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A Bacterial Factor Capable of Solubilizing Cholinesterase from Plaice Body Muscle

S. J. LUNDIN

Research Institute of National Defence, Dept. 1, Sundbyberg 4, Sweden

1. A factor capable of solubilizing a cholinesterase from body muscles of plaice (*Pleuronectes platessa*) is produced by a *Cytophaga* strain of bacteria. This factor has been purified 300 fold from the spent culture by ammonium sulphate precipitation, extraction, and separation on Sephadex G 75.

2. The activity of the solubilizing factor was tested by measuring the amount of cholinesterase liberated from plaice muscle under standard conditions.

3. The solubilizing factor was found to be heat sensitive and dialyzable and it could be used repeatedly without losing activity. This indicates that the factor is an enzyme.

4. Preparations of collagenase and phospholipase A produced a moderate cholinesterase solubilization when tested under the same conditions as the investigated Cytophaga factor. The latter, however, seems not to contain significant collagenase or phospholipase A activity.

It has been shown [1] that a bacterial strain, *Cytophaga sp.*,* growing in cultures of homogenized or minced plaice (*Pleuronectes platessa*) body muscles in water, solubilized a structure bound [2] cholinesterase [3] from the muscle tissues in a way allowing further purification of the cholinesterase.[4] It was found that the solubilizing activity could be exterted not only by the bacteria when growing on plaice muscle [1] but also by certain spent culture media.[5] The optimal conditions for producing the solubilizing activity in high yield by continuous cultivation have been investigated.[6] The present work was initiated in order to evaluate an assay method, to purify the active factor(s), and to elucidate its properties.

* Preliminarily classified by Dr. T. G. Mitchell, Director of the National Collection of Industrial Bacteria, Ministry of Technology, Torry Research Station, Aberdeen, Scotland. The *Cytophaga* strain is placed in the National Collection of Marine Bacteria, Torry Research Station, Aberdeen, under the accession number NCMB 1314.

MATERIALS AND METHODS

Source of cholinesterase solubilizing factor. The bacteria *Cytophaga sp.*, were cultivated in a continuous system by the method of Bovallius.[6] When the bacteria had to be removed the culture fluid was centrifuged for 30 min at 18 000 *g* and the supernatant used. 10 l of the whole culture were used for each purification experiment.

Muscle tissues. Body muscles from plaice and cod, *Gadus callarias*, were obtained from commercially available, skin free, deep frozen filets (Winner Co-operative Society, Sweden). The filets were thawed in plastic bags under tap water and rapidly rinsed in an 0.1 % solution of Desivon (alkyldimethylbenzylammonium chloride, Astra, Sweden) to reduce the number of contaminating micro-organisms as much as possible. The effect of the Desivon on the plaice colinesterase activity has been investigated earlier [1] and been found negligible under the conditions used. The filets were then rinsed 10 times in ice cold saline to remove the disinfectant and were minced with a standard meat grinder. 10 g portions were weighed from a batch of pooled muscle and deep frozen at −18°C until used. The portions were used to test the cholinesterase solubilizing effect of different bacterial preparations. The cholinesterase activity varied between different batches as seen in Table 2. The dry weight (after 105°C overnight) of 10 g portions was 1.725 ± 0.001 g (S.D.) as calculated on five different batches.

Frog (*Rana temporaria*) muscles were taken from the limbs of the animal.

Chemicals. All solutions and, except where otherwise stated, muscle homogenates or suspensions were made up with distilled water. Sephadex G 75 and G 25 (40−120 μ, Pharmacia Fine Chemicals, Uppsala), respectively, were used for gel filtrations and desalting. The separations were carried out on an LKB Recychrome unit (LKB Products, Sundbyberg, Sweden). Ammonium sulphate (*p.a.*, from Kebo AB, Stockholm) was used for the fractionations. The following enzyme preparations and substances were used: Collagenase (E.C. 3.4.4.19), Type III, Sigma; phospholipase A * (E.C. 3.1.1.4): purified preparation from venom of *Naja nigricollis* [7] containing only one fraction and displaying only phospholipase A activity, 2 mg/3 ml buffer; phospholipase A from *Naja naja* (Koch-Light); phospholipase A * from *Apis mellifica* venom containing a lytic factor and a mast cell splitting enzyme, hydrolysing 10 mg lecithin per mg and minute as determined by Fredholm.[8] Preparations containing phosphatidyl choline (lecithin) * (N.B.C.) and phosphatidyl ethanolamine (cephalin) (Sigma) were used as substrates for the phospholipases. Collagen (from tendon) and a lysophosphatide preparation (lysolecithin), grade II, were purchased from Sigma.

Ammonium sulphate fractionation. The following procedure was used to purify the cholinesterase solubilizing factor. Ammonium sulphate was added with stirring to saturation to 10 liters of bacterial culture. The operation was performed in a cold room (4°C), where the preparation was left overnight. The bacteria and the precipitate (P_1) formed were collected by centrifugation at 10 000 *g* in a cold room using a Serwall SS-3 centrifuge. The precipitate was suspended in an equal amount of distilled water. The suspension was centrifuged in the same centrifuge at 10 000 *g*. The supernatant (S_2) was discarded and the remaining precipitate (P_2) was suspended in a volume of water equal to that of the precipitate. The new suspension was again centrifuged at 10 000 *g*. The procedure was repeated once more. The supernatants S_3 and S_4 were pooled (S) and were subjected to gel filtration.

Gel filtration. The size of the column containing Sephadex G 75 was 4×69 cm and the elution rate was 1.1−1.5 cm/h. The eluent was saline. The elution was followed by recording the absorbance at 254 nm. Preparation of the gel column was performed according to the directions given by the manufacturer.[9] The K_{av}-values were calculated by dividing the registered elution volumes with the volume of the gel bed, the void volume first having been subtracted from both of the values.[9] The active fractions were retained and deep frozen at −20° until used.

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* The gifts of the *N. nigricollis* and *A. mellifica* venoms from Dr. A. Wahlström, The Biochemical Department, University of Uppsala and Dr. B. Fredholm, The Department of Pharmacology, Karolinska Institutet, Stockholm, respectively, are gratefully acknowledged, as is the gift of lecithin from Dr. S. Friberg, The Laboratory for Surface Chemistry, Royal Swedish Academy of Engineering Sciences Research Center, Stockholm.

Determination of protein. Protein was determined by the biuret method as described by Zamenhof.[10] The absorbance at 280 nm was also measured spectrophotometrically in the gel filtration fractions.

Determination of cholinesterase activity. Cholinesterase activities were determined in duplicate with an electrometric method [11] at 25°C, in barbital-phosphate buffer [12] at pH 8.2. The substrate used was butyrylcholine iodide at a final concentration of 1.6×10^{-3} M.[4] The activities are expressed as μmoles of substrate split per ml reaction mixture per min. The total reaction mixture was 6.6 ml and contained 1.0 ml of the "S-test" suspension or supernatant (see also under Assay of solubilizing activity).

Determination of collagenase activity. Collagenase activity was determined according to Mandl *et al.*[13] on 25 mg collagen (from tendon) in 5 ml of 0.2 M phosphate buffer. Incubation at 30°C lasted for 18 h with 1 mg collagenase preparation or 0.5 ml solubilizing factor in phosphate buffer. The liberation of amino acids was followed with ninhydrin [14] and was expressed in equivalents of leucine.

Determination of phospholipase activity. Phospholipase A activity was tested with lecithin and cephalin according to the hydroxamic acid method of Magee *et al.*[15] as modified by Fredholm.[8] The incubation temperature was 30°C.

Phospholipase C activity was measured with lecithin by the Warburg method of Zamecnik *et al.*[16] as described by Hayaishi.[17] The incubation temperature was, however, 30°C.

Assay of solubilizing activity, "S-test". A method to assay the ability of a preparation of solubilizing factor, or of other enzymes tested, to solubilize cholinesterase activity from plaice body muscles was adopted, and is subsequently referred to as the "S-test" (S for solubilization).

10 g portions of the frozen, minced plaice muscle (see above) were thawed and suspended in 100 ml of distilled water in Erlenmeyer flasks (500 ml). The solution to be tested was added to the suspension. The pH of the suspension was about 6.7 throughout the test. The test suspensions were incubated without shaking in a water bath for 2 h at 30°C.[1] An aliquot of about 8 ml of the sample was centrifuged at 82 000 *g* for 15 min. Cholinesterase activity, and sometimes protein and amino acid concentration, were determined in the supernatant. A control sample with water was usually run as an indicator that normal experimental conditions prevailed.

Units of cholinesterase activity and of solubilizing activity. Spontaneous (control) and solubilized cholinesterase activities were measured electrometrically and the *cholinesterase activity unit* was expressed as 1 μmole of butyrylcholine split per minute at 25°C.

The *unit of solubilization activity* was expressed as the solubilization of 1 cholinesterase unit per minute from 10 g plaice muscle in 100 ml water for 120 min at 30°C. The specific activity is given as the number of solubilizing units per mg substance.

A spontaneous release of cholinesterase was observed in the absence of solubilizing factor (see Table 2). It cannot be taken for granted that the spontaneous release and the solubilization of cholinesterase are results of the same mechanism or that the cholinesterase containing products are identical. The spontaneously released cholinesterase activity in a control sample was thus not subtracted from the one solubilized in a simultaneously run test sample.

RESULTS AND DISCUSSION

Solubilization test method — (S-test). Centrifugation for 15 min at 82 000 *g* was adequate for obtaining the solubilized cholinesterase activity in the supernatant of a test suspension as shown by complete recovery of the activity.

The solubilization of cholinesterase activity was linear with the incubation time whether the solubilization factor was added as supernatant of cultivating medium or in a purified form (fraction from separation on Sephadex G 75). These results are demonstrated in Fig. 1.

The degree of solubilization varied with the amount of solubilization factor added (Fig. 2). When the activity of the solubilized cholinesterase in the super-

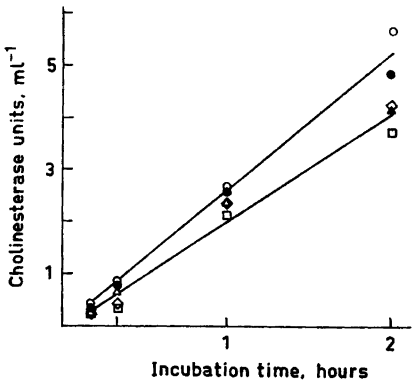

Fig. 1. Solubilizing activity per ml of spent medium (without bacteria) ○, ●, and of purified cholinesterase solubilizing factor (G 75 fractions) □, ◇, △ measured as the number of cholinesterase units solubilized in the supernatants of plaice muscle suspensions incubated for up to 2 h at 30°C with 1 ml preparation.

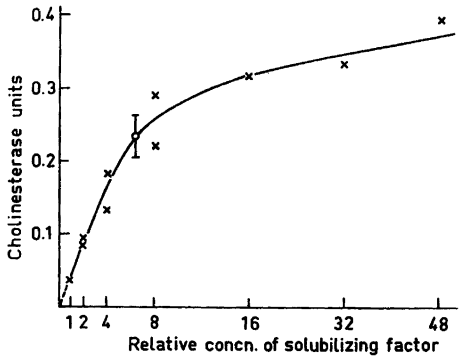

Fig. 2. Amount of cholinesterase activity solubilized per ml of supernatant from S-test suspensions incubated for 2 h at 30°C with increasing amounts of purified solubilizing factor. One point represents one S-test. The vertical bar marks the mean and standard deviation of 11 tests at the indicated concentration of solubilizing factor.

natant of an S-test reached values exceeding approximately 0.25 units ml^{-1}, most of the available cholinesterase activity was released before the end of the incubation time (Fig. 2).

If after an incubation time of 2 h the supernatant of an S-test suspension (containing both the initially added solubilization factor and solubilized cholinesterase) was added to a new portion of plaice muscle, a new and equal amount of cholinesterase activity was solubilized. This could be repeated (Table 1). The result shows that the solubilizing factor retained its activity without being inhibited.

Table 1. Effect of repeated use of cholinesterase solubilizing factor on minced plaice muscle. 5 g minced plaice muscle was incubated on each occasion for 2 h at 30°C in the same solution comprising initially 1 ml solubilizing factor in 49 ml water, and subsequently the supernatant of the previously incubated suspension.

	Number of cholinesterase units solubilized in 1 ml of S-test supernatant during 120 min		
	Control	S-tests	
1st incubation	0.02	0.21	0.21
2nd »	0.04	0.36	0.38
3rd »	0.14	0.72	0.72

Table 2. S-test results obtained with two different batches of plaice body muscle.

	Number of S-tests made on different batches	Number of cholinesterase units solubilized per ml of supernatant of test solution during 120 min		Cholinesterase activity in supernatant in % of activity in suspension
		Average	± S.D.	Average
Control suspensions	10 8	0.023 0.015	0.004 0.004	39
Test suspensions incubated with 1 ml of a preparation of solubilizing factor in saline	14 12	0.23 0.25	0.029 0.010	102

Incubation of the bacterial factor at 30°C for 20 h together with purified cholinesterase obtained by the solubilization process did not affect the cholinesterase activity.

The precision of the S-test is demonstrated in Fig. 2 and Table 2. The results show that the *solubilized cholinesterase activity* in supernatants of test-suspensions was about 10 times higher than in the controls and always was about 100 % of that measured in the suspension. The amount of solubilized cholinesterase activity did not differ significantly when different batches of plaice muscle were incubated with the same amount of a single preparation of solubilizing factor. The precision of the method, expressed as the standard deviation of the mean in per cent (the coefficient of variation) was ±13 % in 14 replicate determinations with one batch and ±4 % in 12 replicate determinations with another batch (Table 2).

Spontaneously appearing cholinesterase activity in control supernatants could sometimes vary when different plaice muscle batches were used. The cholinesterase activity in the supernatants of the control suspensions was appreciably less than 100 % of that in the suspension and usually around 50 %.

On the basis of the results obtained, it was feasible to use the total amount of cholinesterase activity in the supernatants of the S-test suspension after centrifugation for 15 min at 82 000 *g* as a measure of cholinesterase solubilizing activity.

Purification of the cholinesterase solubilizing factor. Three experiments were performed for the purpose of purifying the cholinesterase solubilizing factor from continuously cultivated cultures of *Cytophaga sp.* The results of one purification experiment are shown in Table 3.

The solubilizing activity contained in the bacterial mass of active cultures was found to amount to less than 0.5 % of that of the whole culture. Therefore it was considered unnecessary to remove the bacteria from the culture before adding ammonium sulphate to saturation (see Methods).

Table 3. Purification of solubilizing factor from 10 l of a continuous cultivation of *Cytophaga sp.*

Preparation	Amount of protein (mg)	Total solubilizing activity (units min^{-1})	Yield (%)	Specific solubilizing activity of preparation (units mg^{-1} min^{-1})	Purification (×)
Culture containing bacteria	17 000 [a]	920		0.054	
Pooled extracts (S)	69	156	17	2.3	43
Active fractions collected after separation on Sephadex G 75	5.9	100	11	16.9	312

[a] Measured on supernatant of centrifuged culture.

As is evident from Fig. 3, the presence of bacteria did not significantly affect the solubilizing activity of the culture when assayed with small samples of culture. The increased activity observed when larger amounts were used, might have been due to the growth of bacteria.

Preliminary experiments had shown that stepwise fractionation with increasing ammonium sulphate concentrations was unsatisfactory because considerable precipitation of the bacterial factor occurred at all steps. It was found more satisfactory to precipitate the factor in saturated ammonium sulphate and to extract the resulting precipitate with small volumes of water. This procedure reduced the cholinesterase solubilizing activity substantially. However, the precipitation and extraction steps resulted in a good purification. None or very little solubilizing activity was left in the residue (P_3) after the final extraction.

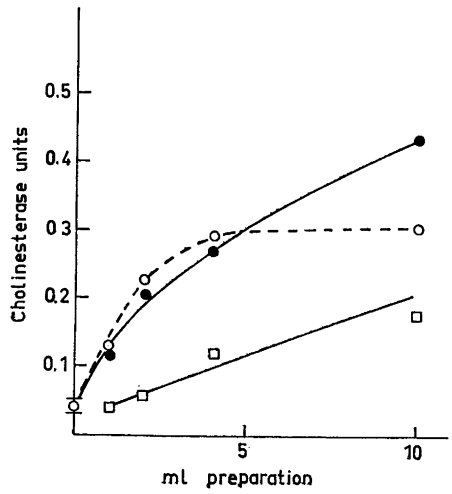

Fig. 3. The influence of the presence of bacteria (*Cytophaga sp.*) on the cholinesterase solubilizing activity of the cultivating medium. Culture = ●, medium freed from bacteria = ○, bacteria in saline in the same concentration as in the culture = □. The solubilizing activity is expressed as the number of cholinesterase units in 1 ml of S-test supernatant after 120 min of incubation at 30°C.

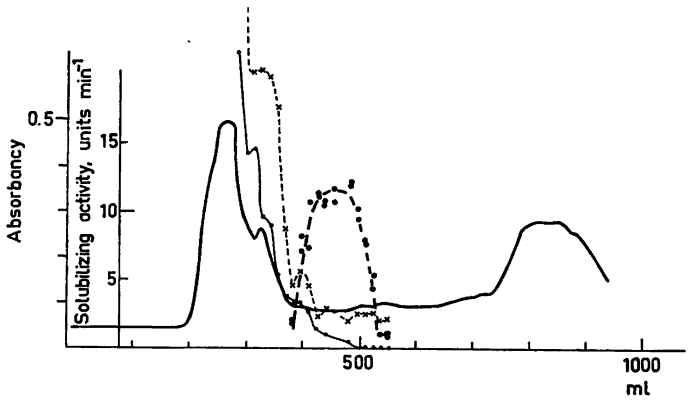

Fig. 4. Separation of Sephadex G 75 of solubilizing factor in extract of ammonium sulphate precipitate. The elution was observed at 254 nm by means of a recorder, ———. Protein concentrations, thin line, —·—, and absorption at 280 nm, ×, were determined in the collected fractions containing solubilizing factor by the methods described in the text. The solubilizing activity, thick hatched curve, ●, was determined as described in Methods.

When gel filtration on Sephadex G 75 was performed the solubilizing activity appeared immediately after the main peak containing the substances of high molecular weights and before the peak containing those of low molecular weights (Fig. 4). The K_{av}-values calculated for the active fractions in the three experiments were 0.35 (see Table 3 and Fig. 4), 0.38, and 0.35, indicating a molecular weight of roughly 20 000.[9]

Properties of the purified solubilizing factor. The solubilizing factor present in the spent medium as well as purified material were destroyed when heated in a boiling water bath for one minute. When frozen it kept its activity for many months and in a refrigerator the activity decreased slowly after several days.

Dialyzing the supernatant of a culture against water or saline for 24 h in a cold room in ordinary dialyzing bags caused no loss of solubilizing activity. When a purified preparation was dialyzed in the same manner, against water or saline, about 40 % of the activity was lost. No activity was found to have escaped into the dialyzing medium when 0.5 ml of purified factor was dialyzed overnight in 10 ml saline. The content inside the bag retained about 50 % of the initial activity. The results indicate that the purified solubilizing factor is deactivated by dialysis in bags. Preparations, which had been kept in the cold room for the same period of time, were used as controls. Desalting on Sephadex G 25 could be accomplished without loss of solubilizing activity.

No significant liberation of proteins or amino acids by the purified solubilizing factor during the solubilization test could be demonstrated as compared to the controls, which is exemplified in Table 4.

The cholinesterase solubilizing factor was tested also on body muscle from another species, cod, which contains butyrylcholine splitting cholin-

Table 4. Liberation of proteins and amino acids into the supernatant during 3 S-tests with purified solubilizing factor.

Solubilizing activity		Protein		Absorbance at 280 nm in TCA extracts		Amino acid equivalents liberated	
Control	Test	Control	Test	Control	Test	Control	Test
(units $ml^{-1}min^{-1}$)		(mg/ml)		(A)		(μmoles/5 ml)	
2.1	11.9	1.5	1.5	0.098	0.154	18	20
1.6	10.8	1.4	1.2	0.117	0.129	18	16
1.3	13.0	1.1	1.3	0.123	0.113	16	18

esterase. The solubilization was small but probably significant, the increase in the S-test supernatant being 1.5 times. Further, preliminary experiments inidicate that it was possible to obtain about twice the amount of cholinesterase in the supernatant when homogenized frog muscles were treated with the purified solubilizing factor.

Possible nature of cholinesterase solubilization. In order to cast some light on the manner in which the cholinesterase was solubilized by the solubilizing factor, several different enzymes were investigated by means of the S-test with respect to their ability to solubilize the plaice cholinesterase. Usually, determinations were made of the increase of cholinesterase activity in the incubated homogenate or suspension that could be ascribed to a solubilizing activity. The amount of protein liberated during the enzymic incubation was measured. The increase in absorbance at 280 nm after precipitation of proteins from the supernatant with TCA was taken as a measure of the amount of amino acids that had been liberated. Positive solubilizing effects of an enzyme led to the performance of experiments aimed at testing the bacterial factor on a substrate for the enzyme in question.

In the screening of selected enzymes for ability to solubilize plaice cholinesterase, only a few enzyme preparations were found to have any effect:

Table 5. Increase in supernatant of cholinesterase activity solubilized by phospholipase A preparations in plaice muscle suspensions. pH = 6.7 – 7.0, incubation for 2 h at 30°C.

Increase of cholinesterase activity as compared with control	Source of phospholipase A			
	Naja nigricollis	*Naja naja*	*Naja sp.*	*Apis mellifica*
Solubilized activity / Control activity	1.7 [a]	2.4	3.8	2.4
	2.2	2.7	2.9	1.9

[a] Measured in suspension.

collagenase preparations from *Clostridium histolyticum* and phospholipase A preparations from various sources.

Collagenase was tested because of the abundant occurrence of collagen threads between the muscle cells,[2] which might possibly prevent solubilization of the sarcolemma bound cholinesterase.[2] The experiments performed showed that a solubilization was obtained by the enzyme preparation used, as the cholinesterase activity was found to increase 2.7 times in the supernatant as compared to that of the control. However, the specific activity was only 0.03 solubilizing units mg^{-1} min^{-1} compared to about 10 units mg^{-1} min^{-1} of purified solubilizing factor, and the latter displayed no splitting activity on collagen from tendon (or plaice skin).

Current theories assume cell membranes to be composed of layers of phospholipid in a central layer with outer layers of proteins on both sides. For a more detailed discussion, see *e.g.* Kavanau.[18] Several other authors also have used phospholipases to solubilize or liberate substances from various cell membranes.[19,20] Electron microscopic observations had shown that the solubilizing factor removes the sarcolemma localized plaice cholinesterase.[2] It thus seemed worthwhile to test the solubilizing effect of various phospholipases.

Various preparations of phospholipase A from snake and bee venoms showed a small but significant cholinesterase solubilizing activity as investigated by means of the S-test. With respect to specific activity, one preparation from *Naja nigricollis* was even found to compare with crude spent cultivation medium. (The venom preparations had been tested for their cholinesterase activity. No such activity was found in them.)

The activity of the purified solubilizing factor in splitting lecithin and cephalin was tested by the method described by Fredholm.[8] No activity was found. This result was confirmed by Fredholm (personal communication [21]) concerning the action of the solubilizing factor on lecithin.

The following enzymes and enzyme preparations have been tested and found to be without solubilizing effect on the plaice cholinesterase: neuraminidase, hyaluronidase, lysozyme, elastase, pronase, trypsin, pancreatine.

The results obtained concerning the influence of various conditions on the activity of purified solubilizing factor may be summarized as follows: The solubilizing factor is heat-sensitive; gel filtration experiments indicate a high molecular weight; when dialyzed, it is retained within the bag, but is also deactivated under certain conditions. These facts suggest that the factor is a protein. It is not consumed when exerting its action and is thus acting as a catalyst, or enzyme. An easily identifiable substance, plaice cholinesterase, appears as a product of the solubilizing action of the factor, indicating the existence of a definite substrate. The cholinesterase moiety may be chemically bound to such a substrate or it may be wrapped in a subcellular structure, which is broken down by the solubilizing factor. In conclusion, it seems justified to characterize the cholinesterase solubilizing factor as an enzyme.[22]

In order to elucidate fully the action of the solubilizing factor, further work regarding substrate specificity, inhibition, pH, and temperature dependence must be carried out.

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