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Acetylcholinesterase from mature *Hymenolepis diminuta* (Cestoda)

Tadeusz Moczoń* and Agnieszka Świetlikowska

W. Stefański Institute of Parasitology, Polish Academy of Sciences, 51/55 Twarda Street, 00-818 Warszawa, Poland

Abstract

Acetylcholinesterase (AChE) sequentially extracted from mature specimens of *Hymenolepis diminuta* was shown to be a globular protein, the monomeric form of which (G_1^a) had molecular mass of 66 kDa as determined by SDS-PAGE. Amphiphilic character of the enzyme was revealed by Triton X-114 phase partitioning. The cestode AChE preferred acetylthiocholine over propionyl- and butyrylthiocholine as substrate, split *N*-acetyl-β-methylthiocholine and myristoylcholine but did not hydrolyze β-carbonaphthoxycholine, a substrate for butyrylcholinesterases. It was sensitive to 10^{-5} M physostigmine and 10^{-5} M BW284C51 but not to 10^{-3} M *iso*-OMPA. No butyrylcholinesterase activity was detected in extracts from the parasite.

Key words

Hymenolepis diminuta, biochemistry, acetylcholinesterase, electrophoresis, phase partitioning

Abbreviations

BW284C51 – 1,5-*bis*-(4-allyldimethylammoniumphenyl)-pentane-3-one dibromide; CHAPS – [3-(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CTAB – cetyltrimethylammonium bromide; DTNB – 5,5'-dithio-*bis*-(2-nitrobenzoic) acid (Ellman reagent); EDTA – ethylenediaminetetraacetic acid; EGTA – ethylene glycol-*O*,*O'*-*bis*-(2-aminoethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid, GPI – glycosylphosphatidylinositol; *iso*-OMPA – tetraisopropylpyrophosphoramide; NEM – *N*-ethylmaleimide; PAGE – polyacrylamide gel electrophoresis; *p*HMB – sodium *p*-hydroxymercuribenzoate; PI-PLD – phosphatidylinositol-dependent phospholipase D; SB-12 – *N*-dodecyl-*N*,*N*-dimethylammonio-3-propane sulfonate; SDS – sodium dodecyl sulfate; TEMED – *N*,*N*,*N'*,*N''*-tetramethylethylenediamine.

Introduction

Cholinesterases can be divided into two major classes: butyrylcholinesterases (BChE) with a high affinity for butyrylcholine as substrate, and acetylcholinesterases that hydrolyze primarily acetylcholine, the natural transmitter substance in brain and excitable tissues. The distinction between these two classes of the enzymes can be made with the aid of a number of cholinesterase inhibitors which selectively block the activity of BChE (*iso*-OMPA) or AChE (BW284C51, ambenonium) and by the use of certain specific substrates, i.e., *N*-acetyl-β-methyl(thio)choline which is hydrolyzed by AChEs and either benzoylcholine or β-carbonaphthoxycholine which are hydrolyzed by BChEs (Ravin *et al*. 1951, Erdös and Debay 1960).

AChE occurs in multiple molecular forms in different tissues of a variety of vertebrates and invertebrates (Massoulié and Bon 1982, Massoulié *et al*. 1999, Massoulié 2002). In vertebrates, AChE molecules are generated from a single gene. In invertebrates up to four AChE genes may occur, like in the free-living nematodes *Caenorhabditis elegans* and *C. briggsae* (Grauso *et al*. 1998). The molecular diversity of AChE results from alternative splicing of corresponding gene(s) and from post-translational maturation. These processes generate four types of catalytic subunits depending on their C-terminal regions (Taylor 1991, Ben Aziz-Aloya *et al*. 1993, Massoulié 2002). The H ("hydrophobic") type that bears an H terminal peptide gives rise to a globular, amphiphilic GPI-anchored form of the enzyme (GPI-G_2^a) which has been found in plasma membrane of certain cell types of vertebrates and invertebrates (Massoulié *et al*. 1999), including the tegumental plasma membrane of *Schistosoma mansoni* and *S. haematobium* (Levi-Schaffer *et al*. 1984, Espinoza *et al*. 1991, Jones *et al*. 2002). The T ("tailed") type that bears a T terminal peptide gives rise to (a) three globular amphiphilic forms: a monomeric, a dimeric, and a tetrameric form (G_1^a, G_2^a, and G_4^a), which

*Corresponding author: moczon@twarda.pan.pl

occupy various tissues of vertebrates and invertebrates, (b) a globular soluble (nonamphiphilic) tetrameric form (G^{na}_1) present in body fluids of mammals, (c) a hydrophobic glycopeptide-tailed tetrameric form (G^{a}_4) which occupies mammalian brain (Gennari *et al*. 1987, Inestrosa *et al*. 1987), and (d) asymmetric, collagen-tailed forms A_4, A_8, and A_{12}, which are nonamphiphilic and synthesized in a number of cell types of vertebrates (Carson *et al*. 1979; Rieger *et al*. 1980; Krejci *et al*. 1991, 1997; Perrier *et al*. 2000). The monomeric soluble S (from "snake") type of the catalytic subunit has only been found in venoms of elapid snakes (Cousin *et al*. 1996a, b), and the monomeric soluble R (from "readthrough") type that results from a lack of splicing is expressed in mammals under psychological, chemical, and physical stress, but it also appears in embryonic and tumor cells of certain mammals (Lev-Lehman *et al*. 2000, Mor *et al*. 2001, Meshorer and Soreq 2002, Deutsch *et al*. 2002, Birikh *et al*. 2003, Brenner *et al*. 2003).

BChE, formerly named "pseudocholinesterase", is expressed in tissues of vertebrates as a product of a separate gene, distinct from that of AChE. Catalytic subunits of BChE are of the T type (Massoulié 2002). Membrane-bound BChE occurs in various tissues of vertebrates and a nonamphiphilic (soluble) form is present in body fluids. Numerous papers published over last 20 years unequivocally indicate that invertebrates synthesize only globular forms of AChE and do not synthesize BChE. The aim of this work was to characterize cholinesterase(s) from the cestode *Hymenolepis diminuta*.

Materials and methods

Mature specimens of *H. diminuta* were isolated from the intestine of laboratory rats. The worms were extensively washed with phosphate-buffered saline at 37°C, rinsed with distilled water and processed immediately or kept frozen overnight at –20°C. Using a whole glass tissue grinder with a motor driven pestle, approximately 3 g of the tissue was homogenized in 5 ml of either 10 mM Tris-HCl or sodium phosphate buffer, pH 7.4, which were supplemented with 1 mM benzamidine hydrochloride and 0.5 mM bacitracin as proteinase scavengers. The homogenate was centrifuged at 10,000 *g* for 5 min, at 4°C and the pellet was discarded. The supernatant was centrifuged at 100,000 *g* for 60 min, at 4°C and the supernatant (low salt extract) was collected. Subsequently, the pellet was re-homogenized in the mixture of 1% Triton X-100 and either a 10 mM Tris-HCl or a sodium phosphate buffer at pH 7.4. Following centrifugation for 60 min at 100,000 *g* the supernatant (detergent-soluble extract) was collected, the pellet was re-homogenized in a 1 M NaCl-25 mM Tris-HCl or a sodium phosphate buffer at pH 7.4, centrifuged as above and the supernatant (high salt extract) was saved. Finally, the three collected extracts were re-centrifuged at 100,000 *g* for 60 min, at 4°C and the volumes of the obtained supernatants were equalized by the addition of appropriate solvents.

Nondenaturing electrophoresis

Nondenaturing PAGE was performed according to Laemmli (1970). Uniform (T6, C2) minigels, 1 mm in thickness, were photopolymerized using sodium riboflavin 5′-monophosphate and TEMED. Anionic detergents (SDS, sodium deoxycholate, sodium glycocholate or taurocholic acid) were added to extract samples, to the stacking gel and to the catholyte at a final concentration of 0.1%. Nonionic detergents (1% Triton X-100, 1% Nonidet P-40, 1% Lubrol PX, 1% Brij-35, 1% *N*-octyl-β-D-glucoside, 0.1% saponin or 0.1% digitonin sulphate), zwitterionic detergent CHAPS (0.1–2%) or zwitterionic sulfobetaine SB-12 (1%) were added to extract samples and to gels. An effect of 5 mM EDTA and 5% (0.7 M) 2-mercaptoethanol on the separation pattern of AChE in the presence of nonionic detergents was also examined. EDTA was added to extract samples and to the catholyte. The reducing reagent was added to extract samples 60 min prior to electrophoresis.

Following separation the gels that contained anionic detergents were extensively washed with a mixture of 0.05 M maleic acid-NaOH buffer and 1% Triton X-100 at pH 6.0, and with the buffer alone (Heussen and Dowdle 1980). Gels that contained other detergents were washed with a Triton-free maleate buffer. Subsequently, all gels were incubated under mild continuous shaking in a medium comprising 0.05 M maleate buffer at pH 6.0, 5 mM $CuSO_4$, 25 mM glycine and 5 mM acetylthiocholine iodide. Following 4–6 h of the incubation the gels were extensively washed with tap and distilled water, transferred to tight capped plastic boxes and sealed with a "developing" mixture comprising 0.25 M 2-amino-2-methyl-1,3-propanediol-HCl buffer at pH 9.0, to which a small volume of ammonium sulfide was added just before use. The gels were kept in this slightly yellow mixture until white bands of the product of the histochemical reaction became perfectly black. After washing with 5% acetic acid and distilled water the reaction patterns were recorded and then copied onto graphically prepared backgrounds.

Rates of hydrolysis of different cholinesterase substrates were evaluated after incubation of gels in 0.05 M maleate buffer at pH 6.0 that contained 5 mM iodides of acetyl-, propionyl-, butyryl- or *N*-acetyl-β-methylthiocholine. In order to ascertain whether or not, the enzyme hydrolyzes esters of choline and long chain fatty acids, some gels were incubated in a mixture comprising 0.03 M Tris-maleate buffer at pH 7.6, 0.0125 M cobaltous acetate, and 20 mM myristoylcholine chloride (Gomori 1948). Detection of butyrylcholinesterase activity in gels was attempted by means of a simultaneous azo-coupling method. The incubation medium comprised 0.05 M sodium phosphate buffer at pH 7.3, 1 mM β-carbonaphthoxycholine iodide (initially dissolved in a small volume of *N*-dimethylformamide), and either Fast Garnet GBC·BF_4 salt or Fast Red TR·$ZnCl_2$ salt (1 mg ml^{-1}) and was filtered before use. Because of a relatively low stability of diazonium salts in solution, the medium was changed every two hours. To examine of the importance of ionized –SH groups

for the activity of AChE, the enzyme in extract samples was treated with either 1 mM *p*HMB or 1 mM NEM prior to electrophoresis. Sensitivity of the separated enzyme to cholinesterase inhibitors was evaluated by preincubation of gel strips in 0.05 M maleate buffer at pH 6.0, which was supplemented with physostigmine sulfate (10^{-6} M and 10^{-5} M), BW254C51 (10^{-6} M and 10^{-5} M), ambenonium chloride (10^{-6}–10^{-4} M), edrophonium chloride (10^{-5}–10^{-3} M), *iso*-OMPA (10^{-5}–10^{-3} M, from a stock solution prepared in isopropanol), decamethonium iodide (10^{-4} and 10^{-3} M), quinine sulfate (10^{-4} M and 10^{-3} M), 10 mM sodium amobarbital, 10 mM atropine sulfate or 10 mM theophylline. Following 45 min of the preincubation under mild shaking, the gel strips were transferred to acetylthiocholine containing histochemical media with the above inhibitors, incubated for 4 h, washed and examined.

Treatment with PI-PLD

As it has been shown that glycosylphosphatidylinositol-anchored AChE occurs in the tegument of schistosomes (Tarrab-Hazdai *et al*. 1984, Espinoza *et al*. 1991, Camacho *et al*. 1996, Jones *et al*. 2002), we exposed the low salt extract and the corresponding pellet (i.e. the fraction of plasma membranes and microsomes), to the action of human serum PI-PLD in order to record a change in the pattern of the electrophoretic separation of AChE, resulting from removal of diradylglycerol from an eventual GPI anchor of the enzyme molecules. The relevant mixtures comprised 50 µl of low salt extract or cell membranes, 10 mM Tris-HCl buffer at pH 7.4, 10 µl of freshly obtained human serum (pretreated or not pretreated with 10^{-4} M *iso*-OMPA), 0.05% Triton X-100, and either 2.6 mM $CaCl_2$ or 5 mM EGTA (Davitz *et al*. 1987, 1989; Low and Huang 1991). Control mixtures did not contain serum. Following incubation for 12 h at 37°C samples of all mixtures were electrophoresed in the presence of 1% Triton X-100 whereupon the gels were processed for histochemical detection of AChE activity.

Triton X-114 phase partitioning

In order to ascertain whether or not, the cestode enzyme is an amphiphilic protein, 1 ml of the low salt extract was supplemented with NaCl (0.15 M) and 200 µl of precondensed 12% Triton X-100. The mixture was kept at the temperature of melting ice for 10 min. Phase separation was promoted at 37°C for 10 min, and the sample was centrifuged at 14,000 *g* for 30 sec. The two phases were transferred to separate tubes and submitted to a new phase separation (Bordier 1981, Brusca and Radolf 1994). Following a third cycle of phase separation, the volumes of the final two phases were equilibrated by addition of 1% Triton X-100 to the Triton X-114 phase. Samples of low salt extract-NaCl mixture (control), final upper (hydrophilic) phase, and final bottom (hydrophobic) phase were electrophoresed in a 1% Triton X-100 containing polyacrylamide gel. Following electrophoresis the gel was processed for histochemical detection of AChE.

Determination of relative molecular mass

Relative molecular mass (M_r) of the cestode AChE was determined by SDS-PAGE (Laemmli 1970) in a photopolymerized linear gel gradient from T5, C2.6 to T14, C2.6, under reducing and nonreducing conditions. The heating step was omitted. Fully reduced standard proteins of known molecular mass were separated in parallel to the low salt extract. Following separation the gel fragment that contained standard proteins was cut off and routinely stained with Coomassie Blue. The remaining fragment was washed with the maleate-Triton X-100 buffer, with the buffer alone and, subsequently, it was processed for detection of the activity of AChE using acetylthiocholine iodide as substrate.

Spectrophotometric assay

Activity of AChE was measured at pH 7.3 by the method of Ellman (Ellman *et al*. 1961) using 1 mM iodides of acetylthiocholine, propionylthiocholine, butyrylthiocholine, and *N*-acetyl-β-methylthiocholine as substrate. The enzyme in the reference samples was inactivated with 10^{-3} M physostigmine. Sensitivity of AChE to cholinesterase inhibitors was examined using physostigmine, BW284C51, ambenonium, and *iso*-OMPA at final concentrations from 10^{-9} M to 10^{-3} M. Stock solutions of these inhibitors were added to assay mixtures 15 min prior to the addition of acetylthiocholine and optical densities were read in a double beam spectrophotometer following 15 min of an incubation in subdued light. For calculation of the enzyme activity, a molar absorption coefficient of 2-nitro-5-thiobenzoate (the chromophore) was utilized (ε_{412} = 14,150 M^{-1} cm^{-1} at pH 7.3; Riddles *et al*. 1979). Protein concentration was measured with the Coomassie Blue protein quantitation method (Bradford 1976) using bovine serum albumin as standard.

Results

As a result of the sequential extraction, approximately 94% of the extractable activity of AChE was found in the low salt extract (1.1 nmoles of hydrolyzed acetylthiocholine min^{-1} µg of extract $protein^{-1}$), 5.5% in the detergent soluble extract (0.07 nmoles min^{-1} μg^{-1}), and 0.5% in the high salt extract (0.006 nmoles min^{-1} μg^{-1}). Electrophoretic mobility of AChE from the three extracts was identical (Fig. 1, lanes 3–8). The scarcely detectable amounts of the enzyme present in the two last extracts were not subjected to further examinations.

The examined AChE tended to form large aggregates, apparently also with other extract proteins (Fig. 1, lanes 1 and 2). An appearance of milky precipitates in slots after switching on the current (5 mA, 6.7 V × cm^{-1}) indicated that a progressive concentration of sample proteins in slots, which was forced by the advancing zone of glycine ions, was responsible for the clustering of extract proteins. In the absence of a detergent the aggregates that contained almost the whole activity of AChE did not enter the 4% stacking gel. Triton X-

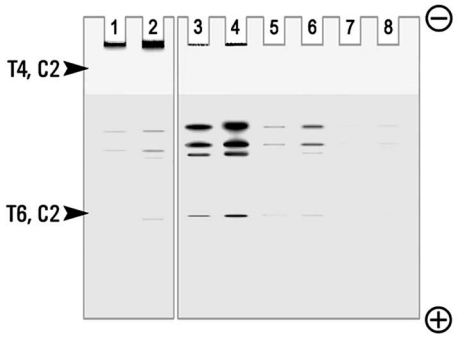

Fig. 1. Nondenaturing electrophoresis of AChE from *H. diminuta*. Single and double volumes of three consecutively obtained extracts were loaded to the even and to the odd slots, respectively. Left fragment of the gel: a separation in the absence of any detergent. **1** and **2.** Low salt extract. Most activity of the enzyme remained at start. Right fragment of the gel: a separation in the presence of 1% Triton X-100. **3** and **4.** Low salt extract that contained almost the whole extractable activity of AChE. **5** and **6.** Detergent-soluble extract. **7** and **8.** High salt extract

100 disrupted most of these protein clusters and this resulted in the appearance of four AChE-positive bands in polyacrylamide gels (Fig. 1, lanes 3 and 4). None of other nonionic detergents as well as the zwitterionic detergent CHAPS and the zwitterionic lauryl sulfobetaine SB_{12}, produced better resolution and deaggregation (Fig. 2). Both EDTA and 2-mercaptoethanol did not influence the separation pattern in the presence of Triton X-100, thus indicating that neither divalent cation ionic bridges nor spontaneously oxidized –SH groups in proteins were involved in the aggregation. It was suspected, therefore, that the three slow migrating bands represented oligomers of the enzyme molecules that were held together by hydrophobic forces, whereas the fastest band represented the monomeric form of the enzyme. This supposition was supported by the observation that after a prolonged storage of low salt extract supplemented with 1% Triton X-100, the activity of AChE in the fastest band gradually decreased. This was most likely due either to a spontaneous oligomerization of monomeric AChE molecules or to their incorporation into the existing oligomeric forms.

Incubation of the low salt extract and of the plasma membrane microsome fraction with human serum produced no changes in the separation pattern of AChE as compared with controls (Fig. 3). The presence of calcium ions (which activate human serum PI-PLD) or EGTA (a strong complexane of Ca^{2+}) in some of the incubation mixtures also produced no effect. These results indicated that a GPI-anchored, strongly aggregating form of AChE does not exist in the organism of the mature cestode. The Triton X-114 phase partition experiment evidenced an amphiphilic nature of the enzyme, the bulk activity of which was found in the hydrophobic phase (Fig. 4).

Following electrophoresis in the presence of either SDS or a bile salt, a single AChE-positive band was observed in the gel (Fig. 2). However, it was not known whether this single band represented the whole and completely deaggregated AChE, or some of presumptive oligomeric forms of AChE (Fig. 2, lane 2) were irreversibly inactivated by the ionic detergents. Spectrophotometric assays failed to resolve this question, because zero activity of AChE that was recorded in the Ellman mixture supplemented with 0.1% SDS could result from binding of either thiocholine or the chromophore or both, by SDS. Consequently, a separate experiment consisting in the following steps was performed: (a) PAGE in the presence of 1% Triton X-100, (b) washing (2 × 10 min) to remove excess Triton X-100 from the gel, (c) incubation (3 × 15 min) in 0.1% SDS, (d) washing (3 × 10 min) in 1% Triton X-100 to renature the enzyme, (e) washing (15 min) to remove excess Triton X-100, and (f) a prolonged histochemical reaction (some of the enzyme became eluted from the gel) which resulted in the appearance of four bands in the gel. Thus, it became evident that no an AChE species irreversibly denatured by SDS occurred in the gel, and that the single AChE-positive band observed after nonreducing SDS-PAGE (Fig. 2,

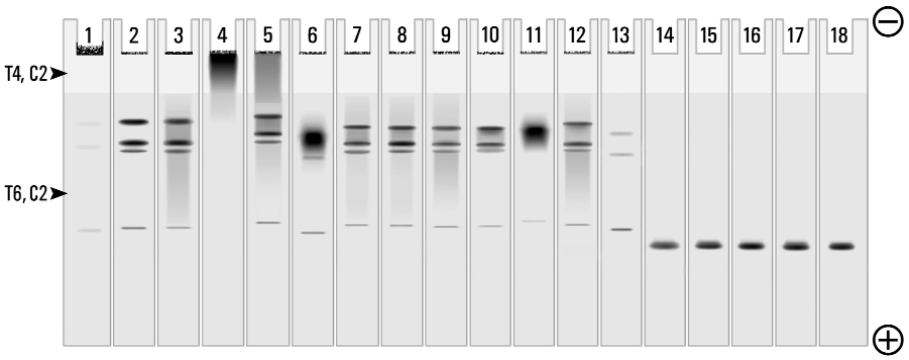

Fig. 2. Effect of various detergents on the separation pattern of AChE from the cestode. **1.** No detergent. **2.** 1% Triton X-100. **3.** 1% Nonidet P-40. **4.** 0.5% Lubrol PX. **5.** 0.5% Brij-35. **6.** 1% *N*-octyl-β-D-glucoside. **7.** 0.1% saponin. **8.** 0.1% digitonin. **9.** 1% Triton X-100 and 0.1% CHAPS. **10.** 1% Triton X-100 and 0.5% CHAPS. **11.** 2% CHAPS. **12.** 1% Triton X-100 and 0.1% SB-12. **13.** 1% SB-12. **14.** 0.1% SDS. **15.** 0.1% deoxycholate. **16.** 0.5% Triton X-100 and 0.2% deoxycholate. **17.** 0.1% glycocholate. **18.** 0.1% taurocholate

lane 14; Fig. 5, lane 3) represented a monomeric form of the enzyme that had relative molecular mass of approximately 66,000 (Fig. 5).

Fig. 3. Results of the treatment of the cestode AChE with human serum PI-PLD for 12 h at 37°C. Prior to electrophoresis in the presence of 1% Triton X-100 the buffered samples of the low salt extract (lanes 1–6) were incubated as follows: **1.** In the absence of serum, $CaCl_2$, and EGTA (control I). **2.** In the absence of serum but in the presence of 2.6 mM $CaCl_2$ (control II). **3.** In the absence of serum but in the presence of 5 mM EGTA (control III). **4.** In the presence of both serum and 2.6 mM $CaCl_2$ (the activity of serum BChE dimers is visible). **5.** In the presence of serum and 5 mM EGTA. **6.** Serum pretreated with 10^{-4} M *iso*-OMPA was applied into this slot. **7.** A buffered sample of the plasma membrane and microsome fraction (obtained after the first ultracentrifugation), which was incubated in the absence of serum. **8.** Same as 7 but incubated in the presence of serum and 2.6 mM $CaCl_2$

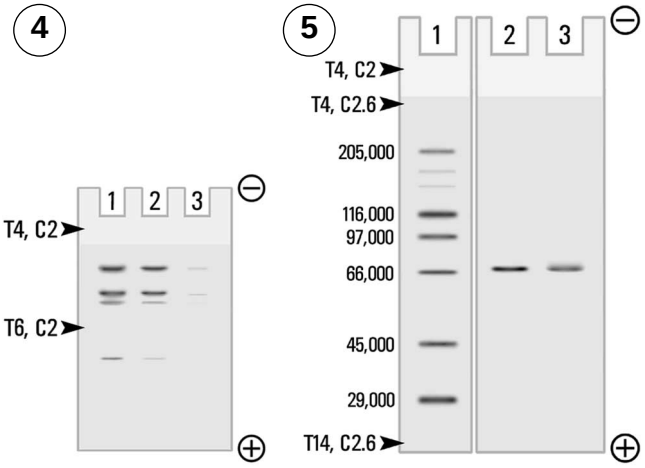

Fig. 4. Amphiphilic nature of the cestode AChE. The three-cycle phase partitioning was followed by a nondenaturing electrophoresis of two final phases, in a 1% Triton X-100 containing gel. **1.** A sample of the low salt extract that was not submitted to the phase partitioning (control). **2.** A sample of the hydrophobic phase. **3.** A sample of the hydrophilic phase. **Fig. 5.** Determination of M_r of the cestode AChE by means of SDS-PAGE. **1**. Standard proteins. From top to bottom: myosin from rabbit muscle, β-galactosidase from *Escherichia coli*, phosphorylase b from rabbit muscle, bovine serum albumin, albumin from chicken egg white, and carbonic anhydrase from bovine erythrocytes. **2**. AChE from the cestode. SDS (2%) and 2-mercaptoethanol (5%) were added to the sample of the low salt extract 60 min prior to the run. The heating step was omitted. **3.** Same as 2, except that no mercaptoethanol was added to the sample

Incubation of gels in histochemical media comprising various substrates revealed that the examined enzyme hydrolyzed acetylthiocholine at a highest rate and did not hydrolyze β-carbonaphthoxycholine, the substrate for butyrylcholinesterases (Fig. 6). Myristoylcholine was hydrolyzed at a very low rate, since first precipitates of the reaction product appeared in gels following 24 h of the incubation period, whereas those resulting from the hydrolysis of acetylthiocholine became visible following 90 min of the incubation. The longer the carbon chain in thiosubstrates, the lower was the rate of their hydrolysis. Quantitative measurements revealed that the activity of AChE towards acetylthiocholine was approximately 3-fold and 7-fold higher than towards propionylthiocholine and butyrylthiocholine, respectively. The activity of the enzyme towards *N*-acetyl-β-methylthiocholine, a substrate that is not hydrolyzed by BChEs (except BChE from chicken serum), was approximately 16-fold lower than that towards acetylthiocholine. Qualitative studies on the inhibition showed that neither *p*HMB nor NEM inhibited the activity of the enzyme. Physostigmine (10^{-5} M) and BW284C51 (10^{-5} M) produced a complete inhibition of AChE in the gels, while the treatment with 10^{-3} M *iso*-OMPA produced no visible loss in the activity towards acetyl- and butyrylthiocholine as substrates. Ambenonium and edrophonium (10^{-5} M) slightly reduced the activity of AChE but inhibited it when used at the concentration of 10^{-4} M. Decamethonium (10^{-3} M) and quinine (10^{-3} M) reduced the activity approximately by a half, whereas 10 mM amobarbital, 10 mM atropine and 10 mM theophylline were not inhibitory at all. Figure 7 illustrates results of spectrophotometric assays of the activity of AChE treated with physostigmine, BW284C51, ambenonium, and *iso*-OMPA.

Discussion

The presence of acetylcholinesterase activity in *Hymenolepis diminuta* was demonstrated histochemically by Lee *et al*. (1963), Schardein and Waitz (1965), Wilson and Schiller (1969), Douglas (1966), and Bogitsh (1967). In certain tissues of the parasite some of these authors observed a slight activity of "pseudocholinesterase" which was insensitive to BW284C51 but sensitive to *iso*-OMPA. Similar observations were made by Roy (1980) on *Raillietina johri*, by Krvavica *et al*. (1967) on *Fasciola hepatica* and by Fripp (1967) on some schistosome adults. It seems unlikely that the artefactual activity of "pseudocholinesterase" in the examined helminths was due to the use of aldehyde-fixed material. Gunn and Probert (1981), who examined AChE in homogenates from the cestode *Moniezia expansa* did not find an evidence for the occurrence of butyrylcholinesterase activity in the parasite. Evidence reported here indicates that butyrylcholinesterase is also absent from *H. diminuta*. The enzyme examined by us proved to be "true" acetylcholinesterase which hydrolyzed acetylthiocholine at a highest rate, split *N*-acetyl-β-methylthiocholine, was sensitive to the specific inhibitor BW284C51

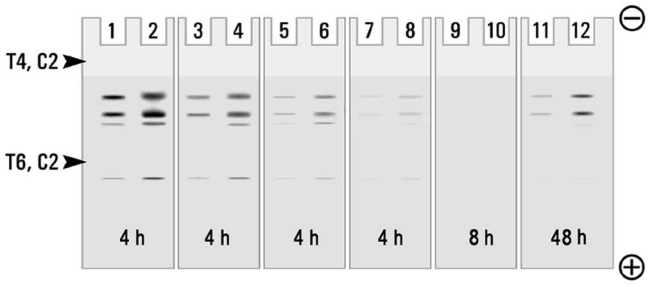

Fig. 6. Activity of the electrophoresed AChE as a result of the incubation of gel strips in histochemical media comprising the following substrates: acetylthiocholine iodide (**1** and **2**), propionylthiocholine iodide (**3** and **4**), butyrylthiocholine iodide (**5** and **6**), *N*-acetyl-β-methylthiocholine iodide, (**7** and **8**), β-carbonaphthoxycholine iodide (**9** and **10**), and myristoylcholine chloride (**11** and **12**). Single and double volumes of low salt extract samples were loaded to the consecutive pairs of slots. Numbers at the bottom of gel strips indicate the duration of their incubation in the histochemical media

(10^{-5} M), did not hydrolyze β-carbonaphthoxycholine, and was insensitive to 10^{-3} M *iso*-OMPA, an organophosphate which inactivates most of vertebrate butyrylcholinesterases when used at the concentration range from 10^{-6} to 10^{-5} M. The examined AChE was weakly sensitive to ambenonium and edrophonium, which produced a complete inhibition at the concentration of 10^{-4} M. However, such a low inhibitory power could result from a relatively high concentration of acetylthiocholine used in the incubation medium for gels (5 mM) and in the Ellman assay mixture (1 mM). This may also apply to 1 mM decamethonium, which only partly inactivated the cestode enzyme, whereas mammalian AChEs become fully inhibited by 5×10^{-5} M decamethonium. It

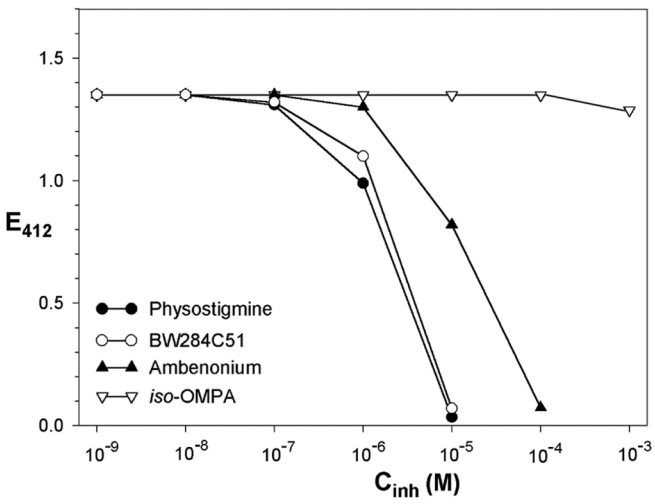

Fig. 7. Sensitivity of AChE to four cholinesterase inhibitors as examined with the method of Ellman *et al*. (1961) with acetylthiocholine as substrate

seems doubtful that certain tissue components present in the low salt extract from the cestode could bind ambenonium, edrophonium, and decamethonium in a manner that might drastically reduce the effective concentrations of these inhibitors. Ambenonium, a reversible *bis*-quaternary inhibitor that binds to the peripheral anionic site and to the anionic subsite of AChEs, exhibits mixed competitive and uncompetitive inhibition with acetylthiocholine as substrate. Edrophonium and decamethonium, the *mono*-quaternary reversible ligands bind only to the anionic subsite and act essentially as competitive inhibitors. In the case of AChE from human erythrocytes the inhibition constants for the competitive components are 0.12 nM for ambenonium and 470 μM for edrophonium (Hodge *et al*. 1992).

There are few reports on the sensitivity of AChEs to –SH-blocking reagents. Mounter and Whittaker (1953) presented results of treatment of AChEs from various sources, with a number of reagents more or less specifically reacting with free (i.e., ionized) sulphydryl groups. They came to the conclusion that the activity of the examined enzymes is rather independent on the presence of these groups. Zahavi *et al*. (1972) found that both DTNB and *p*HMB (but not iodoacetate) inhibit the activity of AChE from green peach aphid *Myzus persicae*. According to Mutus *et al*. (1983) a single –SH group (cys-231) that occurs in the catalytic subunit of AChE from *Torpedo*, is essential for activity. Moreover, a reversal of the blockade of this group does not result in reactivation of the enzyme (Dolginova *et al*. 1992). Our experiments showed that the activity of AChE from *H. diminuta* is insensitive to both *p*HMB and NEM, so the enzyme belongs to the majority of AChEs whose activity is independent on free –SH groups.

The cestode AChE was shown to be an amphiphilic protein, the monomeric form of which has relative molecular mass of approximately 66,000. The efficient solubilization of it in a 10 mM phosphate or Tris buffer in the absence of any detergent suggests that the enzyme in the parasite cells was tethered hydrophobically to the peripheral layer of plasma membranes. As the invertebrates do not synthesize asymmetric forms of AChE, and since the occurrence of a GPI-anchored form of the enzyme in the parasite body was ruled out, one should conclude that only the amphiphilic G_1^a form and its oligomers (G_2^a and G_4^a) remained as candidates for the native forms of AChE in *H. diminuta*. However, because of a possible aggregation during homogenization and because it is not known whether or not, the enzyme was completely freed from other proteins by such a "mild" detergent as Triton X-100, it is impossible to guess which AChE-positive bands in our electrophoregrams correspond exactly with these native oligomeric forms. It is known that during homogenization amphiphilic AChEs form complexes with laminin through strong electrostatic interactions (Johnson and Moore 2003) and certain cell components mediate the formation of complexes of AChEs with acetylcholine receptors (Wallace *et al*. 1985). At low ionic strength such complexes are strong enough to withstand certain procedures of purification of

AChEs, e.g., molecular filtration and affinity chromatography. Hydrophobic interactions between proteins are often strong enough to withstand the solubilizing power of a number of nonionic and zwitterionic detergents. In our experiments the anionic SDS and bile salts dissociated AChE oligomers into the monomeric form, while not irreversibly denaturing of the enzyme. This also applies to the cationic detergent CTAB, as we have found by a single electrophoretic run of the low salt extract in the presence of 0.05% CTAB, followed by removal of this cationic detergent with Triton X-100 prior to the histochemical reaction. In contrast with SDS and CTAB, which are of high solubilizing power due to their long and flexible hydrophobic tail and charged head, the bile salts with their flat and rigid hydrophobic steroid head and a short charged tail are considered as "mild" ionic detergents. In spite of these differences, which have a bearing on effectiveness of "unsticking" of hydrophobic domains of aggregated proteins, both these surfactant types dissociated the parasite AChE oligomers into the monomeric form. On the contrary, nonionic polyoxyethylene detergents with their rather long hydrophobic tails (Triton X-100, Lubrol PX, Nonidet P-40, Brij-35) as well as digitonin and saponin with their glycosylsteroid and glycosyltriterpene core, respectively, and also the zwitterionic CHAPS with its zero net charge, were all incapable to do so. This suggests that electrostatic forces in addition to hydrophobic interactions were involved in the assembly of the enzyme oligomers, at least under the conditions of electrophoresis, particularly at the solid (gel)-liquid (buffer) interface. We believe that sedimentation analysis in sucrose gradients at a low and a high ionic strength, combined with a nonionic detergent, will help in the identification of the particular native forms of the cestode acetylcholinesterase.

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