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Phosphomonoesterases and cholinesterases from *Taenia pisiformis* cysticerci

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Summary

Phosphohydrolase and cholinesterase activities were found in the cytosolic fraction of *Taenia pisiformis* cysticerci from naturally infected hares. By using p-nitrophenyl phosphate as substrate, mainly acid (pH 5.0) but also alkaline (pH 10.0) phosphatase activities were quantified. Cholinesterase activity was determined in the presence of acetylthiocholine iodide. Both hydrolases were also detected by native poliacrylamide gel electrophoresis, and preliminary characterized by inhibition assays. The possible biological roles of these enzymes in cestodes are discussed.

Key words: acid phosphatase; alkaline phosphatase; acetylcholinesterase; metacestode; *Taenia pisiformis*

Introduction

Phosphohydrolases and cholinesterases (ChE) are hydrolytic enzymes widely distributed in helminths that have recently become a focus of attention in parasitology. In the most of the papers found in the literature, both activities are related to nematodes, but ChE (Balasubramanian *et al.*, 1984; Maule *et al.*, 1993; Giménez-Pardo *et al.*, 2000) and phosphohydrolases (Jones *et al.*, 1979; Pappas, 1981; Ajayi *et al.*, 1985; Ženka and Prokopič, 1986; Sarciron *et al.*, 1991; Lawton *et al.*, 1994; Kwak and Kim, 1996) have also been demonstrated in cestodes.

Relative few papers have been found about *Taenia pisiformis* Bloch 1780 (Cestoda: Cyclophillidea: Taeniidae). This is a common intestinal tapeworm of carnivores. Cysticerci of this cestode are localized in the peritoneal cavity of rabbits and hares, waiting to develop adults upon ingestion by their definitive hosts.

The aim of this study was to make a preliminary characterization of the phosphohydrolase and cholinesterase activities in cysticerci of this cestode, because of a better knowledge of basic cestode biology could in a future lead to development of new control measures against these parasites.

Material and Methods

Parasite

T. pisiformis cysticerci were removed from the ommentum of naturally infected hares (*Lepus granatensis*) and washed five times in 0.9 % NaCl with added antibiotics (1 mg/ml penicillin and 2 mg/ml dihydrostreptomycin sulphate). Cysticerci were homogenized at 4ºC in a glass Potter-Elvejem homogenizer with periodic pauses for cooling in an ice bath. Homogenates were centrifuged at 100,000 *g* for 30 min in a Beckman XL-100 ultracentrifuge. The supernatant was collected and recentrifuged under the same conditions, being this final supernatant the cytosolic fraction. Protein concentrations were determined by Bradford (1976) using bovine serum albumin as standard and adjusted to 12 mg/ml. Aliquots were either used immediately or frozen at -80ºC until use.

Phosphatase activity measurement

Quantification of the phosphohydrolase activity was performed by the method of Moulay and Robert-Gero (1995). The reaction mixture (final volume: 0.4 ml) contained 50 mM sodium acetate buffer at several pH (3.5 – 6.0), 50 mM Tris-HCl (pH 7.0 – 9.0) or 50 mM diethanolamine (DEA, pH 10.0 – 11.5), p-nitrophenyl phosphate (pNPP, Sigma, St. Louis, Mo, USA) as substrate (1 mM, 5 mM, 10 mM or 20 mM) and 100 µl of sample (100 µg protein). The mixture was incubated at 37ºC for 30 min, 1 h, 2 h, 3 h or 4 h, and the reaction was stopped by addition of 0.6 ml of 0.3 M NaOH. The resultant yellow product was measured at 405 nm using an Ultrospec III Spectrophotometer (Pharmacia, LKB, Uppsala, Sweden). Appropriate media and reagent blank were run in parallel and test values corrected using these blanks. Acid and alkaline phosphatases were also assayed in the presence of different potential phosphatase inhibitors and divalent cations. Final concentrations were: 10 mM and 1 mM L (+) tartaric acid, 1 mM sodium fluoride, 10 mM ethylenediaminetetraacetic (EDTA), 0.5 mM and 1 mM ethylene glycol-bis (2-aminoethyleter)-N,

N, N', N'-tetraacetic acid (EGTA), 100 µM sodium orthovanadate, 1 mM KCN, 10 mM levamisole, 9.2 M urea, 1 mM SDS, 10 mM cysteine, 10 mM and 5 mM L-phenylalanine, 10 mM L-homoarginine, 1 mM $ZnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$. These compounds were preincubated with the samples in the reaction buffer at room temperature for 10 min before the addition of the substrate.

Cholinesterase activity measurement

Spectrophotometric quantification of the ChE activity was determined by a modified Ellman test (Ellman *et al.*, 1961; Rathaur *et al.*, 1987), using acetylthiocholine iodide (AT CI) as substrate. To do this, 260 µl of 0.1 M PBS buffer pH 8.0, 10 µl of 10 mM 5-5'-dithi-bis-(2-nitrobenzoic acid) (DTNB) solution (39.6 mg DTNB, 15 mg of sodium bicarbonate and 10 ml of 0.1 M PBS pH 7.2), 2.5 µl of 75 mM substrate and 25 µl of the sample (20 µg protein) were added to a microplate and the OD measured at 405 nm after 10 minutes at room temperature (22ºC ± 1ºC) in a EXL 800 spectrophotometer (BIO TEK instruments). All readings were corrected by blanks for non-enzymatic hydrolysis. The rate of enzyme activity in mol L^{-1} min^{-1} was calculated using the extinction coefficient of DTNB (Ellman *et al.*, 1961). One unit of activity represents the hydrolysis of 1 nmol of substrate per minute. Specific activity is defined as the units of activity per mg of protein. Butyrylthiocholine iodide (BTCI, 75 mM) and propionylthiocholine iodide (PTCI, 75 mM) were used as substrate to establish the substrate specificity. Excess substrate inhibition tests were performed with ATCI added to give a final concentration range of 0.1 – 25 mM. Susceptibility of the enzyme to inhibition by eserine, 1,5-bis (4-allyl (dimethylammoniumphenyl) pentan-3onedibromide) (BW284C 51), and tetraisopropyl pyrophosphoramide (iso-OMPA) was determined over a range of inhibitor concentrations (10^{-3} – 10^{-8}). For these assays, the samples were previously incubated with the inhibitor at room temperature for 10 min.

Polyacrylamide gel electrophoresis

Electrophoretical detection of phosphohydrolases was carried out by native electrophoresis on 15 % PAGE (20 µg of protein per lane, diluted 1:4 in sample buffer; 40 % sucrose and 0.05 % blue bromophenol) at 150 V constant. Electrophoresis buffer were 25 mM Tris and 129 mM glycine (pH 8.8). To detect AP activity we followed the methods of Burstone (1962) and Lawrence *et al.* (1960). Gels were incubated in absence of light for 3 h at 37ºC in 40 ml acetate buffer pH 5.2, 10 mg of sodium α-naphtyl acid phosphate, 20 mg Fast Blue RR and 0.3 ml of magnesium chloride (10 %). Alkaline phosphatase (ALP) activity was determined by the method of Gamble and Pappas (1981). After electrophoresis, gels were incubated in absence of light for 15 minutes at 37ºC in 40 ml 100 mM Tris pH 9.0, 0.25 mM sodium α-naphthyl acid phosphate, 0.25 mg/ml Fast Blue RR and 10 mM $MgCl_2$. Phosphatase activity was detected as blue/green bands in the brown gel. Finally, gels were fixed in 7.5 % acetic acid and 5 % methanol.

Cholinesterase activity was determined on nondenaturing 10 % PAGE (20 µg protein per lane, diluted 1:4 in 40 % sucrose, 0.05 % bromophenol blue) at 150 V. After electrophoresis the gel was cut into strips and the tracks stained separately for ChE activity following the method of Karnovsky and Roots (1964). ATCI and BTCI were used as substrates. The gels were immersed in the reaction mixture for 24 h at room temperature and cholinesterase activity was detected as brown bands in the gel.

Results and Discussion

The present study has revealed that phosphatase activity occurs over a wide range of pH in which two distinct peaks of activity, one in the acidic range at pH 5.0 for AP and the other in the alkaline range at pH 10.0 for ALP have been observed. All subsequent determinations were conducted at these pH values. Pennoit-De-Cooman and Van Grembergen (1947) only detected AP activity (optimum pH 4.5) but Erasmus (1957a) demonstrated distinct acid and alkaline phosphatases in *T. pisiformis* cysticerci. The optimum pH for AP is in accordance with pH optima reported for other cestodes (Parshad and Guraya, 1978; Barrett, 1981). The optimum pH for ALP is higher than that previously reported for *T. pisiformis* cysticerci i.e. about 8.0 – 9.0 (Erasmus, 1957a), as well as for *Echinococcus granulosus* (Lawton *et al.*, 1994) and *Echinococcus multilocularis* (Sarciron *et al.*, 1991) metacestodes, but it is the same than that observed for *Cysticercus tenuicollis* (Erasmus, 1957b). Preliminary assays were also realized to insure that the reaction rate was a linear function of assay time and protein concentration in the assay mixture. The enzymatic activity of the cytosolic fraction was directly proportional to the protein concentration up to 0.5 mg/ml in the reaction mixture at pH 5 and 10. On the other hand, the rate of hydrolysis remained constant for at least 3 hours and the reactions were allowed for 2 hours, as this time was sufficient for releasing enough chromogenic p-nitrophenolate to be measurable colorimetrically. Activity of both phosphohydrolases was found to increase with the corresponding increase in substrate concentration up to 10 mM. This concentration was employed in all assays. Pappas (1982) reported significant substrate inhibition of the alkaline phosphatase in "TRIS-disrupted fraction" of *Hymenolepis diminuta* adults at pNPP concentrations greater than 2.5 mM, but no such inhibition was noted in our solubilized preparation. In contrast to *Taenia crassiceps* cysticerci (Ženka and Prokopič, 1986), optimum pH of the ALP was the same with 1 mM or 10 mM pNPP.

The activity (mean ± SD) of AP (18.27 ± 1.74 nmol min^{-1} mg^{-1}) was found to be 3.3 fold higher than that of ALP (5.47 ± 0.06 nmol min^{-1} mg^{-1}). In adult cestodes, alkaline phosphatase is usually the most active, but the relative activities of acid and alkaline phosphatases in the tegument of cestodes change during development (Barrett, 1981). In the aqueous extracts from *T. pisiformis* cysticerci and *C. tenuicollis*, AP activity became predominant in contrast to the major activity of the adult stages, which was exhibited by

ALP (Erasmus, 1957a, b). Histochemical studies in cysticercoids of *H. diminuta* showed more acid than alkaline phosphatase (Moczoń, 1973). On the other hand, it should be noted that ALP activity is associated to membranes. Ženka and Prokopič (1986) studied the ALP present in *T. crassiceps* cysticerci; approximately a half of the total activity was free and the remaining part was bound to membranes. We have only examined soluble fractions.

The effects of several potential inhibitors on phosphatase activity from soluble fractions are summarized in Table 1. At pH 5, phosphatase activity was inhibited by sodium fluoride and tartaric acid, two well recognized AP inhibitors. At pH 10, the enzymatic activity was inhibited by the specific inhibitor of ALP, levamisole. Both enzymatic activities were affected by orthovanadate, more at pH 5. AP activity, but not ALP activity, was markedly inhibited by SDS. This detergent has been employed for the solubilization of ALP in cestodes (Gamble and Pappas, 1980) but also inhibits soluble AP of *H. diminuta* (Pappas, 1982; Bumbulis and Pappas, 1991). EDTA and EGTA are well recognized inhibitors of the ALP, and their inhibitory action at pH 10 indicated the presence of divalent cations in the cytosolic fractions. The addition of Ca^{2+} or Mg^{2+} to the reaction mixture had no or little effect on ALP activity but in the presence of 1 mM $ZnCl_2$ the enzymatic activity was reduced to 25 % of the initial value. ALP from *H. diminuta* (Pappas, 1991) and *E. granulosus* cyst membranes (Lawton *et al.*, 1994) are also inhibited by Zn^{2+}. Well known alkaline phosphatases are Zn (II) metalloenzymes; the role of this metal activator is related to the saturation of Zn^{2+} binding sites. The inhibition observed may be a consequence of excessive zinc replacing magnesium at binding sites in the ALP (Lan *et al.*, 1995).

Table 1. Effects of various compounds on the phosphate activity of *Taenia pisiformis* cysticerci

	Relative activity[a]	
	pH 5	pH 10
L(+) tartaric acid 10 mM	82.8 ± 4.5	43.0 ± 2.3
L(+) tartaric acid 1 mM	86.9 ± 3.0	85.5 ± 0.8
NaF 1 mM	35.3 ± 5.2	93.4 ± 2.3
Sodium orthovanadate 100µM	25.2 ± 3.4	57.1 ± 5.4
Levamisole 10 mM	133.5 ± 9.2	29.3 ± 0.3
EDTA 10 mM	145.0 ± 13.9	0
EGTA 1 mM	103.1 ± 2.1	19.1 ± 1.1
EGTA 0.5 mM	100.6 ± 1.4	56.1 ± 0.8
SDS 1 mM	0.5 ± 0.1	87.4 ± 3.4
$ZnCl_2$ 1 mM	97.9 ± 1.5	26.8 ± 0.5
$CaCl_2$ 1 mM	97.0 ± 0.4	99.0 ± 6.2
$MgCl_2$ 1 mM	94.7 ± 0.7	121.3 ± 8.4
Urea 9.2 M	98.0 ± 1.5	95.4 ± 4.6
L-cysteine 10 mM	95.8 ± 2.5	5.4 ± 0.1
L-phenylalanine 10 mM	ND	69.0 ± 10.2
L-phenylalanine 5 mM	ND	98.8 ± 18.7
L-homoarginine 10 mM	ND	123.7 ± 20.2

a – Phosphatase activity is expressed as a percentage of that measured under control condition, i.e. without other additions; The values represent the mean ± SE of three independent determinations, which were performed in duplicate

Assays of amino acid inhibition of ALP showed slight inhibition (i.e. 31 %) of enzymatic activity with L-phenylalanine and 95 % inhibition by cysteine. The *E. granulosus* and *E. multilocularis* enzymes were moderately inhibited by L-homoarginine and totally intensive to L-leucine and L-phenylalanine (Lawton *et al.*, 1994, 1995). Urea that has been used for differentiating ALP isoenzymes in human serum (Statland *et al.*, 1972) had no discernible effect on *T. pisiformis* ALP.

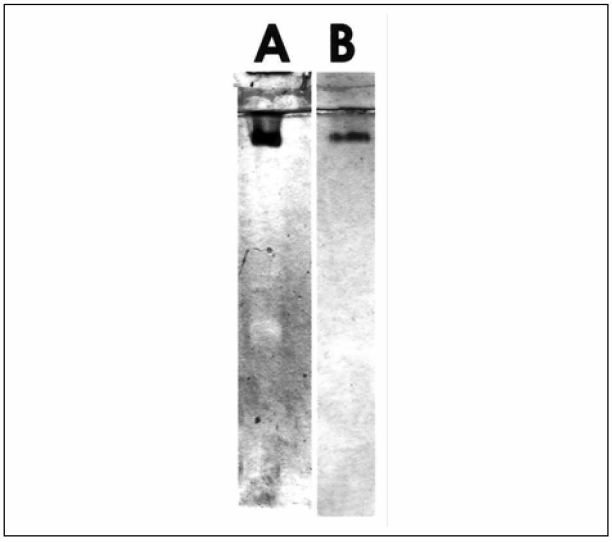

Fig. 1. Gels stained for phosphatase activity. Native electrophoresis was carried out as described in methods in 10 % polyacrylamide gel slabs. 20 µg protein per lane from soluble material from homogenates of *Taenia pisiformis* cysticerci was stained after electrophoresis for 2 h at 37ºC. Staining was developed for acid phosphatase (A) and for alkaline phosphatase (B)

Furthermore, both phosphohydrolase activities were detected at the top of the running gel under native conditions (Fig. 1). In agreement with those colorimetric assays, acid phosphatase band was more intensively stained than alkaline phosphatase band.

On the other hand, soluble fraction of the parasite showed ChE activity. At a substrate concentration of 75 mM ATCI, the specific activity was 24.8 ± 5.7 U mg^{-1}. The enzyme showed a marked preference for ATCI as substrate, the rate of hydrolysis of BTCI and PTCI relative to ATCI were 33.3 % and 53.6 %, respectively. Martínez-Zedillo *et al.* (1984) detected true cholinesterases in the cysticerci of *Taenia solium* and their inhibition by excess substrate was reported. The inhibition of AChE by excess substrate is one of the key features that distinguishes it from butyryl cholinesterases (BuChE) and other esterases, however our preparation did not show this property. This absence of substrate inhibition may be due to a modification of hydrophobic interactions, as it has been observed in mosquito samples (Dary and Wedding, 1990) although the treatment

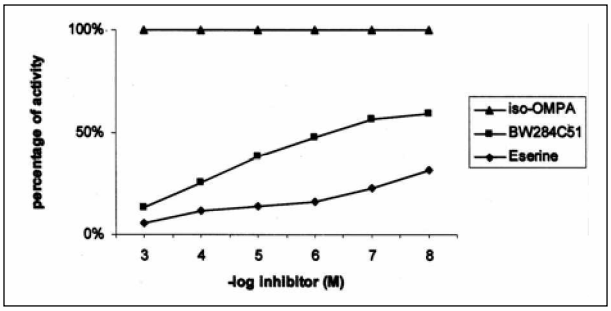

Fig. 2. Inhibition of cholinesterase (ChE) activity in *Taenia pisiformis* cysticerci. ChE activity was evaluated using ATCI as substrate. Assays were performed in triplicate and standard errors (< 10 %) are omitted

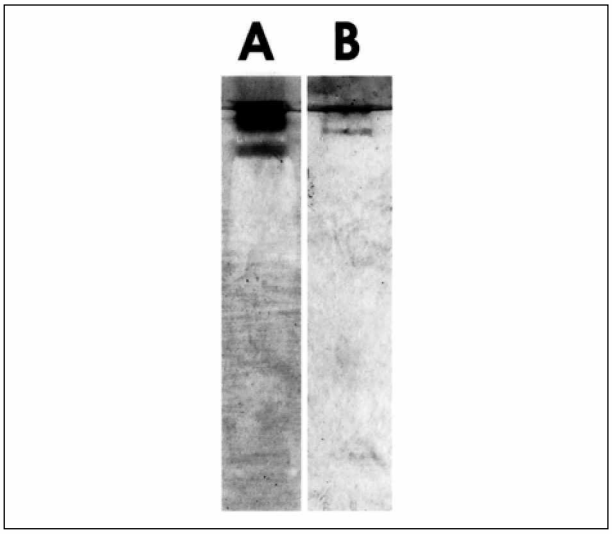

Fig. 3. Gels stained for cholinesterase activity. Native electrophoresis was carried out as described in methods in a 10 % polyacrylamide gel slabs. 15 µg protein per lane from soluble material from homogenates of *Taenia pisiformis* cysticerci was stained after electrophoresis for 24 h at room temperature by Karnovsky and Roots (1964) method. ATCI (A) and BTCI (B) were employed as substrate

of the cysticerci extract with 0.25 M NaSCN (a chaotropic anion) did not change this condition. Inhibition studies showed that cholinesterase activity was sensitive to the cholinesterase inhibitor eserine and to the AChE-specific inhibitor BW 284C51 (Fig. 2). In addition, the enzymatic activity was unaffected by the BuChE inhibitor iso-OMPA at concentrations up to 1 mM.

Confirmation of the presence of AChE was achieved on native gels by the method of Karnovsky and Roots (1964). Two closely zones of activity were visualized when ATCI was used as substrate (Fig. 3) whereas only one specific band was observed with BTCI.

AChE is an enzyme with widespread tissue distribution that hydrolizes acetylcholine in the synaptic cleft and thus regulates the transmission of impulses in the central nervous system of vertebrates, but the enzyme is also present in skeletal muscles and non-excitable tissue such as erythrocytes (Ott *et al.*, 1975). Despite the fact that Bueding (1952) had suggested previously that the presence of acetylcholinesterases in helminths may be related to motor activity, the possibility that this enzyme hydrolizes substance P and encephalines has been raised (Chubb *et al.*, 1983). It seems that AChE exhibits a trypsin-like proteolytic activity (Small *et al.*, 1987), and data suggest that this enzyme could have a role in addition to that related to the cholinergic systems.

AChE is an important tegument protein (Espinoza *et al.*, 1988; Camacho *et al.*, 1995; 1996), and tegument is the major point of metabolic interchange. The possible role of this enzyme as a surface peptidase of the worms, in breaking complex polypeptides into small fragments that are more easily absorbed remains speculative (Samuelson and Cauldfield, 1982). Phosphatases are also associated with the absorptive surfaces. Borges *et al.* (1975) proposed that hydrolases are synthesized in the tegumental cells and are transported towards the surface, where they are associated with extracellular digestion and subsequent uptake of nutrients (Verheyen *et al.*, 1976). Perhaps as happens with *Hymenolepis diminuta* cysticercoid, ChE and phosphatase activities may indicate membrane transport in the metacestode, a function subsequently lost in the adult (Bogitsh, 1967). Further studies characterizing the hydrolases of *T. pisiformis* cysticerci and their functional significance at this localization may help to clarify the biology of this parasite, but now it remains as a future point of action.

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