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Mayfly Cholinesterase: Solubilization and Partial Purification

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ABSTRACT — A method has been developed for the solubilization of cholinesterase from whole mayflies (*Hexagenia bilineata*, Say). Solubilization is enhanced through the use of: (1) protamine sulfate, (2) sephadex, (3) sodium taurocholate, and (4) ammonium sulfate-sodium bicarbonate treatments. Partial purification of the enzyme is also reported. Fractions representing a final activity of 246 units and a purification factor of 17.79 are presented.

Extensive use of insecticides has led to an intensified search for a more purified form of cholinesterase (EC3.1.1.7) from insect sources. In this effort solubilization, an ideal vehicle for purification of membrane-bound enzymes, has proved a stubborn block. Release of the enzyme from cockroaches (Lord, 1961) and flyheads (Dauterman *et al.*, 1961) was in range of 50-60% of total activity. Isolation of cholinesterase (ChE) from sources other than insects, such as *Torpedo marmorato* (Leuzinger and Baker, 1967) have been more successful. In spite of successes such as those of Leuzinger and Baker, relatively pure forms of insect ChE have been not totally realized. Multimolecular forms of the enzyme as indicated from molecular studies (Kremzner and Wilson, 1964; Krysan and Chadwick, 1966; and Lawler, 1963) may be at the root of these purification problems.

The experiments reported here were undertaken in order to provide additional information on precipitation techniques available for further purification studies on whole insect ChE.

Materials in experimental procedure

Chemicals used in this study were obtained from the following sources: acetylcholine bromide, bovine serum albumin, eserine, and Sephadex, Sigma; lipase powder (pork pancreas), protamine sulfate (Salmine), Mann Research Laboratories; sodium bicarbonate, Mallinckrodt; sodium taurocholate, General Biochemicals; and superfine glass beads, type 150-5005, Minnesota Mining and Manufacturing.

Mayfles (*Hexagenia bilneata*, Say) were obtained from the LaCrosse, Wisconsin, Mississippi River Flood Plain during their peak emergence period in July 1968. Mayflies were frozen within 12 hours of capture and stored at −20° until used. All subsequent extractions and purifications were conducted on this stock supply.

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Assay Procedure

ChE activity was determined by the use of a Radiometer TTT-1 pH stat with end point pH of 7.01. Reaction mixture consisted of 12.0 ml of 1.0 M NaCl, 1.0 ml ChE extract, and 2.0ml of 0.001M acetylcholine bromide (AChBr) as substrate (Kruckeberg, 1968). The titrant, 0.05M NaOH, was added to the reaction mixture in a ratio equalling the hydrolysis rate of AChBr. Reaction rates were followed for a 5 minute period with triplicates asayed for all samples. Activity was expressed as μ m of AChBr hydrolyzed/hour/ml sample with specific activity expressed as μ m of AChBr hydrolyzed/hour/ml sample/mg protein. True ChE activity was determined by the addition of 0.0001 M eserine to reaction mixture which permitted calculation of amount of hydrolysis due to esterases other than ChE. Protein determinations were conducted according to the method of Lowry *et al.* (1951).

Solubilization Procedure

A five-step procedure for solubilization of mayfly ChE is described below.

1 — Extraction — Thirty grams whole, frozen mayflies were homogenized at high speed in a Waring Blender, with monel attachment, for a period of 4 minutes. Homogenization was conducted in 100 ml of sodium taurocholate-phosphate buffer (NaCl, 58.45 g; KH_2PO_4; 3.84 g; NaOH, 1.0g; sodium taurocholate, 0.4 g; and water to 1 liter), pH 6.8 (Kruckeberg, 1968). The homogenized material was then subjected to centrifugation of 1800*g* for 15 minutes at 0°.

2 — Additional Mechanical Disruption — The supernatent was further solubilized by the addition of 50.0g of glass beads (type 150-5005) in a ratio of 50.0g beads to 150.0ml of supernatent. Disruption was accomplished with the aid of a Waring Blender followed by centrifugation as prescribed in step 1 above.

3 — Removal of Extraneous Molecules — A 40% protamine sulfate solution in amounts of 0.25 volumes of supernatent (step 2) was employed to remove nucleic acids and other related molecules. After addition of protamine sulfate the extract was allowed to stand for 1 hour at 5° followed by centrifugation at 14,000*g* for 15 minutes.

4 — Volume Concentration — Sephadex (G-100-120) was used to concentrate volume by employing a ratio of

TABLE I. The effect of sodium taurocholate concentration on ChE solubility. Reaction vessel consisting of a 15ml sample volume was as follows: 12.0ml of 1.0M NaCl, 1.0ml ChE extract, and 2.0ml of 0.001M AChBr. Reaction rates represent average of three determinations of 5 minutes each.

Per Cent Sodium Taurocholate [a]	Per Cent Activity Supernatent [b]	Per Cent Decrease Activity Supernatent [b]
0.40	59.60	00.00
1.00	52.25	12.25
1.50	46.20	22.48

[a] Per cent sodium taurocholate in buffer.
[b] Activity determined in μ moles/hour/ml sample.

1.0 g Sephadex to 100 ml of supernatent. The supernatent was exposed to this Sephadex treatment for a period of 10 minutes with Sephadex subsequently removed by centrifugation at 17,000*g* for 30 minutes at 0°.

5 — Ammonium Sulphate Precipitation — The supernatent obtained from step 4 was then precipitated with 23% ammonium sulfate. The ammonium sulfate and sodium bicarbonate treatments are essentially those of Lord (1961). Precipitation was conducted at 20°, and involved, initially, the slow addition of ammonium sulfate to the supernatent with constant stirring over a 30 minute period. The precipitate was allowed to settle for 2 hours at 20°, then centrifuged at 4000*g* for 1 hour. The precipitate was then redissolved in 0.2M $NaHCO_3$ (1 ml $NaHCO_3$ to 20 ml enzyme solution) and allowed to stand for 1 hour at 20° and then centrifuged at 4000*g* for 15 minutes. The supernatent was retained, along with the pellet, re-extracted, and centrifuged as above yielding a second supernatent. The two supernatents were recombined and Sephadex was added (as above) to remove excess sodium bicarbonate and ammonium sulfate. The solution was then centrifuged at 1800*g* for 30 minutes and the supernatent retained.

Enzyme activity assessed

Initial homogenization of whole mayflies resulted in a specific ChE activity of 5.32 units (Table III). Approximately 60% of this activity was located in the supernatent. At all steps of purification both supernatent and pellet were examined for enzyme activity to determine degree of solubilization. Utilization of the glass beads increased specific activity from 5.32 units to 13.05 units resulting in approximately a 2.5 fold purification. The addition of 0.04% sodium taurocholate to buffer was also a direct cause to increased activity.

The sample was then dialyzed against 25% polyethylene glycol for 6 hours at 2°, with constant stirring, followed by overnight treatment against 10% polyethylene glycol, in attempts to increase solubility above the 50-60% level. However, dialysis did not increase sample activity and the procedure was subsequently discontinued.

Sodium taurocholate, a bile salt, was then added to the buffer system to aid lysis in the manner of Lord (1961). Enzyme activity was noted to increase approximately 50% by the addition of sodium taurocholate (0.04%) and all future extractions contained this additive in the buffer (Table I). Examination of the sodium taurocholate effect revealed that activity decreased as the per cent of sodium taurocholate in the buffer was increased (Table I). These results tend to confirm those of Lord (1961) who examined cholinesterase from cockroaches. Further attempts at increased solubilization were directed at combining the effects of sodium taurocholate treatment with autolysis. These treatments resulted in loss of activity and autolysis was subsequently discontinued (Table II). In further evaluation of the 0.4% sodium taurocholate treatment, it was determined that these samples, when centrifuged at 1800*g*, had significant activities remaining in the sediment. Approximately 40% of activity remained in the sediment. Consequently the sediment was resuspended with buffer and reground with the supernatent. Assay of supernatent indicated that 75% of activity was present. All further attempts at solubilization above 75% levels were not successful.

TABLE II. Relationship between sodium taurocholate[a] and autolysis treatments on ChE solubility. Reaction vessel consisting of a 15ml sample volume was as follows: 12.0ml of 1.0M NaCl, 1.0ml ChE extract, and 2.0ml of 0.001M AChBr. Reaction rates determined as in Table I.

Length of Autolysis [b] Treatment (hours)	Percent [c] Activity Supernatent	Per Cent Decrease Activity Supernatent
0.00	59.60	0.00
24.00	58.65	1.60
48.00	54.72	8.21
72.00	38.86	34.80
96.00	36.12	39.40

[a] 0.4% sodium taurocholate in buffer.
[b] Autolysis temperature 23°.
[c] Activity determined in ω moles/hour/ml sample.

TABLE. III. Fractionation of Mayfly Cholinesterase.

Fraction	Protein (mg/ml)	Activity (μm/hour/ml sample)	Specific Activity (μm/hour/ml sample/mg protein)	Purification
Raw	7.52	40.00	5.32	1.00
Sodium Taurocholate	4.62	59.95	13.05	2.45
Protamine Sulfate	3.68	90.54	24.60	4.62
Ammonium Sulfate	3.76	222.24	59.10	11.11
Ammonium Sulfate-Sodium Bicarbonate	2.60	246.00	94.62	17.79

Cholinesterase purifications for a typical set of experiments are reported in Table III. Data indicate that a specific activity of 94.62 units representing a 17.79 fold purification was obtained. This compares more than favorably with earlier reports of ChE purifications from other insect sources (Lord, 1961; and Dauterman *et al.*, 1961), at least at this stage of purification.

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