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Esterases in the zebra mussel *Dreissena polymorpha*: activities, inhibition, and binding to organophosphates

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Abstract

Cholinesterase activities of *Dreissena polymorpha* were measured colorimetrically. In homogenates of whole control mussel, activities of 125 ± 29 μmol min^{-1} kg^{-1} were found ($n = 6$). Neither after exposure of *Dreissena* to organophosphates (thiometon, disulfoton, demeton-*S*-methyl) nor after addition of demeton-*S*-methyl (the activated oxygen analogue of thiometon) in vitro was the measured mussel esterase activity inhibited. Esterases of rat, mouse and human tissue showed a 90–100% inhibition. Radiolabelling of the active serine site of esterases in muscle homogenates with ^{3}H-diisopropylfluorophosphate and subsequent separation on polyacrylamide gels revealed similarities as well as differences between rat and mussel esterases. Coomassie-stained muscle proteins of *Dreissena* showed a different distribution pattern than those of rat. Proteins of rat as well as proteins of mussel with molecular weights between 66 and 97 kDa showed best labelling (highest radioactivity). Proteins with molecular weights greater than 97 kDa were not labelled. Additionally, in *Dreissena* but not in rat, proteins of around 45 kDa were labelled. The results indicate that the esteratic enzymes in *Dreissena* were labelled but not inhibited by organophosphates.

Keywords: *Dreissena polymorpha*; Organophosphate; Acetylcholinesterase; Radiolabelling; Diisopropylfluorophosphate

1. Introduction

The freshwater mollusc *Dreissena polymorpha* (zebra mussel) was shown to be highly resistant towards organophosphate insecticides and their activated oxygen

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analogues (Dauberschmidt et al., 1996). This is true, in spite of an observed accumulation of the organophosphates thiometon and disulfoton in the tissue of these molluscs. In vertebrates, organophosphates inhibit the acetylcholinesterase at the motoneural endplates, which among other effects may lead to lethal failure of respiratory muscles. Metabolism of commercial agricultural organophosphates takes place very quickly in mammals, usually to highly toxic and potent cholinesterase-inhibiting oxygen analogues followed by hydrolytic steps to inactive compounds. At high concentrations however, the parent compound itself inhibits the cholinesterases as well (Gallo and Lawryk, 1991). Accumulation of organophosphates such as observed in *Dreissena* as well as in some fish (Zinkl et al., 1991) indicates slow metabolism and consequently questions an activation of the parent compound to a powerful cholinesterase inhibiting intermediate.

Although there is no doubt that organophosphates exert their toxicity by inhibiting cholinesterases of homeotherm vertebrates, there seems to be less evidence when looking more specifically at several different groups of animals. Fish for example usually show acetylcholinesterase inhibition upon exposure to organophosphates, but some fish species do not (Zinkl et al., 1991; Arnold and Braunbeck, 1992). Frog acetylcholinesterases show extremely low inhibition rates and therefore high resistance towards organophosphate inhibition compared to chicken and rat (Andersen et al., 1977). Furthermore, the inhibition of acetylcholinesterase even in insects is not fully understood despite the fact that organophosphates have served worldwide as efficient insecticides for years (Fest and Schmidt, 1970).

Species differences in organophosphorus pesticide toxicity have been related to lower esterase activities, such as in ticks (Wharton and Roulston, 1970) and some bird species (Thompson et al., 1991), as well as to cholinesterase modifications which evolved in organophosphate-resistant insects (Hemingway and Georghiou, 1983).

Cholinesterases are found in all animal phylas (Walker and Thompson, 1991) including molluscs, but the knowledge about structure, function, and distribution is very scarce in the case of invertebrates. It appears as if insects and molluscs present a higher diversity of cholinesterases than what is known for mammals (Edwards and Fisher, 1991; Habig and Di Giulio, 1991). From the variety of cholinesterase forms found in the animal kingdom and their inherent characteristics, it can be postulated that cholinesterases from invertebrates might represent an intermediate evolutionary level between the more evolved forms of vertebrates and a possible ancestral esterase (Johnson, 1990). Furthermore, it has been shown that the separation between acetyl- and butyrylcholinesterase genes occurred long after the divergence of vertebrates and insects (Chatonnet and Jbilo, 1990).

Due to the above-described resistance of zebra mussels exposed to organophosphates, the interaction of organophosphorus insecticides with zebra mussel acetylcholinesterase was of interest. The aim of this study was to elucidate some characteristics of cholinesterases in *Dreissena* and determine whether these findings would allow a better understanding of the effects of organophosphates on the enzyme systems of these molluscs.

Measurements of cholinesterase activity of *Dreissena* in the presence of inhibitors

were carried out. Additionally, staining of active serine sites of esterases in muscle homogenates with radiolabelled inhibitory organophosphate and subsequent protein separation on polyacrylamide gels was performed.

2. Materials and methods

2.1. Measurement of cholinesterase activity

2.1.1. Collection of Dreissena and pretreatment

Mussels were collected from Lake Zurich. Exposure to the organophosphates thiometon, disulfoton and demeton-*S*-methyl followed as described in Dauberschmidt et al. (1996). Briefly, mussels (valve length 1.6–2.3 cm) were placed into covered glass aquaria containing 7.5 L of dechlorinated tap water (18°C) which was continuously recirculated. The experiments were carried out by adding the same amounts of the respective organophosphates to the aquaria once every 24 h for a total of four times (Table 1). Dead mussels (lacking shell closing reflex) were removed daily, and mussels surviving the 96 h experimental period were stored at −20°C until further processing.

2.1.2. Cholinesterase assay

Determination the cholinesterase activities was based mainly on the method of Ellman et al. (1961) with minor modifications as described in Hill (1988). The reagents were purchased from Boehringer diagnostics, Mannheim FRG as a kit for measuring serum cholinesterase activity. Esterase activity of human plasma can be measured directly without prior preparation. In order to measure activities of tissues, homogenates were prepared as described in Hill (1988). Mussels were thawed, shucked and the excess water was drained off. Weighed tissue (single whole mussels, pooled dissected posterior adductor muscle of *Dreissena*, leg muscle tissue from rat and mouse) was homogenised in 0.05M Tris buffer (pH 8) at a ratio of 1 g ml^{-1} with a power driven homogeniser.

For assaying cholinesterase activity, acetylthiocholine iodide was added as substrate to mixed tissue homogenate with the buffer/chromagen reagent dithiobisnitrobenzoic acid. Reactions were conducted in 2 ml disposable polystyrene photocells with a 1 cm light path. After stirring, the solution was allowed to stabilise for 4–6 min. The sample was placed in a Perkin Elmer 554 spectrophotometer set at a wavelength of 405 nm and change of absorbance was measured for 2–3 min at room temperature. The average change of absorbance per minute was calculated using the following equation (Fairbrother et al., 1991):

$$\frac{\Delta\text{conc}}{\text{min}} = \frac{\Delta\text{abs} \times \mu\text{mol} \times \text{Rxn}}{\text{min} \times E \times \text{Smpl} \times \text{ml}}$$

where Δconc, abs is the change in concentration, absorbance, respectively, Rxn,

Smpl is the reaction mixture volume, sample volume, respectively, E is the extinction coefficient (13.6×10^3 M^{-1} cm^{-1}).

Cholinesterase activity is usually reported in µmol of acetylcholine iodide hydrolysed per min per g (or kg) of tissue (wet weight, ww) at room temperature (1 U = 1 IU = 1 International Unit = µmol g^{-1} min, 10^3 U kg^{-1}, respectively).

2.1.3. Inhibition

Cholinesterase activity inhibition was measured upon addition of 0.1 mg ml^{-1} demeton-*S*-methyl (the activated thiometon analogue) together to human plasma or the respective tissue homogenates into the cuvette. Demeton-*S*-methyl was obtained from Dr. Ehrenstorfer GmbH, Augsburg, FRG (purity 95.2%). It was dissolved at 1 mg ml^{-1} in *n*-hexane.

2.2. Gels and labelling

2.2.1. Sample preparation, protein assay

The homogenates were prepared as described for the cholinesterase assay. The protein contents of the homogenates was determined by the method of Bradford (1976), using bovine serum albumin as standard. Measurements were carried out automatically on a Cobas Fara chemistry analyser (Roche, Switzerland).

2.2.2. Active site labelling of esterases

Tritiated diisopropylfluorophosphate (^{3}H-DFP), an inhibitor of serine esterases, was used to specifically radiolabel cholinesterases present in homogenates for detection. DFP was obtained from Dupont, NEN Products (1 mCi ml^{-1} in propylene glycol). The reference esterases acetylcholinesterase (AChE, EC 3.1.1.7, specific enzyme activity 2 U mg^{-1}), butyrylcholinesterase (BChE, EC 3.1.1.8, 5 U mg^{-1}) and carboxylesterase (CbE, EC 3.1.1.1, 130 U mg^{-1}) were purchased from Boehringer Mannheim. One enzyme activity unit of the reference esterases and 300–400 µg homogenate proteins were incubated for 1 h at room temperature with 0.01 µCi ^{3}H-DFP in a final volume of 200 µl. Thereafter, 10 µl cold DFP (10^{-4} M, Fluka) and 0.8 ml of 30% ice-cold trichloroacetic acid (TCA, Sigma) was added. After centrifugation (10 000*g*, 5 min, 4°C), precipitates were washed sequentially in 1 ml of 10% and 1% TCA by centrifugation. Precipitates were finally dissolved in urea sample buffer as described below.

2.2.3. Protein separation (SDS-PAGE)

Homogenates were mixed at an approximate ratio of 1 : 3 (v/v) with lysis buffer (150mM Tris-HCl pH 6.8, 200mM dithiothreitol, 6% w/v SDS, 0.3% w/v bromphenolblue, 30% v/v glycerol), and boiled for 5 min. SDS-PAGE was performed according to Laemmli (1970), using a 4% stacking gel and a 6 or 7.5% separating gel. High and low molecular weight (mol. wt.) marker were purchased from Sigma. Electrophoresis was run in BioRad mini-vertical gel cells. The gels were stained 30 min in methanol : acetic acid : water (4 : 1 : 5 v/v/v) containing

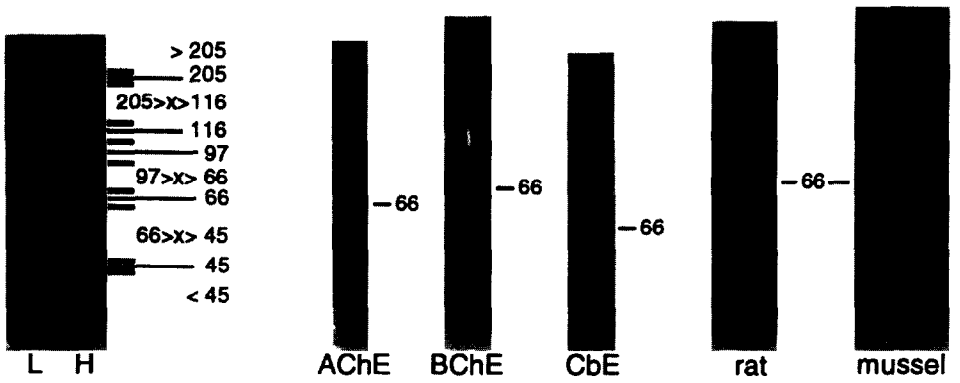

Fig. 1. Coomassie-stained gels of rat and mussel muscle protein and the reference esterases AChE, BChE, CbE. The bold lines beside the gel of the low (L) and high (H) mol. wt. marker indicate where the lanes were cut in order to obtain 10 different mol. wt. (kDa) bands for radioactivity counting.

0.25% coomassie blue, and the background subsequently destained over 12 h by several changes of methanol : acetic acid : water (5 : 7 : 88 v/v/v).

2.2.4. Detection of DFP-binding proteins

After destaining, the gels containing radiolabelled proteins were dried on blotting-paper under vacuum at 80°C for 2 h. The single sample lanes of the dried gels were cut into several mol. wt. bands (Fig. 1). Apparent mol. wt. was determined from electrophoretic mobilities compared with the markers. The bands were then combusted completely with a Canberra Packard oxidiser 306. The condensed tritium water was mixed in liquid scintillation cocktail and the activity was counted with a Beckman LS 6000LL Series Liquid Scintillation System.

Table 1
Cholinesterase (ChE) activities of homogenates of *Dreissena polymorpha* exposed to thiophosphates and demeton-*S*-methyl, the active oxygen analogue of thiometon

Exposure			ChE activity (μmol min^{-1} kg^{-1} ww)	*n*
duration	chemical	amount added daily (mg L^{-1})		
control			125 ± 29	6
96 h	thiometon	6	121 ± 69	3
96 h	disulfoton	10	91 ± 40	3
96 h	demeton-*S*-methyl	6	113 ± 42	3
> 72 h [a]	thiometon	12 or 50	110 ± 37	3

[a] Mussels died last day of exposure.

Table 2
Cholinesterase activities of respective tissues of human, rat, mouse, and *Dreissena* and inhibition in presence of 0.1 mg ml^{-1} demeton-*S*-methyl ($n = 2$)

Tissue	ChE activity (μmol^{-1} min kg^{-1} ww)	Inhibition (%)
human plasma	2507 ± 62	100
rat muscle	610 ± 41	89
mouse muscle	562 ± 2	100
mussel muscle	106 ± 4	0

3. Results

3.1. Enzyme activities and inhibition

The mussel homogenates of differently exposed mussels showed no significant inhibition of their cholinesterases, irrespective of the organophosphate they were exposed to and whether the exposure caused death (Table 1). Furthermore, the acetylcholinesterase activity remained unchanged even in *Dreissena* that presumably had died of organophosphate exposure after 72 h.

Normal activity and activity after application of demeton-*S*-methyl were measured in muscle homogenates of *Dreissena*, rat and mouse (Table 2). Cholinesterase activities of vertebrate muscle were around six times higher than those of the mussel muscle. Addition of 0.1 mg ml^{-1} demeton-*S*-methyl caused an almost total inhibition of the vertebrate plasma and muscle cholinesterase activity. No inhibition of the mussel cholinesterase of whole animal or muscle homogenate could be observed.

3.2. Protein separation and enzyme labelling

In Fig. 1, the coomassie-stained bands of subunits of AChE, BChE and CbE can

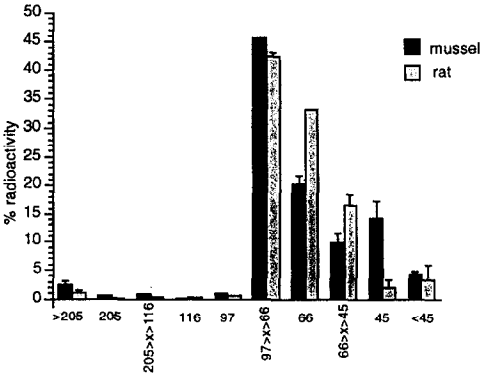

Fig. 2. Molecular weight estimation (x in kDa) for DFP-binding proteins in rat and mussel muscle homogenate. Average ($n = 2$) relative radioactivity of bands combusted (summed radioactivity of whole lane $\Sigma = 100\%$).

be seen. Muscle protein of *Dreissena* show a different pattern of distribution than those of rat. Whereas some bands in the rat homogenate could account for esterases, in the mussel lane no stained bands are detectable at the mol. wt. height of the reference esterases. Comparing mol. wts. of proteins is only a very rough approach however, which must be rendered more sensitive with the results of specific labelling of esterases with DFP.

On SDS-PAGE, peaks of radioactivity (best labelling) of CbE and AChE were at mol. wt. $66 > x > 45$. For BChE they were at mol. wt. $97 > x > 66$. The peaks correspond to the mol. wts. of these enzymes, being 58–61 kDa in the case of CbE, 62 for the AChE subunit, and between 86 and 92 kDa for the subunits of BChE. Radioactivity in rat muscle was highest at mol. wt. $97 > x > 66$. No activity was found at mol. wt. > 97 kDa, and constantly decreasing activities at mol. wts. < 66 kDa (Fig. 2). The peak of activity of mussel protein was also at mol. wt. $97 > x > 66$. The activity in subsequent bands decreased faster than in rat and an additional peak was apparent around mol. wt. 45 kDa.

Due to the relatively high amounts of proteins used (300–400 μg), it was not always possible to resolve the whole TCA precipitated pellet in the urea sample buffer and load it onto the gel. A relative quantification was nevertheless accurate as completely dissolved and loaded proteins showed the same activity pattern as when not the whole pellet was dissolved. Absolute quantification of the binding sites was only possible between samples with similar remaining activity in the pellet (determined by combustion as well) or completely dissolved and loaded proteins. A first estimation showed that *Dreissena* has around one-half to one-third fewer binding sites per mg protein incubated than rat.

4. Discussion

4.1. Enzyme activity

Positive results obtained with a colorimetric method require careful interpretation. Other components able to induce the colour reaction have to be considered. As no more activity could be detected when the proteins were denatured (boiled) prior to measurement in the experiment reported here, it is very probable that the positive reaction was enzyme induced. It can therefore be concluded that also in mussels an enzyme able to hydrolyse cholinesters caused the positive reaction.

The activities of the whole mussel tissue are comparable to earlier measurements in other bivalves (Salanki et al., 1966). Activities of specific tissues show a haemolymph esterase activity in *Mytilus* of 94 U (Von Wachtendonk and Neef, 1975). In the central nervous system of the bivalve *Anodonta* an activity of 230 U kg^{-1} was measured, which is approximately twice the activity measured in the whole mussel tissue of *Dreissena*.

The fact that the cholinesterase activities in tissue of *Dreissena* was around six times below the activities measured in rat and mouse tissue (Table 2) corresponds to generally low enzyme activities in molluscs (Payne et al., 1987). Comparisons be-

tween unrelated species however need careful interpretation. Measuring enzyme activities in species for which the test methods were not originally designed usually does not take into account species specificities such as optimal enzyme reaction temperature.

Almost all the research that has been carried out so far on cholinesterase inhibiting capabilities of organophosphate, has involved vertebrate species. In invertebrate species, inhibition of the acetylcholinesterase by pesticides and other cholinergic compounds were reported for insects, mites, molluscs, earthworms and nematodes (Edwards and Fisher, 1991), in *Planaria* (Villar et al., 1994) and in snails (Varanka, 1968; Singh and Agarwal, 1983). A freshwater mussel die-off was attributed to poisoning by the organophosphate orthene and the carbamate lannate as the cholinesterase activities of the adductor muscles were depressed up to 73% (Fleming et al., 1995). However, the findings from this study demonstrate that the individual variation of mussel cholinesterase activity between single specimens of *Dreissena* was larger than the influence from exposition to organophosphates or type of organophosphate (Table 1). This means that a potential inhibition of the mussel cholinesterase by organophosphates is zero or low enough to be hidden by factors such as individual variation of enzyme activities and, if exposed, their unspecific reactions towards the chemicals tested. As an esterase inhibition by organophosphates could not be detected in the freshwater mollusc *Dreissena* even after adding high amounts of active thiometon metabolite to the measuring system in vitro (Table 2), it may be concluded that esterases of *Dreissena* are not inhibited by organophosphates in vivo either.

Low inhibition rates of cholinesterases can be caused by differences in phosphorylation and dephosphorylation rates of the active serine of the esterase molecule, and differences in the affinity of the inhibitors to the active site (Andersen et al., 1977). A lower affinity and/or higher dissociation of the mussel esterase–substrate bond, the (de)phosphorylation rate, could therefore to some extent explain lower inhibition and thus higher resistance towards organophosphates (see Introduction), but not the observed complete lack of inhibition in mussel homogenates (Table 2).

On the non-enzymatic level, other additional features may further explain resistance. As filter-feeding organisms, the respiratory system in *Dreissena* is, due to its biology of ciliar movements, almost independent from muscles (Czihak et al., 1981; Morton, 1969). The existence of cholinergic transmission in the peripheral nervous system in molluscs has so far not been demonstrated. Acetylcholine (Gerschenfeld, 1973; Heyer et al., 1973; Mercer and McGregor, 1982) or cholinesterase activity may therefore not be essential (Lloyd, 1991; Rosza, 1984).

4.2. Labelling

The comparison of the coomassie-stained pattern with the occurrence of labelled proteins shows the selective DFP-binding to specific proteins (Figs. 1 and 2). It must however be considered that beside cholinesters (Blackburn and Selkirk, 1992), other hydrolases such as proteases contain active serine sites and are labelled with DFP as well (Daja et al., 1993). Figs. 1 and 2 further demonstrate that proteins not

visible with coomassie staining are revealed with radioactive labelling. In earlier studies, the DFP-binding of proteins were made visible by autoradiography (Blackburn and Selkirk, 1992; Benyon et al., 1993; Daja et al., 1993). Burning the gel bands and counting the activity of the obtained tritium water in scintillation liquid has, partly because of the weak energy radiation of tritium, several advantages compared to radiographic methods. First of all, activities down to a few dpm per band of around 20 mm^2 can be counted. Secondly, the long exposure times necessary for autoradiography can be avoided. Finally, the absolute decays obtained allow for a much better quantification than black spots on films. The disadvantage of this method was that one loses some of the information on the definition of the bands.

Supposing that all the DFP-labelled proteins were esterases, the estimated two to three times fewer active binding sites to DFP per mg protein of *Dreissena* compared to rat (see Results) indicate that only part of the six times lower activity can be explained by lower enzyme concentration. The other part may be explained for example by slower hydrolysis of the enzyme acetate bond (dissociation).

The DFP-labelled proteins of *Dreissena* showed in comparison to rat an additional activity peak at a position around 45 kDa (Fig. 2). This compares well with the findings of Blackburn and Selkirk (1992), who observed that separation of nematode acetylcholinesterase with SDS-PAGE revealed two isoforms. Blackburn and Selkirk suggested that the catalytic subunit of 39 kDa was a processed derivate from the 74 kDa isoform. Processing of a higher mol. wt. acetylcholinesterase into smaller subunits has been observed also in other invertebrates such as trematodes, *Drosophila*, house fly (Blackburn and Selkirk, 1992) and in the bivalve mollusc *Mytilus* with subunits of about 39 kDa as well (Von Wachtendonk and Neef, 1975).

5. Conclusions

The esteratic enzymes in *Dreissena* were not inhibited by the active organophosphate demeton-*S*-methyl. This corroborates the earlier observed resistance of zebra mussels towards exposure to organophosphates (Dauberschmidt et al., 1996). Consequently, the classical model for toxic action of organophosphates by acetylcholinesterase inhibition is not applicable to zebra mussels. An independent evolution of mollusc esterases and vertebrate cholinesterase going back to the early phylogenetic divergence (Johnson, 1990) may have led to sterical differences and thus to a sterical protection of the active sites of the mussel esterases (Andersen et al., 1977). Why and which mussel proteins were nevertheless radiolabelled by organophosphates remains to be understood.

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