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Presence and Properties of Thymidine Phosphorylase in *Echinococcus granulosus* Protoscoleces

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ABSTRACT

The present investigation indicates the presence of thymidine phosphorylase (TPase) (EC.2.4.2.4) and its activity in supernatant fraction of *Echinococcus granulosus* protoscoleces extract, furthermore some of its kinetics properties were investigated.

It was found that deoxyuridine might be an alternative substrate for thymidine during catalyzed reaction by enzyme TPase. The result also indicated that TPase activity was extremely sensitive to inhibite by thymine(product reaction), cytidine, 5-flourouracil and albendazole with inhibitory percentages of: 36%, 68%, 52% and 3% respectively.

The presence of TPase activity indicates the presence of salvage pathway for thymine nucleotide which was essential to deoxyribonucleic acid (DNA) synthesis in this organism, in addition to *de novo* pathway.

Keywords: *Echinococcus granulosus*, cestoda, thymidine phosphorylase, inhibitors, nucleotides, salvage pathway.

(EC.2.4.2.4) TPase

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( ) .

.TPase

%36 -5 ( )

%3 %52 %68 .

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DNA

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INTRODUCTION

Echinococcus granulosus belongs to the phylum: Platyhelminthes, class: Cestoda, Order: Cyclophyllidea, family: taeniidae (Roberts and Jenovy,1996). It is medically important parasite that causes unilocular hydatid disease to man and herbivores intermediate hosts,as they harbor the larval stage, while canines harbor the adult worm as a definitive host (Beaver and Junk,1985).

E.granulosus is widely distributed throughout temperate and subtropical regions. In areas contiguous to sheep farming and dogs. Human infection is common in southern south America, much of Africa, Eastern and southern Europe, the Middle East, southern Australia, New Ziland and extensive areas of Asia, including Iraq (Andersen *et al.*,1997).

It has become increasingly apparent that understanding parasite biomolecular metabolism and the present differences and similarities in mammalian hosts is very important. It is the target from which researchers may put treatment strategies for parasitic diseases, for instance the formation of thymine nucleotide is an important step in the complex of metabolic events, which supply nucleotide building blocks for DNA replication (Hassan and Coombs,1988). Nucleotide salvage pathway ensures that the pyrimidine nucleotide is sufficiently large for efficient DNA repair and replication (Hassan, 1979).

Thymidine phosphorylase (TPase) is thymidine orthophosphate deoxyribosyl transferase, EC.2.4.2.4 (Schwartz, 1971). The TPase catalyzes reaction in both eukaryotic and prokaryotic, thymidine is synthesized from thymine and deoxyribose-1-phosphate in a reaction catalyzed by TPase (Shaw *et al.*, 1988).

The enzyme TPase catalyzes the reversible synthesis of thymidine and inorganic phosphate from thymine, using deoxyribose -1-phosphate. It is revealed that mutation in thymidine phosphorylase gene as a result of autosomal recessive will lead to the accumulation of toxic levels of thymidine and deoxyuridine in blood. This may lead to fatal disorder (Doussis-Anagnostopoulou *et al.*,1997; Ioachim, 2008; Lee *et al.*, 2010; Wallace *et al.*, 2010).

TPase was first described almost 56 years ago by Friedkin and Roberts (1954) as enzyme involved in nucleic acid homeostasis, and purified in the mid 1970s from *Escherishia coli* and *Salmonella* (Brown and Bicknell,1998). Kurnova *et al.*,(2011) isolate TPase as a crystal protein from *Escherichia coli*. Eukaryotic TPase was first purified from human amniochorion (placenta) by Kubilus *et al.*, (1978).

TPase activity has been studied in some microorganisms (Restaiono and Frampton, 1975 ; Schwartz,1978; Mc-Elwain *et al.*,1988). Some researchers studied TPase activity in mammalian tissues (Williams and Tuchman,1989; Brown and Bicknell,1998; Al-Abachi, 2006 and Ioachim, 2008). Other researchers detected the activity of TPase in free living and parasitic protozoa (Al-Chalabi and Gutteridge, 1977; Miller and Miller, 1986; Janker,1992; Krungkrai *et al.*, 2003; Al-Hammoshi, 2006; Al-Juwary, 2006). In some Cectoda, TPase activity has been detected by Janker (1996).

The present study aimed to detect thymidine phosphorylase activity and to study some of its kinetic properties in *E. granulosus* protoscoleces.

MATERIALS AND METHODS

Organisms collection and suspension

E.granulosus protoscoleces were collected in cold Phosphate Buffer Saline (PBS)(pH 7.8) from freshly slaughtered sheep livers at a municipal abattoir in Mosul city, Iraq. Protoscoleces were removed from the cysts by aseptic techniques, washed in several changes of sterile (PBS) (Farjou and Al-Hussainawi,1984). Protoscoleces were then suspended in 50mM Potassium-Phosphate Buffer (pH 7.8). The suspension was then put in siliconized test tubes, 2ml\tube which contain 50000 protoscoleces\ml of the suspension.

The mean viability of protoscoleces was 96% according to their movement at negative staining with 0.1% aqueous eosin stain (Smyth and Barrett,1980).

Preparation of enzyme extract

E.granulosus protoscoleces were homogenized using MSE homogenizer. The homogenate was sonicated for 60 seconds in MSE ultrasonic disintegrator at setting of 10 (1200 vibration/second) at 4 ºC. The sonicate was then centrifuged at 45000xg for 60 minutes, and the supernatant fraction was used as a source of enzyme extract (Janker,1996).

Enzyme assay

Assay system used to determine protoscoleces TPase activity was modified from that used by Al-Chalabi and Gutteridge (1977) and Janker (1996). The produced deoxyribose-1-phosphate was assayed using diphenylamine method (Burton,1956). The reaction mixture (2ml), contained 50mM Potassium-Phosphate Buffer,10mM thymidine,10mM inorganic phosphate, 1mM EDTA and completed by enzyme extract.

Reaction mixture was incubated at 35 ºC, for 20 minutes,0.4ml of the samples was added to 1ml of 0.5N perchloric acid in an ice bath, then centrifuged at 12000rpm for 30 minutes and 1ml of the supernatant was mixed with 2ml of diphenylamine reagent and kept overnight at room temperature in the dark place. The control tube contained Potassium-Potassium-Phosphate Buffer instead of thymidine, TPase activity was estimated at the absorbance 600 nm to determine deoxyribose-1-phosphate concentration against blank.

Total protein was determined to estimate TPase specific activity depending on Lowry method in which Folin –Phenol reagent is used (Lowry *et al*., 1951).

The kinetic properties for TPase were studied by estimation the effect of different concentrations of enzyme then substrate under different conditions of incubation periods, temperature, buffer solutions by (PH) then the activity of TPase was estimated by inhibitors to this enzyme (Janker,1996).

RESULTS

Thymidine phosphorylase activity was estimated for *E.granulosus* protoscoleces. The assay system described by (Al-Chalabi and Gutteridge,1977) was used. Thymidine phosphorylase activity was detected in protoscoleces homogenate supernatant and pallet, respectively Table (1).

The result showed that the 45000xg supernatant fraction was the cellular fraction of choice, in which protoscoleces TPase activity was found.

Table 1: Thymidine phosphorylase activity in different fractions of *E.granulosus* protoscoleces homogenate

Fractions	Δ Absorbance 600nm /20 min	%Enzyme activity
Homogenate	0.037	100
Supernatant	0.031	84
Pallet	0.006	16

The complete assay system consisted of 50mM Potassium-Phosphate Buffer, 10 mM thymidine, 10mM inorganic phosphate, 1mM EDTA, and 0.4ml enzyme, all incubated at 35 ºC for 20 minute.

(Fig.1) shows that the maximum of TPase activity was at 60 µg protein. It was obviously found that the speed of enzyme reaction increased with the increasing in protein concentration. Thus, in all further experiments (60-65)µg proteins were used as enzyme concentration.

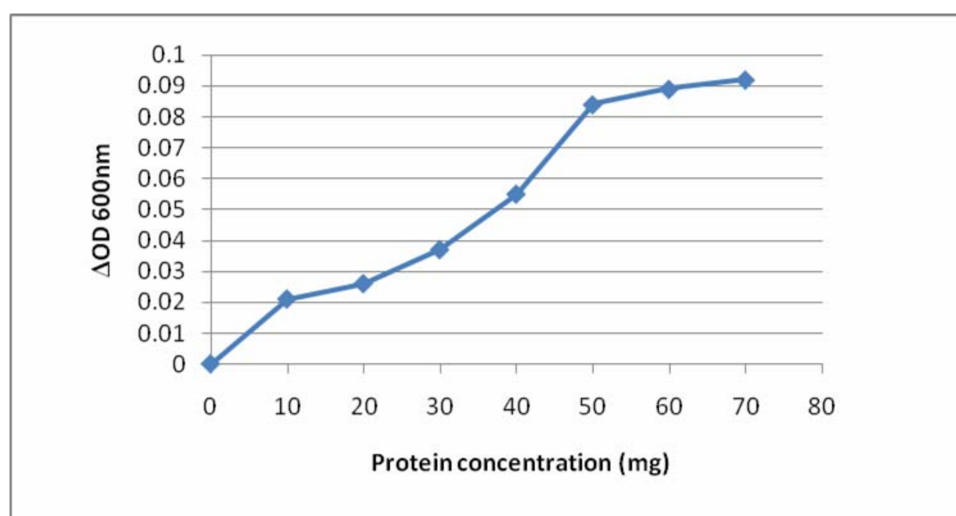

Fig.1: Effect of different protein concentrations (enzyme concentration) on TPase activity from extract of *E.granulosus* protoscolices

The activity of TPase from *E.granulosus* protoscoleces was assayed at a range of different temperatures, (Fig. 2) showed that the enzyme reaches its maximum activity at 35 ºC, then declines beyond that, whereas it loses its activity at 50 ºC. Thus in all further experiments incubation was carried out at 35 ºC.

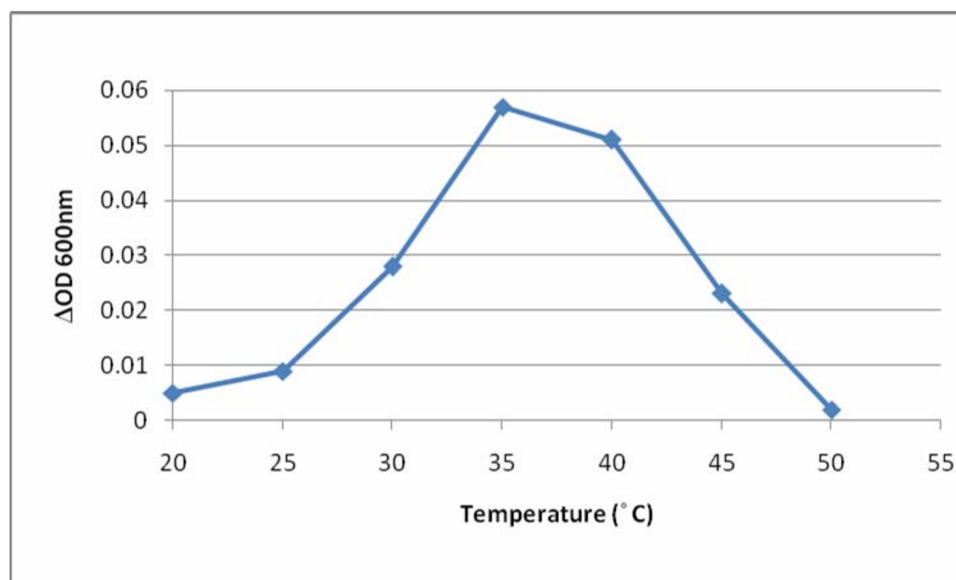

Fig. 2: Effect of temperature on TPase activity from extract of *E.granulosus* protoscolices

Thymidine phosphorylase activity from *E. granulosus* protoscoleces was determined at various time intervals using the same assay system. (Fig. 3) shows that activity was linear for 20 minutes and remains constant. Thus in all further experiments incubation was carried out at (20-30) minutes.

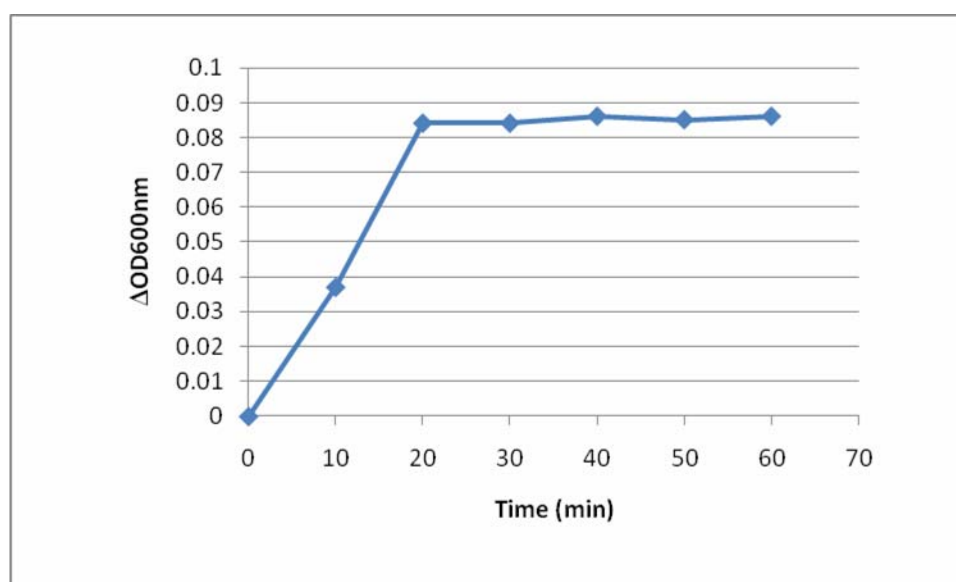

Fig. 3: Thymidine phosphorylase activity as function of time

Table (2) showed the activity of TPase from *E.granulosus* protoscoleces assayed at different buffers. Its activity in 50mM phosphate buffer (pH 7.8) was higher (about 4 folds) than in 50 mM Tris-HCL buffer (pH 7.8). Accordingly, phosphate buffer had more competence as buffer solution for TPase assay system.

Table 2: The effect of different buffers on TPase activity from extract of *E.granulosus* protoscoleces

Buffer	Concentration mM	pH	Δ Absorbance 600nm /20 min	% enzyme activity
* Potassium Phosphate buffer	50	7.8	0.046	100
Tris-HCL buffer	10	7.8	0.012	26

*Potassium phosphate buffer K_2HPO_4(0.87gm) and KH_2PO_4(0.272gm)/100ml

(Fig. 4) showed that the maximum TPase activity was at pH 7.8. The activity of TPase declined sharply at pH 8.0 .

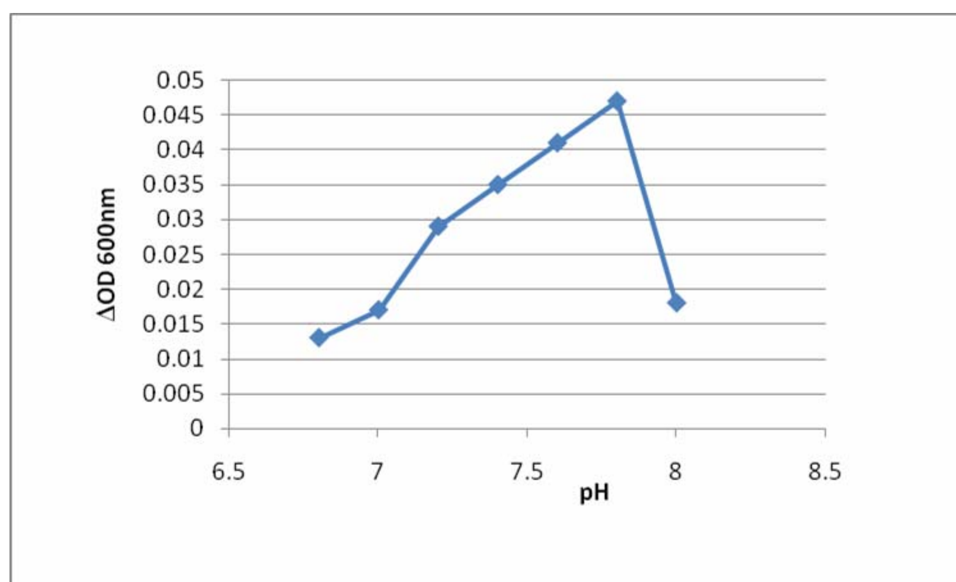

Fig. 4: Effect of different pH of 50mM Phosphate buffer on TPase activity from extract of *E. granulosus* protoscolices

Thymidine is the known substrate for thymidine phosphorylase activity, 2-deoxyuridine was found to have the same effect on TPase activity from extract of *E. granulosus* protoscoleces Table (3), it might be an alternative substrate for TPase.

Table 3: Alternative substrate for thymidine phosphorylase activity from extract of *E.granulosus* protoscoleces

Substrate	Δ Absorbance 600nm/20min	% Activity
10mM Thymidine	0.064	100
10mM 2-Deoxyuridine	0.064	100

The enzyme assay system was measured in reaction mixture which contain TPase substrate or the alternative (10mM) in addition to 1mM EDTA, 10mM inorganic phosphate in 50mM potassium phosphate buffer (pH 7.8) and enzyme extract, all incubated at 35 º C for 20 minutes.

The activity of TPase from *E.granulosus* protoscoleces was assayed at range of different thymidine concentrations (0-20mM of thymidine as substrate of TPase). The

results in (Fig. 5) showed that the enzyme reach its maximum activity at 10 mM thymidine. TPase activity started to be steady after 10mM thymidine.

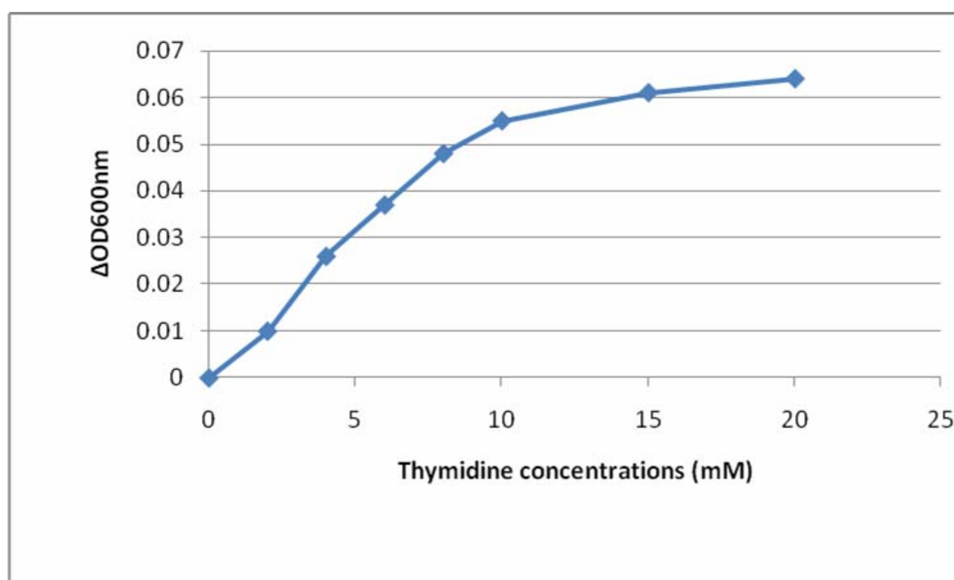

Fig. 5: Tpase activity at different concentrations of thymidine(Substrate)

The effect of pyrimidine bases, analogous and drugs were tested as inhibitors of enzyme activity. The bases used were thymine, cytidine, 5-fluorouracil and albendazole, each at 10mM concentration Table (4). The results showed that cytidine had the highest inhibitory effect on TPase activity (68%), 5-fluorouracil (52%), thymine (36%) and the lowest inhibitory effect was for albendazole (3%).

Table 4 :Effect of some inhibitors on thymidine phosphorylase activity from extract of *E.granulosus* protoscoleces

Inhibitors	Inhibitors concentration (mM)	% activity	% inhibition
Control	0	100	0
Thymine	10	64	36
Cytidine	10	32	68
5-fluorouracil	10	48	52
Albendazole	10	97	3

The complete assay system contained 50mM phosphate buffer pH 7.8,10 mM thymidine, 10mM inorganic phosphate 10mM EDTA, enzyme extract and inhibitors, all incubation were at 35 ºC for 20 minutes.

DISCUSSION

The activity of TPase has been investigated in the supernatant fraction of *E. granulosus* protoscoleces and the optimum conditions of echinococcal TPase activity were determined, at 35ºC (temperature), (pH 7.8), 20 minutes (Incubation time), 10mM thymidine (substrate concentration), 60 µg (protein concentration) and 50mM of potassium phosphate buffer (The better echinococcal TPase buffer).

Echinococcal TPase optimal conditions are similar to some extance with TPase kinetic properties of other organisms like *Escherichia coli* (Schwartz, 1971), *Salmonella typhimurium* (Blank and Hoffee,1975), some trypanosomes (Al-Chalabi and Gutteridge, 1977), *Leishmania* ssp. (Hassan, 1979), *Tetrahymena pyriformis* (Janker, 1992), *Giardia lambelia* (Miller and Millar, 1986), and *Moniezia expansa* (Janker,1996).

On the other hand, 2-deoxyuridine was the alternative substrate to thymidine that gave 100% TPase activity. The result that may refer to the presence of uridine phosphorylase in echinococcal pyrimidine salvage pathway. This result consisted with those of Al-Chalabi and Gutteridge (1977); Janker (1992) and (1996), who investigate TPase activity in protozoa and cestodes.

In mammalian cells, thymidine is known to be incorporated in to nucleic acid via salvage pathway under abnormal conditions (Cha,1989). Hammond *et al*., (1981) revealed that *de novo* pyrimidine biosynthesis take place in most parasitic protozoa, but they may lead salvage pathway under abnormal conditions like low oxygen tension or abnormal pH .

Wang *et al*., (1983) indicate that *Tritrichmonas foetus* (anaerobic flagellated protozoa) take up exogenous uracil, cytidine and thymidine then converted them to nucleotide by salvage pathway. The same as Aldritt *et al*., (1985) who indicate the presence of TPase in *Giardia lamblia*. Al-Chalabi and Gutteridge (1977) and Hassan (1979) referred to the presence of TPase activity in *Trypanosoma* and *Leishmania spp*.

Toxoplasma gondii tachyzoites (Apicomplexa) are also capable of specific pyrimidine salvage pathway (Iltzsch, 2007), while *Plasmodium spp*., lack the ability to salvage performed pyrimidines (Koning *et al*., 2005).

The synthesis pyrimidine in salvage pathway is limited in flatworms (Maule and Marks, 2006). El Kouni *et al*., (1988) referred to the presence of uridine phosphorylase instead of TPase in *Schistosoma mansoni* yet, Al-Chalabi *et al*.,(1994) stated that there is no activity of TPase in Adult *Fasciola hepatica*.

The activity of TPase is present in cestoda, that is indicated in *Moniezia exponsa* (Janker, 1996) and *Hymenolepis dimenuta* (Drabikowska,1996).

The presence of TPase activity in *E.granulosus* protoscoleces may be related to the fact that this parasite lives under low oxygen tension, since that the end product of carbohydrates catabolism in *E. granulosus* protoscoleces is the ethanol but not CO_2 (Chappell,1980) knowning that hydatid fluid pH in the hydatid cyst is about 7.8 (measured in the present work).

As for TPase inhibitors like several cyclopyrimidines which were tested against those enzymes incorporated in pyrimidine synthesis in mouse liver and human liver; *E.coli*; *Schistosoma mansoni* (Park *et al*., 1986; Chu *et al*.,1988).

Al-Hammoshi (2006) synthesizes several heterocyclic compounds that contain pyrazoline ring and pyrimidinone ring. These compounds proved to inhibit TPase activity $\geq$ 50% in *Leishmania tropica* promastigotes.

Furthermore, Al-Juwary (2006) proved that aqueous extract of *Melia azedarach* inhibits 70% of TPase activity in *Trichomonas vaginalis* trophozoites.

Diab *et al*., (2012) synthesize a new class of potential TPase inhibitors, which contain a difluoromethyl phosphonate function, as phosphate mimic.

In the present study, thymine, 5-fluorouracil, cytidine inhibit echinococcal TPase activity 36%, 52%, 68%, respectively. These compounds also found to inhibit TPase activity in mammalian tissues (Blank and Hoffee,1975), and in protozoan organisms

(Hassan and Coombs,1988; Janker,1992), and in cestodes (Janker, 1996) these results indicate that *E. granulosus* protoscoleces contain TPase that have the same properties of iso functional enzyme in other organisms, with simple differences.

On the other hand, Albendazole (the antiechinococcal of choice) was found to inhibit only 3% of TPase activity, this may relat to the fact that TPase is not targeted by the drug. Albendazole interferes with microtubule formation in the parasitic helminthes (Karch, 2005).

RECOMMENDATIONS

1. Echinococcal TPase should be purified, then the accurate kinetic properties of TPase were determined.
2. Other enzymes in pyrimidine salvage pathway like uridine phosphrylase should be investigated, that would make it possible to put a diagram for echinococcal pyrimidine salvage pathway.

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