. Malikov, in: Questions of Medicinal Chemistry and the Action of Physiologically Active Substances [in Russian], Tashkent (1970), p. 76.
4. E. A. Zeller, Advan. Enzymol., 8, 459 (1948).
5. C.-Y. Lee, C.-C. Chang, and K. Kamijo, Venoms (First International Conference, December 27-30, 1954, Berkeley, Calif.), Washington (1956), p. 197.
6. D. K. Chowdhury, Ann. Biochem. Exp. Med., 4, 77 (1944).
7. Ya. Kh. Turakulov, D. N. Sakhiborv, V. M. Sorokin, and L. Ya. Yukel'son, Biokhimiya, 34, 1119 (1969).
8. V. M. Sorokin, Z. Nigmatov, and L. Ya. Yukel'son, Biokhimiya, 37, 1 (1972).
9. A. N. Panyukov, Vopr. Med. Khim., 12, 88 (1966).

10. B. J. Davis and L. Ornstein, Ann. New York Acad. Sci., 121, 321, 404 (1964).
11. M. L. Belen'kii, Elements of the Quantitative Evaluation of Pharmaceutical Effects [in Russian], Leningrad (1963).
12. J. P. Changeux, T. R. Podleski, and J. C. Meunier, J. Gen. Physiol., 54, 225 (1969).
13. V. L. Kretovich, Introduction to Enzymology [in Russian], Moscow (1967).
14. E. Heilbron, Acta Chem. Scand., 8, 1368 (1954).
15. D. K. Chowdhury, Ann. Biochem. Exp. Med., 9, 85 (1949).
16. W. Björk, Biochem. Biophys. Acta, 49, 195 (1961).
17. D. K. Chowdhury, Ann. Biochem. Exp. Med., 9, 73 (1949).
18. J. L. Webb, Enzyme and Metabolic Inhibitors, Academic Press (1963-1966).
19. S. N. Golikov and V. I. Rozengart, Cholinesterases and Anticholinesterase Agents [in Russian], Leningrad (1964).
20. C.-C. Yang, W.-C. Chin, and K.-C. Kao, J. Biochem. (Japan), 48, 706 (1960).
21. Ya. Kh. Turakulov, V. M. Sorokin, S. A. Nishankhodzhaeva, and L. Ya. Yukel'son, Biokhimiya, 35, 1282 (1971).