PRESENCE OF CHOLINESTERASES IN *ECHINOCOCCUS GRANULOSUS* PROTOSCOLICES

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Summary :

Cholinesterases were detected in protoscolices of *Echinococcus granulosus* spectrophotometrically and electrophoretically. To characterize these activities as acetylcholinesterases or pseudocholinesterases, BW284C51 and the organophosphate anthelmintic Neguvón® were assayed as specific inhibitors of acetylcholinesterases, while Iso-OMPA was employed as specific inhibitor of pseudocholinesterases. We concluded that these cholinesterase (ChE) activities would be considered as possible targets in chemotherapy.

KEY WORDS : cholinesterases, *Echinococcus granulosus*, protoscolices.

Résumé : PRÉSENCE DE CHOLINESTÉRASES AU NIVEAU DU PROTOSCOLEX D'*ECHINOCOCCUS GRANULOSUS*

*Des cholinestérases ont été détectées par spectrophotométrie et électrophorèse au niveau du protoscolex d'*Echinococcus granulosus. *Les activités – acétylcholinestérase et pseudocholinestérase – ont été caractérisées en utilisant des inhibiteurs spécifiques des acétylcholinestérases : BW 284 C51 et l'antihelminthique Neguvon®. L'Iso-OMPA a été utilisé en tant qu'inhibiteur spécifique des pseudocholinestérases. Les auteurs concluent que ces activités pourraient constituer une cible en matière de chimiothérapie.*

MOTS CLÉS : *cholinestérases,* Echinococcus granulosus, *protoscolex.*

There is little information about the neuromuscular system in cestode species of clinical significance (Smyth & McManus, 1989). Acetylcholine, 5-hydroxytryptamine (5-HT) and glutamate have been localized in cestodes (Webb & Eklove, 1989; Samii & Webb, 1990; Solís & De Jong, 1994; Brownlee *et al.*, 1994; Fairweather *et al.*, 1994; Terenina *et al.*, 1998) and there is a consensous that acetylcholine acts as the inhibitory neurotransmitter (Thompson & Mettrick, 1984; Sukhdeo & Mettrick, 1987), 5HT and glutamate stimulate the motility in cestodes working as the excitatory neurotransmitters (Sukhdeo & Mettrick, 1987; Webb & Eklove, 1989) and they have an activating effect on metabolism (Thompson & Mettrick, 1984). It is known that acetylthiocholine is degraded into thiocholine and acetate by means of acetylcholinesterase (AchE) activity (Ellman *et al.*, 1961).

Echinococcus granulosus protoscolices are waiting to become in adults upon ingestion by definitive hosts and their interest may be related to dissemination following the cyst rupture. With the finality to avoid this problem, several authors have worked with different drugs (Casado *et al.*, 1989; Pérez-Serrano *et al.*, 1997). In nematodes the ChE activities have been proposed as targets of some anthelmintics (Blackburn & Selkrik, 1992; Tekwani, 1992), in this paper, these activities in whole extracts of *E. granulosus* protoscolices were studied, and the organophosphate anthelmintic Neguvón® was assayed *in vitro* as an agent that effectively binds to AchE.

MATERIALS AND METHODS

Protoscolices of *E. granulosus* were removed aseptically from hydatid cysts from the liver of twenty naturally infected sheep slaughtered at the municipal abbatoir of Sepúlveda (Segovia, Spain). They were washed with 10mM sterile phosphate buffer solution (PBS pH 7.2) supplemented with penicillin (1 mg/ml) and dihydrostreptomycin sulphate (2 mg/ml), pooled and homogenized once at 4° C using a glass Potter-Elvejem homogenizer. Centrifugation of the homogenates was performed 2 × at 100,000 × g for 30'. The final supernatant was considered to be the cytosolic fraction. Protein concentration was measured by a modified Lowry method (Peterson, 1983) and adjusted to 2 mg/ml. Aliquots were then used immediately or frozen at – 80° C and the results from those experiences did not show differences between them. Cholinesterase (ChE) activity was determined spectrophotometrically according to Ellman *et al.*, (1961) using acetylthiocholine iodide (ATCI) or butyrilthiocholine iodide (BTCI) and electrophoretically corroborated by Karnovsky & Roots (1964) staining method with the same substrates. Briefly, 3 ml of 0.1 M PBS pH 8, 20 µl substrate, 50 µl

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of the sample and 100 µl of dithiobisnitrobenzoic acid solution, (39,6 mg dithiobisnitrobenzoic acid (DTNB), 10 ml PBS pH 7 and 15 mg sodium bicarbonate), were added to each tube and the absorbance was measured at 412 nm. Moreover, it was employed an enzyme-free blank at which 20 µl of substrate were added. The increases in OD were converted to units per litre (Ellman *et al.*, 1961). One unit is equivalent to 1 µmol of substrate hydrolyzed per minute per mg of protein. The inhibition of the acetylcholinesterase or pseudocholinesterase activities, was assessed according to Ellman procedure (Ellman *et al.*, 1961) using a microplate in which 260 µl of 0.1 M PBS buffer pH 8, 10 µl of 10 mM dithiobisnitrobenzoic acid solution, 2.5 µl of 75 mM substrate and 25 µl of the sample (which were 20 µl of protoscolices crude extracts plus 5 µl of each inhibitor, incubated 10' at 37° C) were added to each tube and the OD increment was measured at 412 nm. Inhibitors and concentrations employed were 40,000 nM, 4,000 nM, 40 nM and 4 nM of 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284C51) and 50 mM, 5 mM, 0.5 mM and 0.05 mM of tetraisopropylpyrophosphoramide (Iso-OMPA). Experiences were performed in paralel with their respective controls in which an inhibitor-free blank was employed. These inhibitors were used since they are considered, at least for Vertebrata, as specific inhibitors of AchE (EC 3.1.1.7) and BchE (EC 3.1.1.8) respectively, and they are used in recent studies on invertebrate enzymes (Talesa *et al.*, 1997). Neguvón® ($C_4H_8Cl_3O_4P$) (spot-on, Bayer AG), was employed as AchE inhibitor which irreversibly inhibits the enzyme, at the following concentrations: 4×10^{-3} mM, 0.05 mM, 0.5 mM, 5 mM, 50 mM and 400 mM. The inhibition was calculated as the percentage of ChE activity in the controls. In every case, each test was performed in triplicate and the readings were realized ten times (one per minute).

Electrophoresis in a 6 % polyacrilamyde gel without SDS (100-200 µg protein per lane diluted 3:1 in glycerol) was performed by Laemmli method (Laemmli, 1970) for two hours, and gels were incubated in: 20 mg ATCI or BTCI dissolved in 26 ml of 0.1 M PBS pH 6 for two hours to which the following were added sequentially: 2 ml 0.1 M sodium citrate, 4 ml 30 mM $CuSO_4$, 4 ml distilled water and 4 ml 5 mM potassium ferricyanide. Activity resulted in brown bands in the gel after two hours and it was fixed with 5 % acetic acid in deionized water.

RESULTS AND DISCUSSION

This work has revealed that protoscolices of *E. granulosus* have cholinesterase activities (Fig. 1). These activities appeared when ATCI or BTCI were employed as substrates by Karnovsky & Roots (1964) staining method. Moreover, this activity was also quantified by Ellman test (Ellman *et al.*, 1961). Hydrolysis of ATCI occurs as a higher relative rate than hydrolysis of BTCI as it happens in other helminths (Vos & Dick, 1992). The degradation of ATCI was 6.98 U/mg and the BTCI hydrolysis was 4.12 U/mg, both refered to 2 mg/ml protein concentration. The use of specific inhibitors (BW 284C51 and Iso-OMPA) corroborated that protoscolices have acetylcholinesterases and pseudocholinesterases. Note the presence of AchE, as demonstrate the higher inhibition percentages of this activity by BW 284C51 (Table Ia), while Iso-OMPA (Table Ib) had no effect on cholinesterase activity when the substrate used was ATCI. When BTCI was employed as substrate, boh inhibitors showed inhibitory effect though it could be due to the low activity obtained when we used this substrate as occurs in vertebrates (Massoulié *et al.*, 1993). When Neguvón®, the specific AchE inhibitor was employed, the results have shown that ChE activities are inhibited by the drug, this inhibition being statistically significative only when we employed ATCI as substrate, while significative BTCI hydrolysis happened at high concentrations (Table IIIa, b).

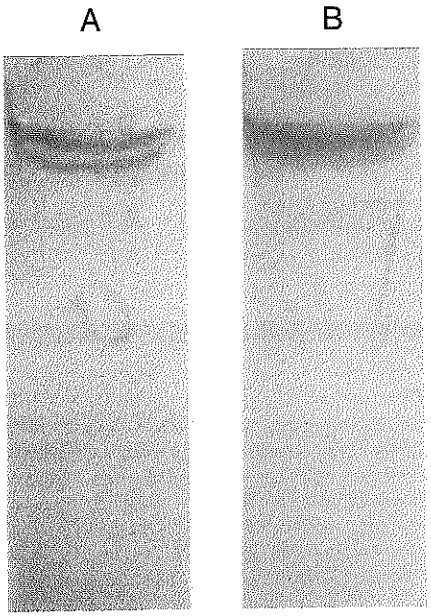

Fig. 1. – Cholinesterase activity in *Echinococcus granulosus* protoscolices by Karnovsky *et al.* (1964) staining method on A) Acetylthiocholine iodide (ATCI). B) Butyrilthiocholine iodide (BTCI).

Perhaps, *E. granulosus* protoscolices could release those cholinesterases in the hydatid cyst. This fact would open a new hypothesis of work since these enzymes could be able to pass through the germinal layer and come to the host-tissues destroying the host acetylcholine. Moreover, all these enzymes (acetyl and pseudocholinesterases) could be attractive targets for chemotherapeutic or immunological intervention in

A

Inhibitor (nM)	ChE activity (U/mg) X ± SD	% inhibition
0	6.98 ± 0.52	0
4×10^4	2.27 ± 0.10	68.42*
1×10^3	2.95 ± 0.14	58.94*
4×10^1	3.67 ± 0.13	47.36*
4	5 ± 0.44	28.42*

* $p \leq 0.05$.

B

Inhibitor (nM)	ChE activity (U/mg) X ± SD	% inhibition
0	4.12 ± 0.85	0
4×10^4	2 ± 0.37	76.58*
1×10^3	2.3 ± 0.63	73.14*
4×10^1	2.94 ± 0.73	66.20*
4	2.94 ± 1.3	66.20*

* $p \leq 0.05$.

Table I. – *Echinococccus granulosus* cholinesterase activity inhibited by BW. A) Substrate: ATCI. B) Substrate: BTCI. U: one unit is 1 µmol of substrate hydrolyzed per minute.

A

Inhibitor (mM)	ChE activity (U/mg) X ± SD	% inhibition
0	6.98 ± 0.52	0
50	4.7 ± 0.30	28.42*
5	7.7 ± 2.98	0
0.5	5.88 ± 0.77	15.78
0.05	5.58 ± 0.63	2.10

* $p \leq 0.05$.

B

Inhibitor (mM)	ChE activity (U/mg) X ± SD	% inhibition
0	4.12 ± 0.85	0
50	2.68 ± 0.72	69.58*
5	2.20 ± 0.67	71.75*
0.5	2.46 ± 0.47	72.08
0.05	3.30 ± 0.64	61.57*

* $p \leq 0.05$.

Table II. – *Echinococccus granulosus* cholinesterase activity inhibited by Iso-OMPA. A) Substrate: ATCI. B) Substrate: BTCI. U: one unit is 1 µmol of substrate hydrolyzed per minute.

hydatid disease as Geary *et al.* (1992) proposed in other helminth infections, and, in future, a new combination of compounds that inactivate the enzyme could allow acetylthiocholine to be accumulated causing abnormally high levels of inactivation and a total blockage of the nerve function (Thompson & Mettrick, 1984; Sukhdeo & Mettrick, 1987), but until now it remains open to speculation.

A

Inhibitor (mM)	ChE activity (U/mg) X ± SD	% inhibition
0	6.98 ± 0.52	0
400	1.50 ± 0.19	59*
50	1.69 ± 0.35	68*
5	2.30 ± 0.40	51*
0.5	3.97 ± 0.32	60*
0.05	3.52 ± 0.18	18
4×10^{-3}	3.52 ± 0.36	18

* $p \leq 0.05$.

B

Inhibitor (mM)	ChE activity (U/mg) X ± SD	% inhibition
0	4.12 ± 0.85	0
400	1.10 ± 0.12	35.54*
50	1.10 ± 0.14	35.54*
5	1.58 ± 0.19	0
0.5	1.47 ± 0.22	6.64*
0.05	1.25 ± 0.32	12.70
4×10^{-3}	0.7 ± 0.019	0

* $p \leq 0.05$.

Table III. – *Echinococccus granulosus* cholinesterase activity inhibited by Neguvón®. A) Substrate: ATCI. B) Substrate: BTCI. U: one unit is 1 µmol of substrate hydrolyzed per minute.

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