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Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals

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Abstract

The acid and alkaline phosphatase activities of the clam *Scrobicularia plana* have been partially characterised in different organs and tissues (digestive gland, gills, foot, siphon and mantle) and the 'in vitro' effect of heavy metals on both types of enzymatic activity have been analysed. The optimal pH ranged between 4.0 and 5.5 for acid phosphatase activity and 8.5 and 9.5 for alkaline phosphatase activity. The apparent optimum temperature was in the 30–60° range for acid phosphatase activity and in the 30–40°C range for alkaline phosphatase activity. The effect of substrate concentration on enzymatic activities in the tissues showed a good fit to the Michaelis–Menten model. For both types of enzymatic activity, the highest values were found in the digestive gland. The effect of heavy metals was dependent on the tissue analysed. Mercury showed the highest inhibition in the organs/tissues and the parameters K_m and V_{max} were modified when the inhibitor concentration increased, thus indicating a mixed type of inhibition.

Keywords: Phosphatases; Kinetic analysis; pH; Temperature; Heavy metals; Clam; Scrobicularia plana

1. Introduction

Coastal and estuary ecosystems are subjected to input from anthropogenic activities. The load of organic and inorganic substances may affect the inhabitant organisms. To determine the effect of these pollutants on the biota, several strategies have been used. Among these, the change of the rates of key enzymatic activities has been employed in fish and molluscs (Depledge et al., 1995; Viarengo and Canesi, 1991).

Acid (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) (AcP and ALP, respectively) catalyse the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at acid and alkaline pHs, respectively. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville et al., 2000), whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Both enzymatic activities have been studied in several organisms and the influence of heavy metals has been reported (Blasco et al., 1993). These enzymatic activities are involved in a variety of metabolic processes, such as molecule permeability, growth and cell differentiation and steroidogenesis (Ram and Sathayanesan, 1985), and their activities have been used to evaluate the effects of crude oil (Krajnovic-Ozretic and Ozretic, 1982) and methylparathion

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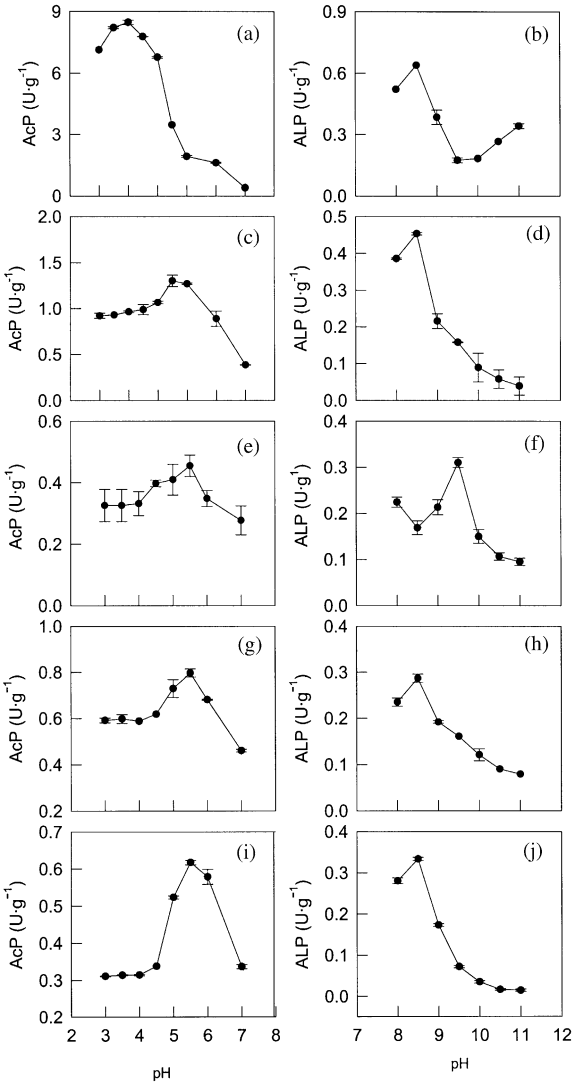

Fig. 1. Influence of pH on phosphatase activity in the acid and alkaline range: (a,b) digestive gland; (c,d) gills; (e,f) foot; (g,h) siphon; (i,j) mantle of *Scrobicularia plana.*

(Reddy and Rao, 1990) on prawns. The measurement of alkaline phosphatase activity is generally carried out in clinical and ecotoxicological studies. In ecotoxicology, this enzyme may serve as an indicator of intoxication because of its sensitivity to metallic salts (Bogé et al., 1988).

Although several phyla have been employed as biomonitor organisms, the molluscs are widely used due to their characteristics (size, wide distribution, easy identification, life cycle, bioaccumulation capacity). Among these, the clam *Scrobicularia plana* has been widely used in estuaries (Langston, 1986).

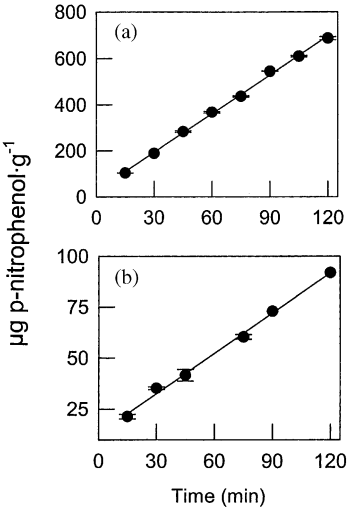

Fig. 2. Influence of incubation time on acid (a) and alkaline (b) phosphatase activators in the digestive gland of *Scrobicularia plana.*

The objective of this paper is to characterise the acid and alkaline phosphatase activities and to analyse the 'in vitro' effect of heavy metals on both types of enzyme activity, to estimate its potential use as a stress biomarker for heavy metals.

2. Materials and methods

Individuals of *Scrobicularia plana* (Da Costa, 1778) were collected in the intertidal zone of the Bay of Cádiz (south-west Spain). Clam size ranged between 38 and 44 cm for all assays. After collection, the clams were immediately taken to the laboratory and kept in a flow-through system with a filter (Whatman GF/C glass-fibre) and aerated seawater at 16 °C for 48 h. At the end of this period, clams were washed and blotted dry. Gills, mantle and digestive gland of the clams were dissected and put into liquid nitrogen and stored at −80 °C until processing. Tissues were homogenised in 100 mM Tris–HCl (pH 7.5), 2 mM DTT and 0.5 mM PMSF. This was carried out in Ultraturrax at 25 000 rev./min for three intervals of 30 s at 4°C. Homogenates were centrifuged at 27 000×*g* for 15 min at 4 °C. The supernatant was collected and used as the enzyme source.

Acid and alkaline phosphatase were determined by the procedure described by Walter and Schutt (1974), with slight modifications: when examining

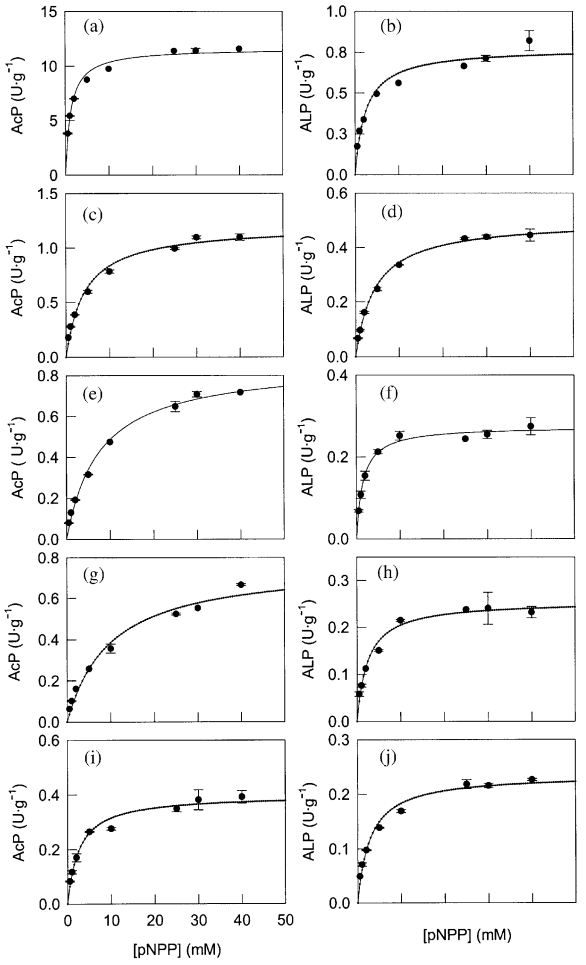

Fig. 3. Saturation curves (substrate *p*-nitrophenyl phosphate) of acid and alkaline phosphatase activities: (a,b) digestive gland; (c,d) gills; (e,f) foot; (g,h) siphon; (i,j) mantle.

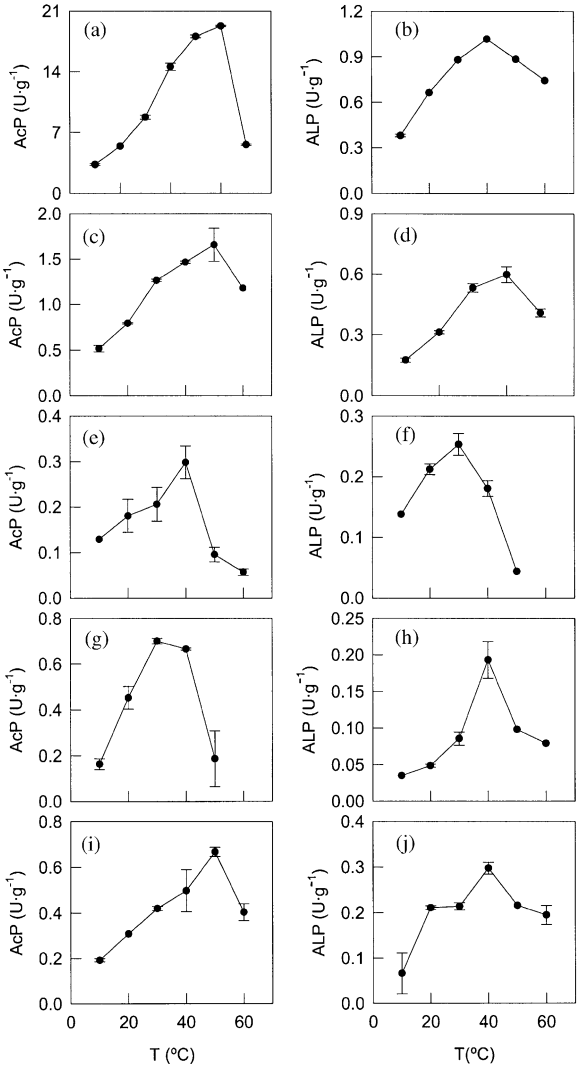

Fig. 4. Influence of temperature on phosphatase activity in the acid and alkaline range: (a,b) digestive gland; (c,d) gills; (e,f) foot; (g,h) siphon; (i,j) mantle of *Scrobicularia plana.*

the influence of pH, we used 80 mM citric-sodium citrate in the range 3.0–6.0 and 80 mM glycine-NaOH in the range 8.5–11; the effect of time was analysed within a range of 0–120 min and the effect of temperature within the range 10–60 °C; these determinations were carried out at optimum pH and at a substrate concentration of 25 mM *p*-nitrophenylphosphate. The thermal denaturalisation of enzyme was analysed in the range 30–70°C with incubation times between 5 and 60 min. To determine the effect of metals (Hg^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}), the concentrations, in the final volume, ranged between 0.05 and 1 mM, except for Hg^{2+}, for which the concentrations ranged between 0.005 and 0.1 mM. The buffers employed in the analysis of heavy metal effects were 50 mM citric-sodium citrate and 190 mM glycine NaOH at the optimum pH for each organ/tissue. To calculate the apparent kinetic parameters (K_m and V_{max}), a non-linear regression was employed at optimum pH and 30 °C, with substrate concentrations between 0.5 and 40 mM. The determinations of enzyme activity in the different organs/tissues were performed at their optimum pH, a temperature of 30°C and an incubation time of 30 min. The substrate concentrations selected were $10 \times K_m$ for each organ/tissue. At the end of incubation period, the reaction was stopped immediately by placing the tubes on ice and adding 2

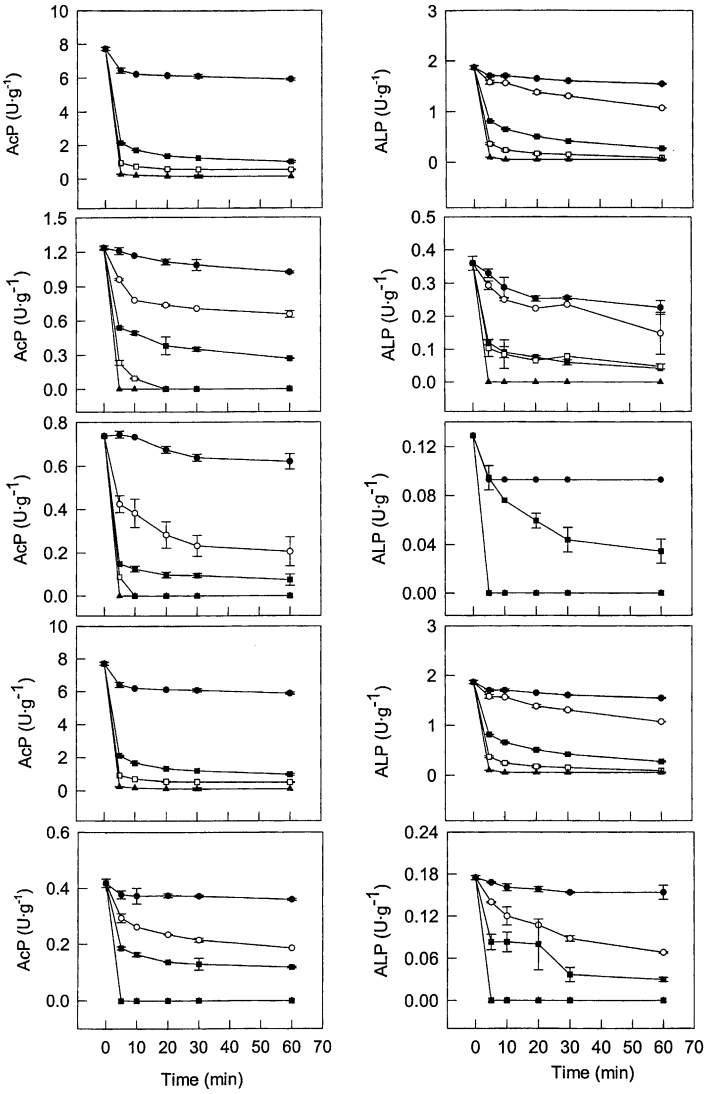

Fig. 5. Thermal inactivation curves (●, 30 °C; ○, 40°C; ■, 50°C; □ 60°C; ▲, 70°C) of acid and alkaline phosphatase activities in the organ/tissue of *Scrobicularia plana*: (a,b) digestive gland; (c,d) gills; (e,f) foot; (g,h) siphon; (i,j) mantle.

ml 0.1 N NaOH. The absorbance of *p*-nitrophenol released was recorded at 405 nm. Results are expressed as units/g (1 unit = 1 μmol *p*-nitrophenol min^{-1}). The molar extinction coefficient of 4-nitrophenol used for the calculation was 18.5 μmol cm^{-2}) (Walter and Schutt, 1974).

Results were analysed using the software package STATGRAPHIC PLUS for Windows 3.3 (Statgraphics, 1998). Data were compared using Student's *t*-test. A probability level of $P \leq 0.05$ was used in all test hypotheses. The kinetic parameters were calculated according to a non-linear regression model.

3. Results

Fig. 1 shows pH vs. enzymatic activity in homogenates of the gill, digestive gland, foot, mantle and siphon of *Scrobicularia plana*. Two peaks were apparent in all tissues, the first at pH 5.5 for gills, foot, siphon and mantle, and pH 4.0 for digestive gland (AcP) and the second at pH

Table 1
Apparent K_m and V_{max} of acid and alkaline phosphatase activities in the organ/tissues of the clam *Scrobicularia plana*

Organ/tissue	AcP		ALP	
	V_m (U g^{-1})	K_m (mM)	V_m (U g^{-1})	K_m (mM)
D. gland	11.62	1.23	0.78	2.48
Gills	1.20	4.41	0.50	4.52
Foot	0.86	7.66	0.27	1.51
Siphon	0.77	9.92	0.25	2.48
Mantle	0.40	2.62	0.24	2.88

8.5 for digestive gland, gills, siphon, mantle and at pH 9.5 for foot (ALP). The activity of acid phosphatase was higher than that of alkaline phosphatase in all tissue analysed.

The influence of incubation time vs. acid and alkaline phosphatase is shown in Fig. 2. In all tissues, both enzymatic activities displayed linear responses in the range 0–120 min. The effect of substrate concentration on enzymatic activities in the tissues is shown in Fig. 3. The results showed a good fit to a Michaelis–Menten model (r^2 ranged between 0.962 and 0.992 for AcP and 0.943 and 0.993 for ALP). The apparent K_m and V_{max} values are reported in Table 1. The lowest K_m is found in the digestive gland for AcP, and the highest in the siphon, whereas for ALP, the lowest value is observed in the foot and the highest in the gills. With relation to the V_{max}, the highest values were found in the digestive gland for both enzymatic activities. Hill coefficient (n_h) (Dixon and Webb, 1979) was obtained from a least squares linear regression plot of log $(v/V_{max}-v)$ vs. log [pNPP]. The results for all organs/tissues showed values close to 1 (for AcP, this ranged between 1.35 in digestive gland and 0.89 in siphon and for ALP, between 0.99 in digestive gland and 0.96 for mantle), indicating that there was no allosteric behaviour for either enzymatic activities.

Table 2
Apparent energy of activation (E_a) for acid phosphatase (AcP) and alkaline phosphatase (ALP) activities in the organ/tissues of *Scrobicularia plana*

Organ/tissue	AcP	ALP
D. gland	7.02	5.75
Gills	5.41	7.43
Foot	4.66	5.21
Siphon	12.48	9.96
Mantle	5.42	8.08

The results are expressed as kcal mol^{-1}.

With regard to the effect of temperature (Fig. 4), the optimum apparent temperature ranged in the intervals 30–60 °C for AcP and 30–40 °C for ALP, the highest value was found for AcP in the digestive gland. The optimum temperatures were higher for AcP than for ALP, in general. The siphons showed the opposite behaviour. The apparent activation energy was determined from an Arrhenius plot. The results expressed as kcal mol^{-1} are shown in Table 2. The thermal denaturalisation for both enzymatic activities is shown in Fig. 5, for all tissues. They are not fitted to a first

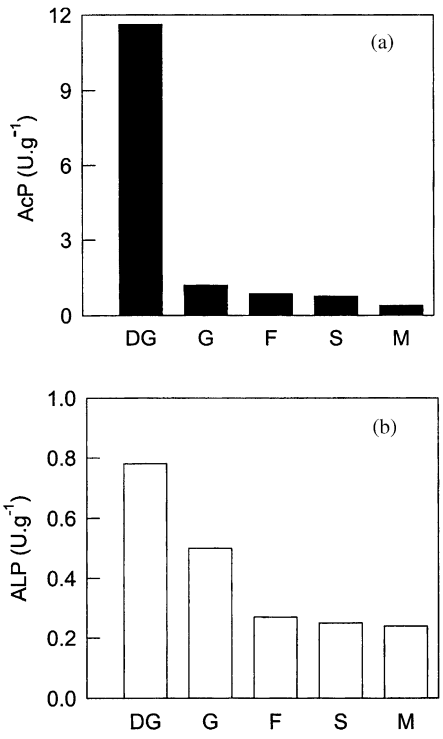

Fig. 6. Distribution of acid and alkaline phosphatase activities in the organ/tissues of *Scrobicularia plana*. DG, digestive gland; G, gills; F, foot; S, siphon; M, mantle.

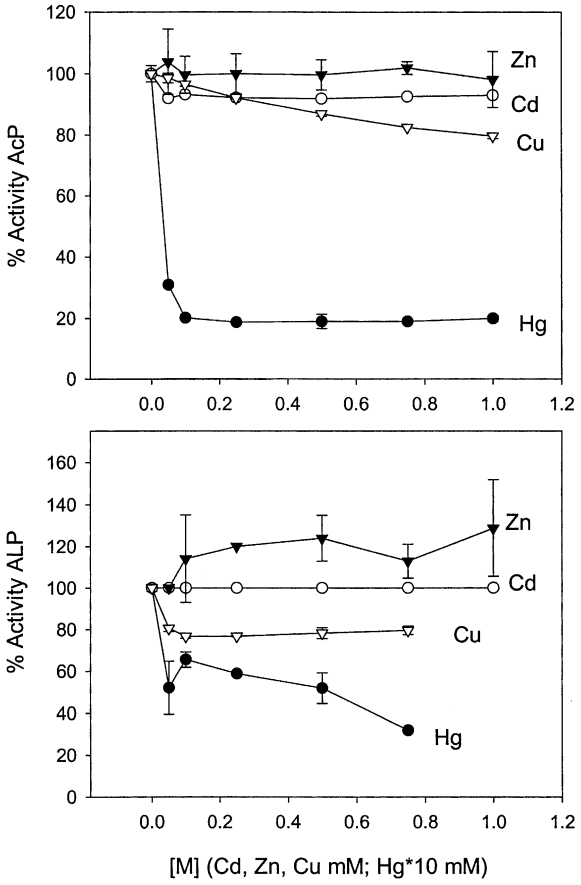

Fig. 7. Effect of heavy metals (Hg, Cd, Cu and Zn) on acid and alkaline phosphatase activities in the digestive gland of *Scrobicularia plana*.

order kinetic model. In foot, siphon and mantle, the exposure at 60 and 70 °C for 5 min provoked the loss of enzymatic activities, while in the gills and digestive gland a decrease of the enzymatic activity was reported at 70 °C, although a residual activity was observed at 60 °C.

The specific activity of AcP and ALP (units mg protein^{-1}) in the organs/tissues is shown in Fig. 6. For both enzymatic activities, the highest values were found in the digestive gland. Nevertheless, for AcP activity, the values in gills were approximately 10 times lower than in the digestive gland, whereas for ALP activity, the values in the gills are lower than in the digestive gland, although of the same order.

The effect of the heavy metals (Hg^{2+}, Cd^{2+}, $Zn^{2+}$and Cu^{2+}) on acid and alkaline phosphatase activities in the digestive gland and gills are plotted in Figs. 7 and 8. For Hg^{2+}, AcP and ALP activities show a significant decrease ($P<0.05$) between control and homogenates incubated with metal. The inhibition was high for both enzymatic activities, in the assayed concentration range, although differences were observed depending on the analysed tissue. For Cd^{2+}, the behaviour was different from that of Hg^{2+}; thus in the digestive gland, the decrease of AcP activity was only 7%, and this metal did not exert any effect on ALP activity. In the gills, at 1.0 mM, the AcP activity remaining was 76% with respect to the control, whereas ALP activity decreased to 39%. For Zn^{2+}, the effect as inhibitor or activator was dependent on the organ /tissue; thus ALP activity decreased by approximately 30% with respect to the control in the gill, mantle and siphon. For Cu^{2+}, ALP activity was inhibited more than AcP activity, except in the

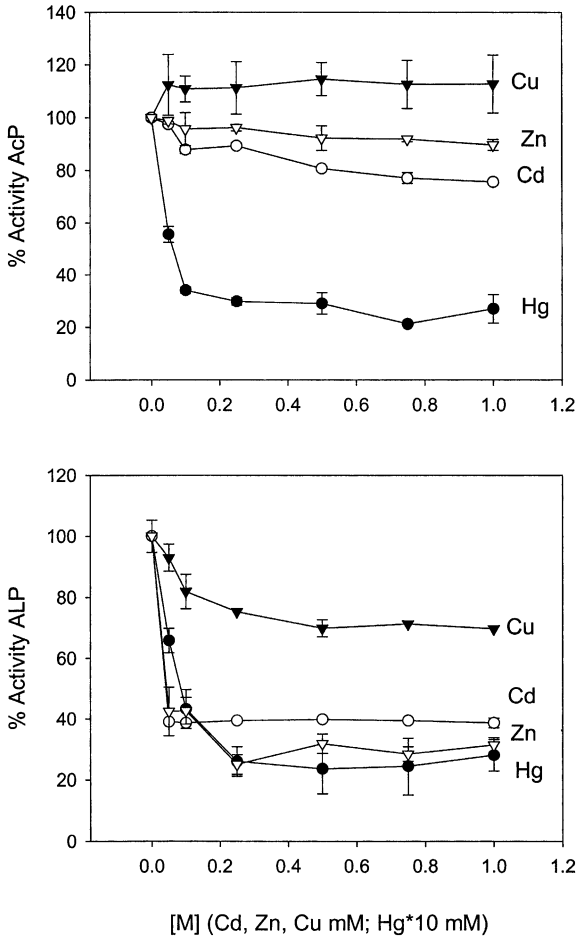

Fig. 8. Effect of heavy metals (Hg, Cd, Cu and Zn) on acid and alkaline phosphatase activities in the gills of *Scrobicularia plana*.

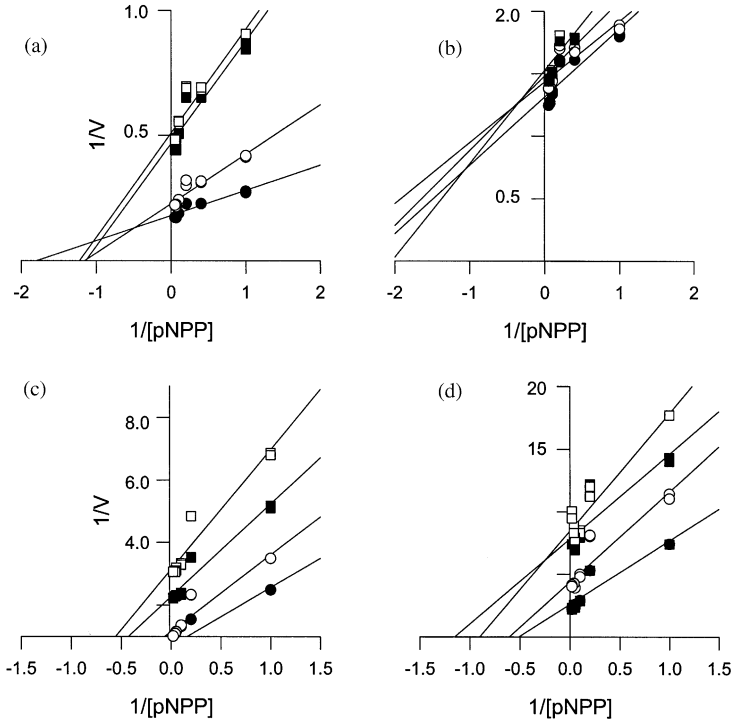

Fig. 9. Lineweaver–Burk plots of acid (a,c) and alkaline (b,d) phosphatase activities in the digestive gland and gills, respectively, at different Hg concentrations (●, 0 μM; ○, 10 μM; ■, 25 μM; □, 50 μM).

digestive gland where a degree of inhibition was observed.

For Hg^{2+} the kinetic analysis of inhibition was carried out in the digestive gland and gills. The Lineweaver–Burk plots for both enzymatic activities and these tissues are shown in Fig. 9. The parameters K_m and V_{max} were modified when the inhibitor concentration increased, indicating a mixed type of inhibition.

4. Discussion

The range of optimum pH values for both enzymatic activities, in each of the tissues, are similar to those found in other species, thus for acid phosphatase, the pH lies between 4.0 and 6.0; e.g. 4.5 in *Ruditapes philippinarum* (Blasco et al., 1993); 4.75 for *Chelon labrosus* gill (Belloc and Gallis, 1980). The peaks for ALP activity in the tissues are similar to those in the same tissues of *R. philippinarum* (10.5) (Blasco et al., 1993) and to the kidney and intestine of carp, eels, mice (Yora and Sakagishi, 1986) and whole body homogenates of *Venus gallina* (Carpenè et al., 1979).

The pH profile is recognised as one of the factors used for characterisation of isoenzymes; the pattern of pH profile was similar in the gills, mantle, foot and siphon, whereas the digestive gland showed a different profile, possibly indicative of another type of isoenzyme. The comparison of optimal pH between species must be carried out with caution because factors such as substrate concentration (Asgeirsson et al., 1995), the nature of the buffer (Curti et al., 1986), and purity of the enzyme all have an influence on the optimum pH.

The high value found for AcP activity in the digestive gland indicates that it plays a functional lysosomal role. This enzyme is also involved in shell deposition. In higher animals, ALP activity is known to be involved in bone formation and in transport membrane activities. In the blue crab, *Callinectes sapidus,* ALP activity is involved in the modulation of the osmoregulatory response (Lovett et al., 1994). The low levels found for ALP activity in clams suggest that membranes are an ineffective barrier to most molecular substances in this species (Chambers et al., 1975). The acid phosphatase activity in the digestive gland of *S. plana* is located in the intestinal epithelium and diverticules and the alkaline phosphatase activity

in the epithelium tubules and digestive diverticules (Mazorra, 1996). These enzymatic activities decrease in line with larger size in the clam *R. philippinarum*, reflecting a decrease in shell deposition as the organisms age (Blasco et al., 1993). K_m values varied between organs for both enzymatic activities, although they are of the same magnitude and similar to those shown by the clam *R. philippinarum* (Blasco et al., 1993) and in the same range as trout intestinal alkaline phosphatase (Whitmore and Goldberg, 1972a,b), although higher than for the crab, *C. spidus* (Lovett et al., 1994). Nevertheless, the comparison of kinetic constants must, in general, take into consideration the buffer substance used and its concentration, since transphosphorylation leads to higher reaction velocities (Asgeirsson et al., 1995).

The optimum temperature for AcP is higher than for ALP activity. This behaviour is the same for the clam *R. philippinarum* (Blasco et al., 1993) and the kidney of *Sparus aurata* (Establier et al., 1984). The optimum values for phosphatase activities are known to be species-dependent. The calculated E_a for acid and alkaline phosphatase ranged between 5.4 and 12.5, and 5.2 and 10.0 kcal mol^{-1} in the tissues and are in the same range as that found by other authors in other species (Blasco et al., 1993; Lien and Knutsen, 1973; Møller et al., 1975; Whitmore and Goldberg, 1972a,b). Low values of activation energy are associated with hydrophobic interactions between substrate and enzyme (Hiwada and Wachsmith, 1974). In *S. plana,* the thermal denaturalisation did not follow a first order kinetic model and the reduced structural stability in the enzymatic activities may be related to optimisation of the balance between stability and activity at low temperatures.

The effect of heavy metals was dependent on the organ/tissue and the enzymatic activity analysed. This finding may be a consequence of the difference in structure. Hg is a strong inhibitor of enzymatic activity due to the high affinity for proteins shown by the –SH group (Sastry and Sharma, 1979) and is the result of the presence of this group in the enzyme. The kinetic analysis of Hg inhibition in digestive gland and gills showed a mixed type of inhibition, indicating that the enzymes may be interacting in different ways with several groups. The cadmium and copper inhibition were quite different between tissues for the two types of enzymatic activity; this may be the result of the different structure of the enzyme molecules and their possible interaction with the metal. All highly purified alkaline phosphatases have proved to be Zn(II)metalloenzymes (Coleman and Gettings, 1983); the role of this metal as activator is related to the saturation of Zn(II) binding sites. Nevertheless, this behaviour was not observed in the tissues analysed, since there were no significant differences between the control and the assayed range of concentrations ($P < 0.05$). However, gill, mantle and siphon showed a slight inhibition. This may be a consequence of excessive Zn replacing Mg at binding sites in the ALP (Lan et al., 1995). For fish, in laboratory conditions, liver alkaline phosphatase activity changes in response to waterborne metal (Lan et al., 1995) making it useful as indicator of heavy metal exposure. The variation in properties between the two types of enzymatic activity suggests that they are not expressed by the same enzyme. More knowledge of the physicochemical and kinetic characteristics of these enzyme activities in the clam *Scrobicularia plana* is necessary before they can be employed as biochemical indicators of stress due to heavy metals.

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