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Purification of a Cholinesterase from the Body Muscles of Plaice (*Pleuronectes platessa*)

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A procedure for the purification of a cholinesterase from the body muscles of plaice, *Pleuronectes platessa*, is described. The enzyme is liberated from the cell structures by incubation of a muscle homogenate in water at 30°C for about 30 h with a bacteria, *Cytophaga* sp. After the liberation the enzyme is fractionated twice with ammonium sulphate and further purified by gel filtration. A 2000 fold purification was reached with a yield of about 7 %. The highest specific activity recorded was 53 μmoles mg^{-1} min^{-1} of butyrylcholine split.

Attempts to purify structure bound acetyl cholinesterase (E.C. 3.1.1.7) from different sources have been undertaken with varying success.[1-8] Working with a firmly bound cholinesterase in body muscles of plaice (*Pleuronectes platessa*) [9] the present author used autolysis to liberate the enzyme for further purification.[10] It could be shown that the liberation was due to the action of at least one bacterial strain, *Cytophaga* sp., which was isolated from a successful autolysis preparation.[11] The present paper describes a procedure to purify to a high degree the plaice muscle cholinesterase.

MATERIALS AND METHODS

Bacterial strain. Cytophaga sp., as preliminarily classified by Dr. T. Mitchell,* 1965, was initially isolated from the bacterial flora developing in a plaice muscle homogenate kept for 24 h at 30°C.[11] The strain was kept in a refrigerator on tilted agar tubes. From time to time it was inoculated on agar dishes from which loops were taken when the strain was to be cultured in sterile Oxoid Nutrient Broth. It was allowed to grow for 48 h, whereafter the bacteria were collected by centrifugation and resuspended in small amounts of broth.

Plaice muscles. Due to difficulties in obtaining fresh material, commercially available, deep frozen filets of plaice muscles (Winner, Co-operative Society, Sweden) were used. Their dry weight (after 24 h at 105°C) was 1.725 ± 0.001 g (S.D. in five tests) per 10 g.

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Chemicals. The substrates used to determine cholinesterase activity were butyrylcholine iodide [12] and 2,6-dichloroindophenyl acetate.[13] Ammoniumsulphate (*p.a.*, Kebo, Stockholm, Sweden) in saturated solutions adjusted to alkaline pH (~7.8) with ammonia was used for the fractionations. The gel filtration was performed on Sephadex G 200, 40—120 μ (Pharmacia Fine Chemicals Co., Uppsala, Sweden) treated according to the directions of the manufacturer.[14]

Preparation of muscle suspensions. Thawed muscle filets were disinfected as described earlier.[11] They were cut into pieces with a pair of scissors and pooled. 1 g was added to 10 ml of distilled water. The muscle suspensions were homogenized for 1 min with an Ultra-Turrax homogenizer, Type TP 45/2.

Bacterial treatment. To the homogenate was added an amount of *Cytophaga* sp. to give about 10^6 bacteria per ml. The cultures were placed in a water bath at 30°C.[11] The liberation of the cholinesterase activity was followed in the supernatant after centrifugation at 82 000 *g* of withdrawn samples. When reaching maximal cholinesterase activity the treatment was stopped, usually after about 30 h. After chilling and adjusting the pH to 8.2, the culture was centrifuged for 30 min at 2500 *g*. The supernatant was kept and, preferably, used immediately because standing sometimes resulted in loss of cholinesterase activity, presumably due to proteolytic enzymes produced by the bacteria.

Fractionation with ammonium sulphate. Saturated ammonium sulphate solution at about 5°C (~3.9 M [15]) was added slowly under stirring to the chilled extract. Three precipitates forming at different salt concentrations, the highest concentration being 2.7 M, were removed by centrifugation. One further addition of saturated ammonium sulphate solution was made, resulting in a concentration of 3.2 M. The precipitate was left to form overnight at 4°C. It was collected by centrifugation at 10 000 *g* for 15 min. The last fraction (F_1) was dialyzed overnight against a barbital buffer, pH 8.2. Undissolved protein was removed by centrifugation. To concentrate the preparation the clear supernatant was again salted out with saturated ammonium sulphate solution. The cholinesterase activity was contained in the third precipitate forming between salt concentrations of 2.2 M to 2.9 M at 5°C (F_2). This precipitate was dissolved in 2—5 ml of an ammonium acetate buffer, 0.1 M, pH 8.2. This solution was used for gel filtration on Sephadex G 200. Samples of the fractions to be tested for cholinesterase activity or for determinations of protein concentration were dialyzed free from ammonium sulphate either in dialyzing bags overnight or on Sephadex G 25.[14]

Gel filtration. Gel filtration on Sephadex G 200 was performed on a column 8 $cm^2 \times 82$ cm. As eluent was used ammonium acetate, 0.1 M, pH 8.2. The applied samples measured about 5 ml and contained about 5 mg protein per ml. The flow rate was about 0.6 cm h^{-1}. The ratio of the elution volume (V_e) to the total volume of the gel bed (V_t) was determined.[14]

Determination of cholinesterase activity. Cholinesterase activities were determined in duplicates at 25°C with an electrometric method.[16] A barbital-phosphate buffer was used.[9] As substrate was used butyrylcholine iodide at a final concentration of 1.6×10^{-3} M (pS = 2.8). Although acetylcholine is hydrolyzed faster,[17] butyrylcholine was used in order to be able to follow the purification of the special muscle cholinesterase avoiding the effect of any acetylcholinesterase which might possibly be present. Specific activities are expressed as μmoles substrate hydrolyzed per mg protein and minute.[18]

Demonstration of the cholinesterase activity in the effluent from the Sephadex columns was performed by mixing 50 μl effluent with 50 μl of 2,6-dichloroindophenyl acetate (1 mg dissolved in 1.5 ml acetone; diluted ten times with water) in the pits of white spot test plates. The inversed time needed for the transition from orange to blue colour was taken as a measure of the cholinesterase activity.

Determinations of protein concentrations. Protein was determined by a biuret method as described by Zamenhof.[19] Standard curves were made on egg albumin (Grave, Sweden).

The method was accurate for determining protein concentrations down to about 0.5 mg/ml. When lower protein concentrations appeared, which was the case especially in the gel filtration eluates, the absorbance at 280 mμ was measured spectrophotometrically assuming that an absorbance of 1.0 corresponded to 1 mg protein per ml. No corrections were made for possibly altered proportions of aromatic amino acids in the different preparations. Comparisons between the two methods indicate that lower protein concentrations are recorded by the biuret method.

RESULTS

The solubilizing effect of the bacteria *Cytophaga* sp. on the muscle cholinesterase was investigated in the following manner. Plaice muscle homogenates were incubated for about 30 h at 30°C and the cholinesterase activity was measured in the homogenates and their supernatants. The results, which are

Table 1. Per cent of cholinesterase activities in homogenates, supernatants, and residues after centrifugation at 82 000 *g* for 15 min measured before and after incubation for 29—33 h at 30°C with *Cytophaga* sp. Means from 7 experiments. Substrate: butyrylcholine iodide, pS = 2.8. Highest and lowest values are given in brackets below the figures (see also Table 2).

Cholinesterase activity	Before incubation	After incubation
in *incubated homogenate* in per cent of initial homogenate activity	—	191 (82—319)
in *supernatants* in per cent of respective homogenates	20 [a] (14—28)	47 [a] (31—62)
in *residues* in per cent of respective homogenates	76 (38—122)	33 (15—50)

[a] The specific activities of the supernatants ranged between 14—44 (mean = 21) and 61—117 (mean = 86) × 10^{-3} μmoles mg^{-1} min^{-1}, respectively.

Table 2. Results from one typical experiment to liberate and fractionate cholinesterase from body muscles of plaice (100 g wet muscle + 1000 g water) with ammonium sulphate. Highest and lowest values in seven experiments between brackets.

	Total amount of (wet) tissue or protein (mg)	Enzymic activity: total amount (μmoles min^{-1})	yield calculated on initial homogenate (%)	specific activity (μmoles $mg^{-1}min^{-1}$ × 10^3)	purification (×)
Homogenate at start	100 000	300		17.3 [a]	
Supernatant [b] at start	2 700	48	16	17.8	
» after 23 h				45	
» » 29 h				54	
» » 33 h				66	
» » 33 h [c]	2 200	300	100	135 (96—215)	7.8
Fraction precipitated between 2.7 to 3.2 M ammonium sulphate	100	225	75 (20—75)	2200 (725—2200)	127 (30—127)

[a] Calculated on the dry weight of the muscle tissue.
[b] After centrifugation at 82 000 *g* for 15 min.
[c] After centrifugation at 2 500 *g* for 30 min and storing for 12 h in a refrigerator.

given in Table 1, imply that almost all the initially demonstrated cholinesterase activity in the muscle homogenates was found in the supernatant of the incubated homogenate.

For further purification, ammonium sulphate fractionation was undertaken. The results from a typical preparation are given in Table 2.

After refractionation with ammonium sulphate, gel filtration on Sephadex G 200 of the cholinesterase fractions resulted in highly active preparations. The highest specific activity recorded was 53 μmoles mg^{-1} min^{-1} (in one of the fractions constituting the second preparation in Table 3). The purification, calculated on the dry weight of the muscle tissue, was, in two experiments, 700 and 2300 with a total yield of 6 and 7 % (Table 3).

Table 3. Results of two experiments to purify cholinesterase from plaice muscle by bacterial liberation, repeated ammonium sulphate fractionation and gel filtration on Sephadex G 200.

Preparation	Total amount of (wet) tissue or protein (mg)	Enzymic activity: total amount (μmoles min^{-1})	yield calculated on initial homogenate (%)	specific activity (μmoles $mg^{-1}min^{-1}$ × 10^3)	purification calculated on dried muscle tissue (×)
Homogenate	225 000	660		3.0 [a]	
	245 000	970		2.8 [a]	
Centrifugate of treated homogenate, 2500 *g* for 30 min.	9 210	295	45	32	
	4 640	490	50	105	
F_1: most active ammonium sulphate fraction 2.7–3.2 M $(NH_4)_2SO_4$	136	64	10	470	30
	168	250	26	1 490	105
F_2: after refractionation, 2.2–2.9 M $(NH_4)_2SO_4$	34	85	13	2 590	170
	17	47	5	2 790	200
Preparation after gel filtration on Sephadex G 200.	4.0 [b]	40	6	10 000	680
	2.0 [b]	65	7	33 000	2 350

[a] As calculated on the dry weight of the muscle tissue.
[b] As calculated on the absorbance at 280 mμ.

The values for V_e/V_t in the two runs of the cholinesterase preparations performed on Sephadex G 200 were 0.40 and 0.40, showing a good consistency. Pooled and frozen at −18°C the cholinesterase preparations kept their activity for several months, even if repeatedly thawed and refrozen.

Preliminary experiments with gel electrophoresis on polyacrylamid of the purest cholinesterase preparation showed that the substance contained at least 4 protein bands, of which the slowest moving one displayed cholinesterase activity.[20]

DISCUSSION

The effect of the *Cytophaga* strain used in this work to liberate cholinesterase activity from the plaice muscle might be due to a destruction of the muscle cell walls where the cholinesterase activity is localized.[25] Another *Cytophaga* strain, *Cytophaga johnsonii*, attacks yeast cell walls.[21] The cholinesterase liberating enzyme contained in the strain used for the present investigation is now being studied further.

A remarkable increase in cholinesterase activity has been noted both when simply extracting, after sonification, the tissues repeatedly with water (unpublished results) and after the *Cytophaga* treatment. To the author's knowledge a corresponding phenomenon does not appear to have been described as occurring when preparing cholinesterase from other sources. However, studying the permeability of different substances into electroplaxes from electric organs and into squid axons, Rosenberg and Dettbarn[22] showed that treatment of the cells with certain snake venoms led to an increased ability of the cells to split acetylcholine or acetyl-β-methylcholine. Further Karlin[23] showed that the acetylcholinesterase activity of gradient centrifuged homogenates of electric organ, which was associated with a membrane containing fraction, could be activated with sodium deoxycholate. Using a magnetic diver technique, Brzin *et al.*[24] could measure the cholinesterase activity of single pieces of squid axon membranes and showed it to increase after sonic treatment. They ascribed this effect to the existence of permeability hindering membranes, which should exclude the substrate from some sites of enzymic activity, and which were broken down by the sonic treatment. Corresponding phenomena could explain the increase in enzymic activity observed in the present investigation.

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