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The Solubilization of a Cholinesterase from Plaice Muscle by Bacteria

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A bacterial strain, *Cytophaga* sp., incubated for 24 h at 30°C on a water suspension of plaice (*Pleuronectes platessa*) muscle, liberates into the supernatant about 100 % of the butyrylcholine splitting activity present in a muscle homogenate. The treatment allows a further purification of this cholinesterase from the muscle.

Autolysis has been used to solubilize a structurally bound cholinesterase from the body muscles of plaice (*Pleuronectes platessa*) and a procedure to purify the solubilized enzyme was briefly described.[1] Further attempts to apply the autolysis technique for large scale preparations were, however, complicated as the solubilization step sometimes failed. As the process had been carried out in the absence of bacteriostatic agents it was decided to investigate its dependence of bacterial interaction. The present work describes the isolation of a bacterial strain that is capable of solubilizing the aforementioned cholinesterase.

MATERIALS AND METHODS

The muscles used were commercially available, deep-frozen filets of plaice (*Pleuronectes platessa*, Winner, Co-operative Society, Sweden). Only skin free filets from the dorsal side were used. After thawing, the whole filets were washed in an 0.1 % solution of Desivon (alkyldimethyl benzylammonium chloride, Astra, Sweden) for 1 min and then rapidly rinsed 10 times in ice cold, sterile saline. This procedure was found suitable to reduce considerably the initial bacterial flora existing on the material.

The filets were cut in small pieces to a size of about half a cubic cm, with a pair of sterile scissors. 10 ml of distilled (and, in the irradiation experiments, autoclaved) water were added per gram. The muscle suspension was inoculated to contain 10^3-10^6 of the bacteria to be tested. The cultivation was carried out in Erlenmeyer flasks covered with sterile aluminium foil and placed in a temperature controlled water bath.

The muscles to be irradiated were cut as above and 10 g portions of the sliced tissue were placed in plastic bags. These were hermetically sealed by gentle heating. The bags were placed directly under the window of the irradiation tube but on an ice cooled support.

The five different bacterial strains used were cultivated in Oxoid Nutrient Broth at +30°C for two days, centrifuged and resuspended in saline before use. The yellow

strain (*Cytophaga* sp.), which was used in this work, was continually recultivated and tested for purity.* The saline suspensions were useable after 14 days storage in the refrigerator. The bacterial growth in the experiments was followed by conventional colony counting technique, viable count, according to Meynell and Meynell.[2] No attempts were made to follow the growth of bacteria adhering to the surfaces of the muscle pieces. Colonies with a yellow colour like that of *Cytophaga* sp. were counted separately in some cases (see Fig. 2).

The cholinesterase activity was followed by an electrometric method [3] at pH 8.2 using butyrylcholine iodide as a substrate [1] at a final concentration of 1.6×10^{-3} M, pS = 2.8. The activity was measured either in the muscle suspension medium, after freeing it from larger tissue particles, or in the suspension, after homogenizing it with an Ultra-Turrax for about 10 sec, and is expressed in relative units. All enzyme activity determinations were performed in duplicate on 1 ml aliquots.

The degree of solubilization was judged by determining the amount of cholinesterase activity that remained in the supernatant after centrifuging the experiment cultures at 82 000 *g* for 30 min in a Spinco L Centrifuge. For calculating the yield of enzymatic activity in the supernatant the relative units were used. The specific activity was expressed as μmoles substrate split by 1 g of protein in 1 h (μmoles $g^{-1}h^{-1}$).

The protein concentration was determined by a biuret method [4] and was expressed as mg protein per ml solution.

The irradiation was performed with an X-ray apparatus Müller MG 300, 260 kV, 10 mA. No filters were used. The self filtering effect of the X-ray tube was equivalent to 4 mm Al. 2200 R/min were given for 2 h and 16 min totalling 300 000 R as measured just below the tube opening with a Philip's universal dose-meter and measuring chamber 37489/10 No. 1924.

The tests were principally performed in the following way: The culture flask was gently whirled and the coarser pieces of tissue were allowed to settle. From the upper parts of the suspension were taken aliquots; first about 1 ml in sterile tubes for viable count of the bacteria and then 5—10 ml for determinations of enzyme activity and protein concentrations. The test volumes were replaced by sterile water. When homogenizing occurred the whole culture was used. The pH of the larger aliquot was always adjusted to 8.2 with 0.1 M NaOH immediately after sampling. Part of the sample was centrifuged as described above and the protein concentration was determined in the supernatant. Enzyme activity was determined in the suspension (homogenate) and the supernatant.

RESULTS

Differentiation of strain, liberating cholinesterase. To investigate the possible bacterial contribution in the cholinesterase solubilization step, the first aim was to correlate the solubilization effect to the presence of some particular bacterial strain of those found to grow during the autolysis. Thus five strains from a successful autolysis preparation were isolated on Oxoid Nutrient Agar. Each strain was inoculated on an aliquot of a suspension of pieces of cleansed plaice body muscle filets in water, and was cultivated for 47 h at 30°C. This resulted in widely different abilities of the culture media to split butyrylcholine (Fig. 1). The strain *Cytophaga* sp., whose presence was correlated with the highest liberation of cholinesterase activity in the medium, was chosen to be investigated further. The growth of yellow bacteria in the corresponding culture as compared to that of the control culture is shown in Fig. 2 which

* The strain was classified by Dr. T. G. Mitchell at the National Collection of Industrial Bacteria, Ministry of Technology, Torry Research Station, Aberdeen, Scotland. His help is gratefully acknowledged.

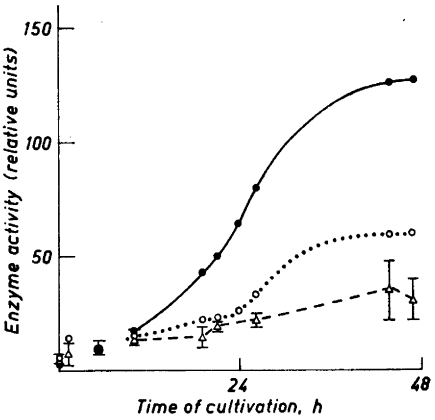

Fig. 1. The butyrylcholine splitting activity developed in five cultures consisting of plaice muscle suspensions in water each inoculated with one of five different, pure bacterial strains. For details, see text. Control culture, ○; *Cytophaga* sp., ●; mean and extreme values from the four other differently inoculated cultures, △.

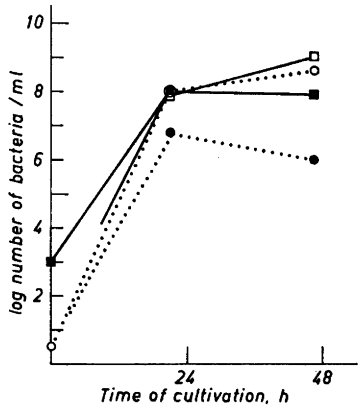

Fig. 2. The growth of inoculated *Cytophaga* sp. (and other yellow strains) during the experiment illustrated in Fig. 1. Control culture, ○ ●; *Cytophaga* sp., □ ■; filled symbols represent yellowish strains including the inoculated *Cytophaga* sp., unfilled symbols represent uncoloured strains. Only yellow bacteria were observed in the inoculated culture at start, therefore the line between start and 22 h is broken for the uncoloured strains.

displays the considerably larger amount of *Cytophaga* sp. in the inoculated culture.

It is also observed that the main accumulation of amino acids in the culture medium of *Cytophaga* sp. as measured as the extinction at 280 mμ in the supernatant after precipitating with trichloroacetic acid, does not start until the cholinesterase liberation is mainly finished (Fig. 3). The early rise

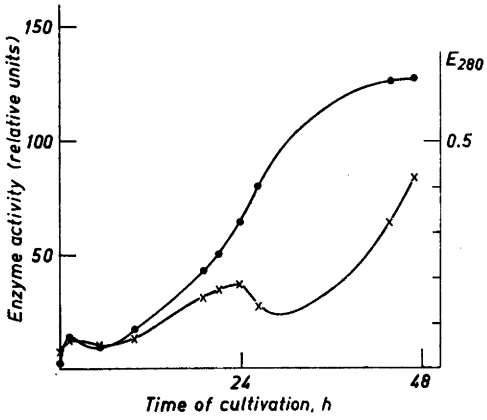

Fig. 3. The liberation of cholinesterase activity by *Cytophaga* sp., shown in Fig. 1 compared to the accumulation of amino acids in the culture medium in the same experiment. The enzyme activity is expressed as explained in Fig. 1. The amount of amino acids is expressed as the extinction, E, at 280 mμ, and is demonstrated by the hatched line.

of the extinction values may originate from an initial break down of more easily available proteins in the sarcoplasma and extracellular fluids.

Solubilization effect by Cytophaga *sp.* Increase in the specific activity and in the yield of the total enzymatic activity in the supernatant after 82 000 *g* occurred in the presence of *Cytohaga* sp., Fig. 4. The yield was determined as the per cent activity remaining in the supernatant of that initially found in the muscle homogenate. In the test culture the inoculated yellow bacteria (*Cytophaga*) increased from 10^6 to 10^8 per ml. No yellow bacteria were detected in the control culture although the number of other bacteria increased from 5×10^3 to 10^8 per ml. It is noticed in both of the typical experiments described above that butyrylcholine splitting activity was developed also in the control cultures. However, it reached considerably lower values and it developed later.

Temperature dependence. In Fig. 5 is illustrated the result of the experiment to investigate the temperature dependence for the enzyme liberating effect in the presence of *Cytophaga* sp. Again it is clear that the presence of these bacteria considerably accelerated the development of the enzymatic activity (Fig. 5) as compared to what happened in the control cultures. This temperature dependence is, however, noticeable also in the control cultures and at 35°C the ability to split butyrylcholine is developed parallel and to the same degree in both test and control culture. An important difference still exists though, between the processes at this temperature, since a lower specific activity is achieved in the supernatant of the control than in that of

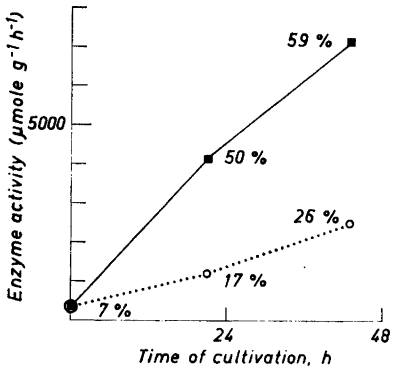

Fig. 4. Specific activity and yield of butyrylcholine splitting activity solubilized during cultivation into the supernatant of a plaice muscle homogenate in water inoculated with *Cytophaga* sp. The yields, as determined in per cent of the initial enzyme activity of the respective homogenates, are given at each point. Control culture, O; *Cytophaga* sp. ■.

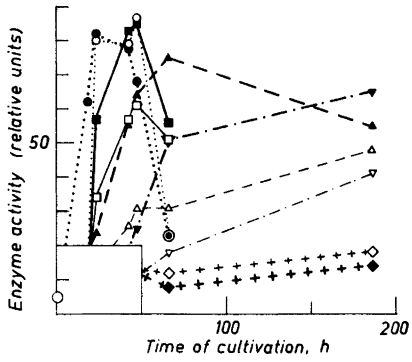

Fig. 5. The temperature dependence for the development of butyrylcholine splitting activity in plaice muscle water suspensions after inoculating with *Cytophaga* sp. and in a control culture. 35°C, O ●, 30°C, □ ■; 20°C, △ ▲; 10°C, ▽ ▼; 5°C, ◇ ◆. Filled symbols connected by coarse lines represent inoculated cultures, unfilled symbols connected by fine lines represent controls. For the sake of clarity the values obtained are not reproduced in the framed area. Also all initial values are only represented by the open circle on the *y*-axis.

Table 1. Results from two experiments combining treatments with X-ray irradiation of plaice muscle and inoculation with the yellow bacteria *Cytophaga* sp. on water suspensions of the muscle tissues. The figures given are the means of the results obtained in the two experiments, which lasted 22.5 h and were performed at 30°C.

	Irradiated material			Unirradiated material		
	control	inoculated	inoculated/ control	control	inoculated	inoculated/ control
Enzyme activity (μmole $g^{-1}h^{-1}$) in homogenates and supernatants of control suspensions before incubation.	105 300*			141 470*		
Yellow bacteria including inoculated *Cytophaga* sp., number/ml:						
at start of cultivation	none observed	7×10^6		none observed	6×10^6	
after cultivation for 22.5 h	»	490×10^6		»	320×10^6	
Other bacteria, number/ml:						
at start of cultivation	10	none observed		3×10^2	none observed	
after cultivation for 22.5 h	3×10^2	»		10^8	60×10^6	
Enzyme activity in the (supernatant medium of the) culture, relative units.	7	39	5.6	10	46	4.6
Specific activity in the supernatant (after 82 000 *g*) of the suspension, μmoles $g^{-1}h^{-1}$	1100**	4600	4.2	1500	5100	3.4
Yield of enzyme activity in supernatant, in per cent.	67**	102		109	118	
Protein in supernatant, mg/ml.	1.0	1.4		1.0	1.3	

* Determined only in one of the experiments.

** In one of the experiments no activity at all was obtained in the supernatant. The figures are those of the second experiment.

the test culture, being 120 and 2300 μmoles $g^{-1}h^{-1}$, respectively (see also Table 1).

Effect on irradiated muscle tissue. The possibility that *Cytophaga* sp. was entirely ineffective and only by coincidence had been correlated with the changes noted, and also that these rather were to be ascribed either to other bacteria strains or to quite other processes was tested in the following way. According to a personal communication from Dr. A. Molin [5] (see also Thornley *et al.*[6]) massive X-ray irradiation should result in a considerable killing off of the bacteria initially present in the material and delay the growth of the remaining ones for a sufficiently long time that the material might be considered very nearly sterile. Thus pieces of muscles were irradiated with 300 000 R as described under Materials and Methods. Control material from the same pool as the irradiated muscle was kept in a refrigerator during the irradiation. Both irradiated and unirradiated muscles in water suspensions were inoculated with *Cytophaga* sp. and then together with uninoculated control cultures cultivated at 30°C for 22.5 h. The results of two experiments are given in Table 1.

A minor loss of about 25 % of the enzyme activity may have occurred in the irradiated material, as measured in homogenized tissue samples before starting the cultivation. Comparable figures were obtained by Serlin and Cotzias [7] irradiating the electric organ from the electric eel by Cobalt-60 γ irradiation. The inoculated bacteria grew equally well in both irradiated and control material. In the latter, however, growing of the natural flora to about the same number of individuals per ml occurred. No such growth of other bacteria could be demonstrated in the inoculated, irradiated material. In the irradiated control material a very moderate and partly abnormal growth from less than 10 to about 300 bacteria/ml occurred during the 24 h of the experiment. This was considered a sufficiently small growth to make the irradiated material be regarded as devoid of interfering bacteria during the experiment, especially so, as compared with the normal growth occurring in the nonirradiated material.

After 24 h a mean of about five times higher butyrylcholine splitting activity was present in the medium of the cultures inoculated with *Cytophaga* sp., whether the muscle tissue was irradiated or not. The specific activity (μmoles butyrylcholine split $g^{-1}h^{-1}$) was about four times higher in the supernatant (after 82 000 *g*) from the inoculated cultures, where the protein concentration was also slightly higher. The yield in the supernatant was below 100 % only in the irradiated control.

In all experiments with *Cytophaga* sp., pH increased from about 6.5 to about 7.9 during incubation.

Cholinesterase activity in Cytophaga *sp.* Enzymes are easily induced in bacteria. Goldstein and Goldstein,[8] Goldstein,[9] and Fitch [10,11] have actually described and purified an enzyme which could be induced in a strain of *Pseudomonas fluorescens* by choline and choline esters and which they claimed to be a cholinesterase, although Fitch [12] later described the acetyltransferase activity of the enzyme and showed that it was not inhibited by organic phosphorus compounds. However, to find out if any butyrylcholine splitting activity could be induced in *Cytophaga* sp., the bacteria were cultivated both

on Oxoid Nutrient Broth and autoclaved plaice muscle suspensions. The butyrylcholine splitting activity was measured both in the bacteria themselves (about 10^8 per ml saline suspension), whole or treated with ultrasonics (20 kc for 5 min with a Branson Sonifier S 110), and in the growth media. Virtually no such activity could be demonstrated, showing that the enzyme activity was not induceable in the bacteria and thus was not derived from them in the cultivated plaice muscle suspensions.

DISCUSSION

Plaice muscle cholinesterase. The occurrence of a special butyrylcholine splitting cholinesterase in plaice muscle was demonstrated by Lundin.[13,1] In this work we have shown that a soluble component carrying butyrylcholine splitting activity appears in the supernatant of plaice muscle suspensions inoculated with *Cytophaga* sp. Although we ascribe this solubilization to bacterial enzymes we do not exclude a possible synergistic effect of truly autolytic enzymes in the fish muscle tissues (*cf.*, *e.g.*, Shewan *et al.*[14]) or of such endogenous, protein solubilizing, muscle enzymes as were described by McCollester *et al.*[15]

The obvious increase (up to a certain time of cultivation of the muscle homogenate) in total enzyme activity described already in a previous paper,[1] was observed also during this work. This observation can be explained if the breakdown of the cell structure by the bacterial enzymes and the accompanying liberating of the cholinesterase exhibits new active sites, which are not available in a homogenate of fresh tissue.

The further purification and the properties of the cholinesterase described in this work will be published later.

Influence of bacterial flora on enzyme solubilization. Discussing the action of bacteria on fish (and other food) after death and during storage one is confronted with the problem of fish spoiling.[16] Spoiling is defined as those changes taking place after the capture of fish making it unsuited as food. Some of these changes are ascribed to so called spoiler strains especially among the genera *Achromobacter* and *Pseudomonas*.[17–19] There are several tests, both objective analyses and judging by taste panels, to determine the degree of spoiling.[14] However, such analytical tests as determination of amino acids seem to measure the effect of rather advanced breakdown processes on proteins. Also methods measuring a decline of enzymatic activity in the flesh may indicate a rather late breakdown process.[20] The liberation of a structurally bound enzyme on the other hand might come comparatively early in the chain of processes breaking down the tissues. We have obtained preliminary results showing the enzyme liberation to start earlier than the main accumulation of amino acids in the cultures inoculated with *Cytophaga* sp. (see Fig. 3). To our knowledge it has not been proposed earlier to use the liberation of some structurally bound enzyme as a test to measure the rate of spoiling in fish. The cholinesterase studied by us may not be the best one for this purpose; the problem may be worthwhile to be studied further.

Different bacterial strains show variations in their occurrence on fish at different times of the year.[21] Also rapid changes occur among the spoiler

strains during storage after catching the fish.[18,19] Together with the fact that at least one of the strains can solubilize the cholinesterase activity investigated, these differences may well explain the earlier shortcomings initiating this work.

Note added in proof: Since our paper was submitted for publication it has become known to us that Gould [22] 1965 proposed to use the changes in malic enzyme activity as a quality test for fish and also has studied nearer the possibilities therefor.[23]

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