Cholinesterases from the common oyster (*Crassostrea gigas*)

Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors

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Abstract Marine bivalves such as oysters and mussels are widely used as bioindicators of contamination in the monitoring of pollutant effects. As filter feeders, these species are known to be good general indicators of chemical contamination. However, the efficient use of decreased acetylcholinesterase activity in the oyster as a biomarker of exposure to neurotoxic compounds requires a definition of the different types of cholinesterases coexisting in this mollusk. This study reports the partial purification, separation and characterization of two cholinesterases extracted from the oyster *Crassostrea gigas*. Differences in apparent molecular weight, type of glycosylation and hydrophobicity, and sensitivity to inhibitors suggest that they are encoded by two different genes. 'A' cholinesterase (apparent molecular weight 200 kDa) is anchored to the membrane via a glycolipid, is not glycosylated but sensitive to organophosphate and carbamate inhibitors. 'B' cholinesterase (molecular weight 330 kDa) is hydrophilic, glycosylated and highly resistant to organophosphate and carbamate inhibitors. The kinetic properties of these two cholinesterases were compared with those of other invertebrate cholinesterases. The presence of a cholinesterase insensitive to insecticides suggests that a significant improvement in the use of oyster cholinesterases as biomarkers of pollutant effects could be achieved by simple separation of the two forms.

Key words: Cholinesterase; Acetylcholinesterase; Pollution monitoring; Insecticide; Oyster

1. Introduction

Anticholinesterase agents such as organophosphate and carbamate insecticides are widely used to control insect pests. After spraying, these toxic compounds enter rivers and ultimately reach the sea. The recent development of biomarkers based on the study of biological responses of organisms to pollutants has provided the biochemical tools essential to the implementation of programs for monitoring of contaminants effects. Cholinesterase inhibition has been used for years as a marker of exposure to insecticides, not only in humans but also in wild life, in order to monitor the effects of contaminants on living organisms. In the marine environment, the choice of a monitoring target species has mainly concerned bivalve mollusks such as the common mussel or the oyster, essentially because of their capacities to accumulate contaminants. However, mollusks, unlike vertebrate species, have proved relatively insensitive to inhibitors [1].

Cholinesterases are highly polymorphic enzymes in most species. The number of genes encoding cholinesterases varies according to species: one in insects, two in vertebrates and three in nematodes. The products of the different genes may be characterized by their catalytic properties. In vertebrate species, the use of acetylcholine and butyrylcholine allows the characterization of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8). For each protein, various molecular forms originate either from alternative gene splicing, oligomerization, association with structural proteins or degradation. Cholinesterases may be soluble or be linked to the basal lamina via a collagen tail or to the membrane via a glycolipid or a hydrophobic peptide [2].

Cholinesterases hydrolyze a large variety of substrates, including choline esters and organophosphate insecticides. The chemical steps consist in a nucleophilic attack on the substrate, generating an acyl-enzyme or a phosphoryl-enzyme intermediate, and its subsequent deacylation or dephosphorylation. Since dephosphorylation is very slow, organophosphates may be considered as irreversible inhibitors. Individuals intoxicated by these compounds show partial cholinesterase inhibition. Cholinesterases are present in the synaptic cleft where they catalyze the hydrolysis of the neurotransmitter, acetylcholine. In this situation, inhibition of the enzyme is lethal. However, the enzyme is also present in other regions where its inhibition does not affect viability [2].

Preliminary studies of cholinesterases from the gills of the common oyster showed that inhibition curves did not follow pseudo-first order kinetic but were biphasic, suggesting the presence of more than one cholinesterase in this species (Fig. 1). To investigate this apparent heterogeneity in the present study, cholinesterases of the common oyster were purified by affinity chromatography, separated by Triton X-114 partitioning and binding to lectin and then characterized. Their specific kinetic behavior with different substrates and inhibitors was determined as well as their distributions in different tissues.

2. Materials and methods

2.1. Animals

Oysters were obtained live directly from a cultivator in Fromentine (Atlantic coast of France). Animals were frozen for 24 h at −20°C to facilitate tissue sampling. Preliminary studies [3] showed that the highest specific AChE activity in the oyster is found in the gills (5000 g of oyster tissues are needed to obtain 300 g of gills).

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2.2. Extraction of cholinesterases from the oyster

Extraction was performed using 20 mM Tris-HCl, pH 7, plus 0.1% Triton X-100. Organs were homogenized 1/1 (w/v) for 1 min using an Ultra Turrax. Extracts were then centrifuged at 10 000×*g* for 30 min, and this operation was repeated with resuspension of the pellet until no cholinesterase activity was detected in the supernatant. The extracts were then concentrated by precipitation with ammonium sulfate which was added to the pooled supernatants from the gills to 70% saturation (0.4 g/ml) and mixed for 4 h. The precipitate obtained after centrifugation at 10 000×*g* for 30 min was dissolved in 20 mM Tris-HCl, pH 7, containing 0.1% Triton X-100 and dialysed overnight against the same buffer.

2.3. Chromatography

Affinity chromatography was performed on procainamide, a ligand specific for the choline binding site. The dialysed extract was directly applied to an affinity column. Procainamide-containing gel was prepared using ECH-Sepharose 4 B (from Pharmacia) as the coupling gel and EEDQ (*N*-ethoxycarbonyl-2-ethoxy 1,2-dihydroquinoline) from Sigma for ligand immobilization. The loading buffer was 20 mM Tris-HCl, pH 7, plus 0.1% Triton X-100 and the elution buffer was 20 mM Tris-HCl, pH 9.5, plus 0.1% Triton X-100 containing 1 M NaCl and 1 mM procainamide. Fractions exhibiting cholinesterase activity were pooled, dialysed against 20 mM phosphate buffer pH 7 and stored at 4°C before use.

For chromatography on concanavalin-A Sepharose, the sample was loaded on the column in 20 mM Tris-HCl, pH 7, plus 0.1% Triton X-100, 1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Following washing with the same buffer, the bound proteins were eluted in 20 mM phosphate buffer pH 7 containing 10% methylmannose and 0.1% Triton X-100.

2.4. Centrifugation

Sedimentation analyses were performed in 5–20% sucrose gradients in low salt buffer in the presence of 0.5% Triton X-100 (LST gradients) or in the absence of detergent (LS gradients). 300 μl of supernatant was loaded on each gradient and centrifuged at 36 000 rpm for 18 h at 4°C in a SW41 rotor (200 000×*g*). Alkaline phosphatase from calf intestine ($s_{20,W}$ = 6.1) and β-galactosidase from *E. coli* ($s_{20,W}$ = 6.1) were used as internal markers of migration. Forty fractions were collected from the bottom of each gradient and assayed for AChE activity according to Ellman et al. [4] with acetylthiocholine concentration of 1 mM in the presence or absence of 1 mM paraoxon. LST fractions displaying the highest AChE activity were recovered and used for non-denaturing electrophoresis.

2.5. Non-denaturing electrophoresis

Electrophoresis experiments were performed under non-denaturing conditions either in 7% gels containing 0.5% Triton X-100 or in 2–23% acrylamide gradient gel. Gradient gels contained either 0.25% sodium deoxycholate or 0.1% Triton X-100. Deoxycholate and Triton X-100 are both non-denaturing detergents which bind to the hydrophobic domain of proteins. In gradient gels, deoxycholate, which is negatively charged, allows migration of proteins toward the anode until they reach an acrylamide mesh corresponding to their sizes. In presence of Triton X-100, amphiphilic proteins remain at the top of the gel. Cholinesterase bands were stained according to the procedure of Karnovsky and Roots [5]. In gradient gels, molecular weights were estimated by comparison with the migration of high molecular weight standard proteins for non-denaturing electrophoresis gels (Pharmacia).

2.6. Triton X-114 partitioning and phosphatidyl inositol-phospholipase C (PI-PLC) treatment

The non-ionic detergent Triton X-114 was used to separate amphiphilic and hydrophilic cholinesterases as previously described [6]. Phospholipase digestion of the amphiphilic form of cholinesterase by PI-PLC (5 U/ml) from *Bacillus cereus* (Boehringer Mannheim) was performed at 37°C in 20 mM phosphate buffer, pH 7, for 1 h. The conversion of the amphiphilic into a soluble cholinesterase was assessed and controlled by phase partition in precondensed Triton X-114 and by migration in a non-denaturing electrophoresis gel.

2.7. Activity, substrate and inhibitor specificities

Cholinesterase activity was routinely monitored using acetylthiocholine at 1 mM as substrate in 20 mM phosphate buffer, pH 7. One unit corresponds to the hydrolysis of 1 nmol of substrate per minute. Bradford's method [7] was used for quantitative determination of proteins with bovine serum albumin as standard. Cholinesterase activities are expressed as units and specific activities are given as units/mg protein. The hydrolysis of three different substrates [acetylthiocholine (AcSCh), propionylthiocholine (PrSCh) and butyrylthiocholine (BuSCh)] was followed for each cholinesterase with concentrations of substrate ranging from 5 μM to 10 mM at 25°C. The Michaelis constant, K_m, was determined by analysis of the Lineweaver-Burk transformation.

We investigated the sensitivity of each cholinesterase to inhibition by three organophosphates [paraoxon, DFP (diisopropylfluorophosphate), iso-OMPA (tetraisopropyl pyrophosphoramide) a specific inhibitor of human butyrylcholinesterase] and three carbamates (eserine, carbaryl and carbofuran). Stock solutions of inhibitors were prepared in ethanol. The bimolecular reaction constant, k_i, which expresses the blocking power of the inhibitor, was calculated by the dilution method of Aldridge [8]. Samples were first incubated with the inhibitor for various times and then introduced into the Ellman medium (1 mM ASCh) to follow the decrease of residual activity versus time. In these conditions, plotting residual enzyme activity A/A_o against inhibitor concentration or time makes it possible to calculate the k_i (ln $A/A_o = k_i[I]t$).

2.8. Distribution of the two cholinesterases in oyster tissues

As the two cholinesterases differed in their reactivity to paraoxon, inhibition with this molecule was used to assess the natural distribution of the distinct cholinesterases in the main organs of the oyster.

3. Results

Preliminary studies of cholinesterases from the gills of the common oyster showed that inhibition was biphasic, suggesting the presence of two cholinesterases in this species (Fig. 1). The sensitive one was named 'A' and the resistant one was named 'B'.

3.1. Extraction and purification

Total cholinesterase activity was extracted using 20 mM Tris-HCl, pH 7, 0.1% Triton X-100. A high ionic strength buffer did not release additional activity. The amount of cholinesterase activity was measured in different tissues of the

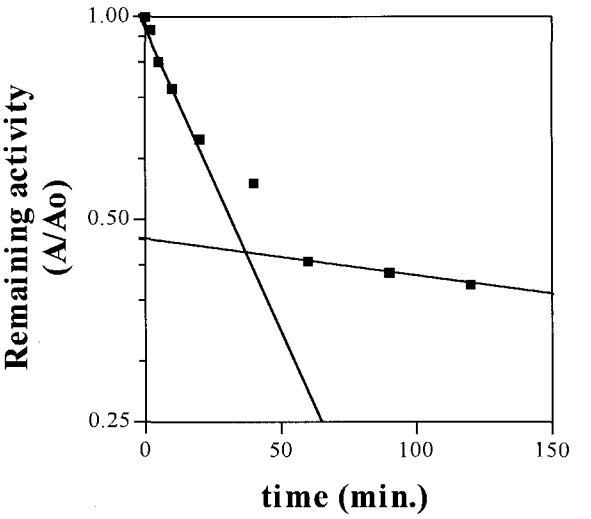

Fig. 1. Residual cholinesterase activity in the gills of the common oyster in the presence of 10 μM DFP as a function of time. (residual activity is expressed as A/A_o, A_o being the original activity at time = 0.)

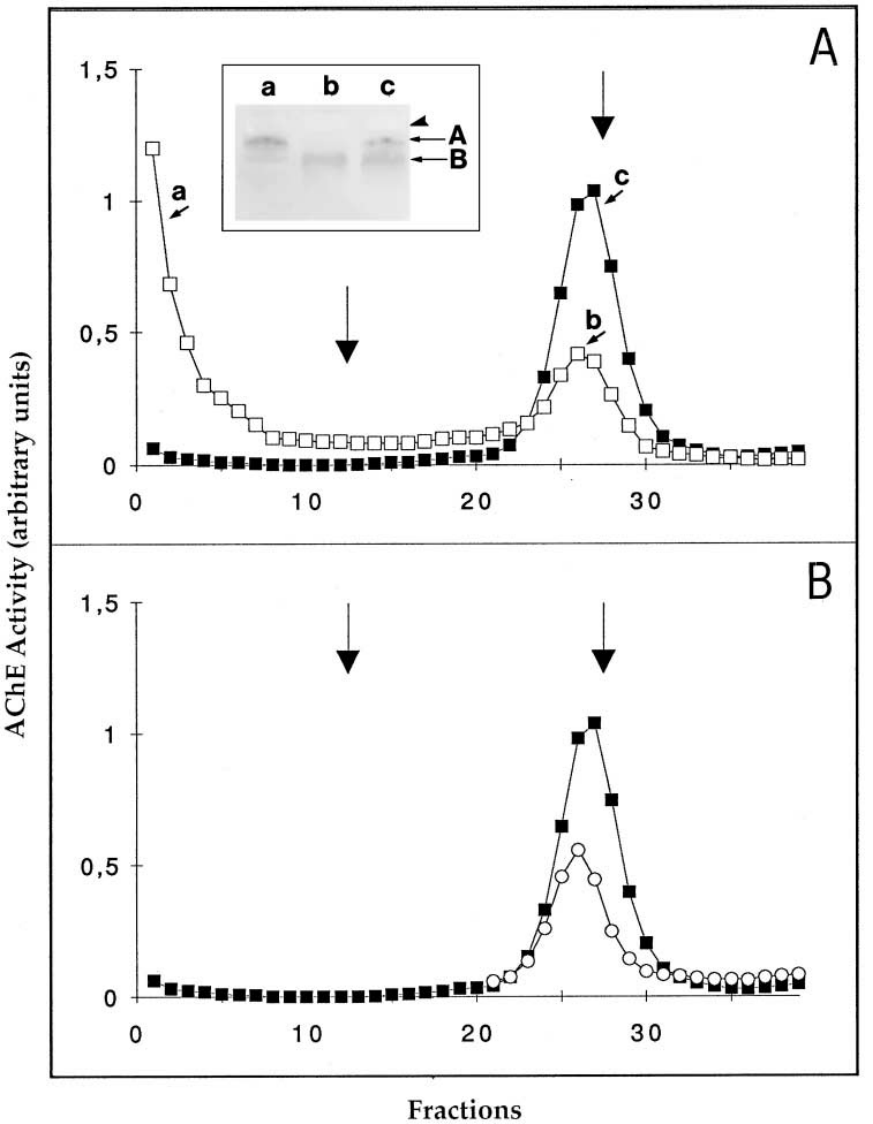

Fig. 2. Sedimentation analysis of AChE activity from gills. A: LST extract analyzed on LS (□) and LST (■) gradients. The single peak at 6.5S in the presence of Triton X-100 in the gradient (c) was converted, in the absence of detergent, into fast-sedimenting aggregates (a, amphiphilic component) and a hydrophilic peak (b). Non-denaturing analysis of samples a, b and c is shown in the insert. The arrowhead indicates the origin of migration. 'A' and 'B' are two AChEs as defined in text. B: LST extract sedimented on a LST gradient as in A but assayed for AChE either in absence of inhibitor (■) or in the presence of 1 mM paraoxon (○). A part of the peak is resistant to this concentration of inhibitor. This component had a sedimentation coefficient slightly higher than the original peak. It corresponds to the hydrophilic component b shown in A. Vertical arrows indicate the position of internal markers of sedimentation: alkaline phosphatase (6.1S, at right) and β-galactosidase (16S, at left).

common oyster (Table 1). Gills, the mantle and the hepatopancreas exhibited the highest cholinesterase specific activity using ASCh as substrate, whereas activities measured in two parts of the adductor muscle (smooth and striated muscle) were unexpectedly low. High concentrations of sulfhydryl groups made a pre-dialysis necessary before cholinesterase activity could be measured in the adductor muscle.

When applied onto a procainamide affinity column, the

Table 1
Specific AChE activity and A/B ratio in the main tissues of the common oyster

Tissue	Total AChE activity (A+B)	A/B ratio
Gills	28.1 ± 2.8	4.7
Hepatopancreas	25.5 ± 3.6	2.8
Mantle	24.3 ± 2.5	0.2
Labial palps	13.0 ± 1.1	0.2
Striated adductor muscle (gray part)	6.9 ± 0.7	2.0
Smooth adductor muscle (white part)	6.2 ± 0.2	1.3

Activity is expressed as nmol AcSCh hydrolyzed/min/mg of protein. Results are given as mean ± S.D. of 4 determinations.

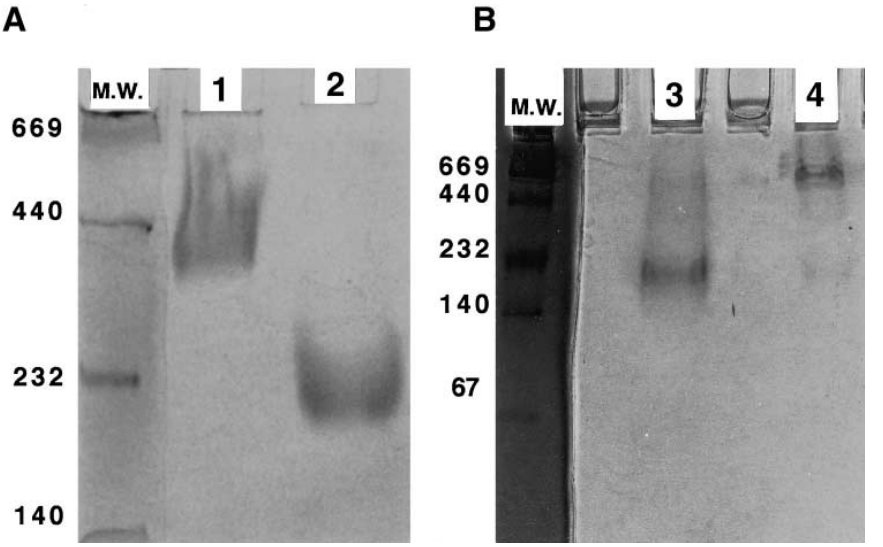

Fig. 3. Electrophoretic analysis of cholinesterases from the common oyster *Crassostrea gigas*. Non-denaturing electrophoresis was performed in gradient polyacrylamide gels in the presence of 0.25% deoxycholate (A) or gel in the presence of 0.1% Triton X-100 (B). Lane 1: AChE 'A'; lane 2: AChE 'B'; lane 3: AChE 'A' previously digested by PI-PLC; lane 4: AChE 'A' incubated without PI-PLC. M.W.: molecular weight standards in kDa.

whole cholinesterase activity remained bound to the column, with recovery ranging from 23 to 57% of the original activity. This yield can be considered relatively poor when compared to purification of other AChEs. Similar ratios were found for the two cholinesterases on crude and purified extracts, indicating that they could be purified on a procainamide column with the same efficiency.

3.2. *Characterization of the two cholinesterases*

Gills were extracted in LST buffer and centrifuged in sucrose gradients in the presence of Triton X-100 or in the absence of detergent (Fig. 2A). This procedure showed that a portion of the activity that sedimented at 6.5S in the presence of Triton X-100 aggregated in the absence of detergent. Another part sedimented as a well defined peak. These two components were therefore amphiphilic (aggregates) and hydrophilic. Non-denaturing electrophoresis of aggregates (a) and hydrophilic peak (b) showed that they had distinct mobility in gels but were both present in the single peak in LST gradients (c). Gradients shown in Fig. 2A were assayed with 1 mM AcSCh as a substrate without inhibitor. They were also assayed in the presence of 1 mM paraoxon. Fig. 2B shows that a part of the peak was resistant for the LST gradient. The LS gradient assay indicated that the hydrophilic component was resistant to paraoxon but that aggregates were sensitive (results not shown).

The crude extract, like the purified extract, showed two bands migrating at ≅200 kDa ('A' cholinesterase) and ≅330 kDa ('B' cholinesterase) when electrophoresis was performed in non-denaturing conditions on gradient gel in the presence of deoxycholate (Fig. 3A). In the presence of this negatively charged detergent, the hydrophobic (or amphiphilic), cholinesterase migrated until the gradient zone corresponding to its apparent molecular weight [9]. When the sample was applied to a gel in the presence of Triton X-100 instead of deoxycholate as detergent, the 'B' cholinesterase appeared to be insensitive to the detergent and migrated to ≅330 kDa as in the case of the deoxycholate gel. Conversely, the 'A' cholinesterase was affected by the nature of the detergent, migrating higher in the gel in the presence of 0.1% Triton X-100.

After Triton X-114 partitioning, there was some activity in both the detergent and the aqueous phases. The detergent phase contained only the 'A' cholinesterase, while the aqueous phase contained 95–98% of 'B' cholinesterase and some traces of 'A'.

Additional phase separations did not extract the 'A' cholinesterase present in the aqueous phase, indicating that this

Table 2
Kinetic characteristics of acetylcholinesterases A and B from the common oyster

	Relative rate of hydrolysis	K_m (μM)	K_{ss} (mM)
'A' cholinesterase			
AcSCh	100%	77.6	13.3
PrSCh	81%	124	N.M.
BuSCh	8.5%	63.5	N.M.
'B' cholinesterase			
AcSCh	100%	18	15.13
PrSCh	93%	39	N.M.
BuSCh	N.D.	N.D.	N.M.

N.M.: not measurable; N.D.: not detected.

protein is a degradation product of the amphiphilic 'A' cholinesterase.

Conversion of the 'A' enzyme into its hydrophilic counterpart by PI-PLC was followed by the monitoring of cholinesterase distribution after phase partitioning in the presence of Triton X-114. After PI-PLC treatment, we found 92% of the cholinesterase activity in the aqueous phase whereas without treatment only 8.8% was recovered. In the same way, the effect of PI-PLC treatment was assessed by non-denaturing gradient electrophoresis in the presence of 0.1% Triton X-100 (Fig. 3B). Migration of cholinesterase 'A' was dramatically increased after PI-PLC treatment. Both methods led to the same results, suggesting that the hydrophobic behavior of this cholinesterase is due to the presence of a glycolipid anchor which probably mediates in situ the association of this form to cell membranes.

'A' cholinesterase was not retained upon loading onto concanavalin A Sepharose, whereas 'B' form remained bound and could be released by a buffer containing a high concentration of methyl mannose. This suggests that only the 'B' cholinesterase is glycosylated.

The specificity of the two enzymes toward AcSCh, BuSCh and PrSCh was measured with 1 mM of substrate (Table 2). 'A' cholinesterase was most active against AcSCh (100%), less active against PrSCh (81%) and relatively inactive against BuSCh (8.5%). 'B' cholinesterase had nearly the same activity for AcSCh and PrSCh (respectively 100% and 93%) but did not hydrolyze BuSCh substrate at all. No activity could be measured for this thiocholine-ester, regardless of the substrate concentration (5 μM to 5 mM).

The two cholinesterases displayed an apparent Michaelian behavior in the 5 μM to 1 mM range, when acetylthiocholine was used as substrate. K_ms were in the same range (Table 2). The K_m of the 'A' enzyme for BuSCh was nearly the same as that for AcSCh, although BuSCh was poorly hydrolyzed. At higher substrate concentration (above 5 mM), both enzymes were inhibited.

Inhibition experiments with organophosphate and carbamate inhibitors underscored the great difference in sensitivity between the two cholinesterases. Except for the carbamate eserine, the 'B' enzyme proved to be almost insensitive to organophosphorus and carbamate compounds, whereas 'A' was highly sensitive. The results expressed in Table 3 show that membrane-bound cholinesterase was 40 000-fold more sensitive to paraoxon and 16 000-fold more sensitive to carbofuran than the soluble enzyme (which was only sensitive to eserine). The insensitivity of the soluble cholinesterase was so great that the k_i for carbofuran reached 135 M^{-1} min^{-1}. The lack of sensitivity of the 'B' enzyme to iso-OMPA is quite consistent with the fact that it did not hydrolyze BuSCh, assuming that accessibility to the acyl pocket controls the reaction with organophosphate inhibitors.

Table 3
Bimolecular reaction constant (k_i in M^{-1} min^{-1}) of six inhibitors on the two classes of oyster AChE

	'A' cholinesterase	'B' cholinesterase
DFP	2.0×10^3	1.3
Paraoxon	3.0×10^5	7.6
Iso-OMPA	3.0	N.I.
Eserine	4.7×10^5	2.7×10^4
Carbofuran	2.2×10^6	135
Carbaryl	6.7×10^4	62.5

N.I.: no inhibition at 1 mM iso-OMPA.

3.3. *Distribution of the two cholinesterases in the main organs of the oyster*

Differential sensitivity of cholinesterases 'A' and 'B' to paraoxon provided an opportunity to analyze the contribution of each form in different tissues. The results (Table 1) show large differences between tissues. Both cholinesterases were present in each tested organ in different proportions but with a clear predominance of the 'A' form in the gills and the 'B' form in the mantle and in labial palps.

4. Discussion

Our results indicate the presence of two distinct cholinesterases in the common oyster. Structural and catalytic differences suggest that these two forms isolated from the gills constitute two separate proteins probably encoded by two different genes. The presence of several different genes coding for cholinesterases in invertebrates has already been reported. In the nematodes *Panagrellus redividus* and *Steinernema carpocapsae*, AChE is encoded by two different genes [10,11], whereas three different genes, *Ace-1*, *Ace-2*, and *Ace-3*, encode three AChE classes (A, B and C) in the nematodes *Caenorhabditis elegans*, *Meloidogyne arenaria* and *Meloidogyne incognita* [12,13]. Two different AChEs have been described in the medicinal leech (*Hirudo medicinalis*) [14]. However, only one gene has been described in insects [15] and *Octopus* [16].

The question still arises as to whether these two enzymes are cholinesterases or non-specific esterases which also metabolize acetylcholine. Although it is not clear whether enzymes actually metabolize acetylcholine in vivo, some data suggest that they can be considered cholinesterases. Both forms in the oyster are active against acetylthiocholine iodide. K_ms for acetylcholine are in the range of 10–100 μM. They are also inhibited by eserine and by an excess of acetylthiocholine iodide as are most cholinesterases.

Vertebrate cholinesterases have been classified into two groups, either AChE or butyrylcholinesterase, depending on substrate hydrolysis and sensitivity to inhibitors. Both cholinesterases from the oyster were most active against acetylthiocholine iodide and showed less activity against propionylthiocholine iodide and butyrylthiocholine iodide. They were also inhibited by an excess of acetylthiocholine iodide and were insensitive to iso-OMPA which has been identified as a specific vertebrate butyrylcholinesterase inhibitor [17,18]. Although it is tempting to classify the two oyster cholinesterases as AChE, this classification has only been clearly established in vertebrate species. Von Wachtendonk and Neef [19] have described a single AChE in the hemolymph of the common mussel (*Mytilus edulis*) as compare to four pseudo-cholinesterases differing in their pharmacological behavior. For Talesa et al. [20] the cholinesterase extracted from the shellfish *Murex brandaris* is a propionylcholinesterase since PrSCh constitutes a better substrate than acetylthiocholine or butyrylthiocholine. Therefore, the situation of invertebrate cholinesterases is still confusing. Determination of sequences will be necessary, (i) to establish whether all enzymes isolated so far fulfil the molecular requirements defining a cholinesterase or an acetylcholinesterase and (ii) to establish their molecular evolutionary relationships.

From a kinetic point of view, both 'A' and 'B' cholinesterases from the oyster exhibit K_ms for acetylthiocholine in the range of 10–100 μM. Similar results have been reported for the same substrate concerning detergent-sensitive AChE from the leech (25 μM), soluble AChE from the leech (70 μM), AChE from *Murex* (78 μM) and classes A and B from most nematodes (in the range of 10 μM). Comparative analyses of the K_m values of the two cholinesterase from the common oyster show characteristics similar to those of other known cholinesterases from both invertebrates and vertebrates.

The soluble cholinesterase from oyster ('B') is reported to be insensitive to most irreversible inhibitors and therefore close to the cholinesterase described as class C in nematodes [21]. This insensitivity to inhibitors makes these cholinesterases ('B' from oyster and C from nematodes) of special interest among known cholinesterases. As the measurement of AChE inhibition in bivalves could be used as a biomarker of the effects of neurotoxic contaminants (including organophosphate and carbamate insecticides), the coexistence of two distinct cholinesterases (one sensitive) has until now been detrimental to the use of bivalves as potential bioindicators of inhibitory effects. Though they exhibit low levels of cholinesterase activities, bivalves are very easy to sample through existing monitoring networks such as the American Mussel Watch or the French National Monitoring Network (Réseau National d'Observation, R.N.O.). Now that the differential properties of each cholinesterase have been determined, isolation of the activity of sensitive membrane-bound cholinesterase from total cholinesterase activity should provide considerable improvement for the use of cholinesterase from the oyster as a marker of inhibitory effects. The activity of the sensitive 'A' form can be obtained by calculating the difference between total cholinesterase activity and the same activity following incubation in 1 mM paraoxon.

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