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.
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 Zealand 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).

\[
\text{Thymine} + \text{deoxyribose-1-phosphate} \xrightarrow{\text{TPase}} \text{Thymidine} + \text{Pi}
\]

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 Cestoda, 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 °C. 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 °C, 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 pellet, 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

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Δ Absorbance 600nm /20 min</th>
<th>%Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.037</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.031</td>
<td>84</td>
</tr>
<tr>
<td>Pallet</td>
<td>0.006</td>
<td>16</td>
</tr>
</tbody>
</table>

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.

![Graph](image)

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

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.
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.

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

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration mM</th>
<th>pH</th>
<th>Δ Absorbance 600nm /20 min</th>
<th>% enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Potassium Phosphate buffer</td>
<td>50</td>
<td>7.8</td>
<td>0.046</td>
<td>100</td>
</tr>
<tr>
<td>Tris-HCL buffer</td>
<td>10</td>
<td>7.8</td>
<td>0.012</td>
<td>26</td>
</tr>
</tbody>
</table>

*Potassium phosphate buffer K$_2$HPO$_4$(0.87gm) and KH$_2$PO$_4$(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* protoscoleces

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Δ Absorbance 600nm/20min</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM Thymidine</td>
<td>0.064</td>
<td>100</td>
</tr>
<tr>
<td>10mM 2-Deoxyuridine</td>
<td>0.064</td>
<td>100</td>
</tr>
</tbody>
</table>

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 °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.

![Graph showing TPase activity at different concentrations of thymidine](image)

**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

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Inhibitors concentration (mM)</th>
<th>% activity</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Thymine</td>
<td>10</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>Cytidine</td>
<td>10</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>10</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>Albendazole</td>
<td>10</td>
<td>97</td>
<td>3</td>
</tr>
</tbody>
</table>

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 °C 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 35°C (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 extant 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 *Tritrichomonas 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 expansa* (Janker, 1996) and *Hymenolepis diminuta* (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) knowing 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 ≥ 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 relate 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 phosphorylase should be investigated, that would make it possible to put a diagram for echinococcal pyrimidine salvage pathway.

**REFERENCES**


