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Study of hepatic tyrosine aminotransferase from *Schistosoma*-infected mice

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Abstract. In the current study, tyrosine aminotransferase (TAT) as a target enzyme was purified from the liver of control and *Schistosoma*-infected mice that was subjected to catalytic investigation. The purified enzyme has a single band on SDS-PAGE with a molecular mass of almost 100 KD from control and infected mice. The kinetic studies of hepatic TAT towards its substrates showed no change in K_m, whereas V_{max} value was increased from 2.3 to 2.9 fold in the enzyme isolated from *Schistsoma*- infected mice. In addition, the K_{cat}/ K_m ratio displayed a higher value for the enzyme from infected mice, indicating that it is more efficient and specific. On the other hand, the *in vitro* effect of praziquantel showed a slight activation of hepatic TAT in both control and infected mice, whereas mirazid (MZD) has an inhibitory effect in a concentration-dependent manner. The response of TAT from infected mice towards MZD inhibition is less than that from controls. These data suggest that there is a change in the catalytic properties of hepatic TAT in schistosomiasis and the *in vitro* effect of the schistosomicidal drugs appears to have inductive or inhibitory effect.

INTRODUCTION

There are 200 million people in many countries, especially in Africa infected with schistosomiasis and about 60% of them display symptoms (Lelo *et al.*, 2014; Chitsulo *et al.*, 2000). Praziquantel (PZQ), a pyrazinoisoquinoline derivative, is currently the mainstay for treatment and plays a critical part in community-based schistosomiasis control programs (Neves *et al.*, 2011). PZQ reliably cures 60 to 90% of patients and substantially decreases the worm burden and egg production in those who are not cured (Neves *et al.*, 2011). Many medicinal plants were studied to investigate their antischistosomal potency and found to be effective. The most successful product is myrrh extract (Mirazid, abbreviated as MZD), an oleo-gum resin extracted from the stem of *Commiphora molmol*, believed to affect schistosome musculature (Sheir *et al.*, 2001).

Tyrosine transaminase (L-tyrosine:2 oxoglutarate aminotransferase, EC 2. 6. 1. 5, abbreviated TAT) is an enzyme present in the liver and catalyzes the conversion of Tyr to 4-hydroxyphenylpyruvate (Rettenmeier *et al.*, 1999). Elevated hepatic and serum TAT levels have been found during *Trypanosoma brucei gambiense* infections in voles (Stibbs & Seed, 1976), however the effect of *Schistosoma* infections on the synthesis of this enzyme in hepatic tissue has not been studied. Alterations in tyrosine metabolism, which may affect the rates of catecholamine biosynthesis could lead to disorders such as mental depression and mania (Schildkraut, 1965) and this could influence both the health and behavior of the host. Stress due to *Diplococcus pneumoniae* infections in rats produces a rise in serum corticosteroids, which in turn induces an eightfold increase in hepatic TAT activity (Shambaugh & Beisel, 1968). However, many transferases were

found to be subject to change in schistosomaisis (Brown & Smith, 1977; Balbaa *et al.*, 2004; Balbaa & Bassiouny, 2006; Kardorff *et al.*, 1997). Since TAT is one of the aminotransferases that has not been completely elucidated and investigated, the present work was designed to isolate and purify this enzyme from *Schistosoma mansoni* – infected mice to study its catalytic and kinetic characteristics. Moreover, a comparative *in vitro* effect of the schistosomicidal drugs PZQ and MZD on the kinetic behavior of this enzyme were also investigated.

MATERIALS AND METHODS

Chemicals

α-Ketoglutarate (KG), Tyrosine (Tyr), ammonium molybedate, pyridoxal- 5-phosphate and Sephadex G-200 were purchased from Fluka, India. DEAE-Cellulose (DE- 52) was purchased from Whatman. Coomassie Brilliant Blue R-250 and molecular weight protein markers were obtained from Sigma Co, Mo, USA. PZQ and MZD were obtained from the Alexandria Company for Pharmaceutical and Chemical Industries. Both are solubilized in ethanol for the required concentration.

Animals

Four-week old male Swiss albino mice (CDI) with an average weight of 20-25 g were obtained from the Theodore Bilharz Research Institute, Schistosome Biological Supply Program (SBSP), Cairo, Egypt. The mice were housed in wire cages in groups of 5 mice per cage. They were kept under conventional conditions of temperature and humidity with a 12 h photoperiod. Food and water were supplied ad libitum. All the experimental procedures were conducted according to the animal protocols approved by the Ethics Committee of Faculty of Science, Alexandria University, Egypt.

Animal infection

Fifty mice were infected with *Schistosoma mansoni* by an intraperitoneal injection of an average of 80 cercariae/mice. The infected mice were grouped (10 mice per group) according to the duration of infection with 15 days interval between each group. Another ten mice were used as the control, corresponding to the infected groups. At the end of each infection period, the infected group and the corresponding control one were sacrificed. The livers were immediately removed, weighed, perfused, rinsed with a chilled saline solution, homogenized in 9 ml of 10 mM sodium phosphate buffer, pH 7.4 and centrifuged at 3000 rpm for 15 min. The obtained supernatant was subjected to enzyme assay.

Alanine aminotransferase (ALT) and aspartate amiontransferas (AST) assays

The activities of ALT (EC 2. 6. 1. 2) and AST (EC 2. 6. 1. 1) were determined as what previously described (Vanikar & Bhatt, 1956). A unit of enzymatic activity is defined as mmol of NADH consumed or disappeared per min at 37 °C. The specific activity is given as mmol of NADH consumed / min / mg protein.

TAT assay and kinetics

Enzyme activity was assayed as previously described (Canellakis & Cohen, 1956) at 37°C. Briefly, the assay was performed at 1.9 mM Tyr, 13 mM pyridoxal phosphate, 3.0 mM KG and an appropriate amount of the enzyme in a final volume of 10 ml at pH 7.3. After the addition of the color reagent (0.2% potassium dihydrogen phosphate and 0.6% ammonium molybedate reagent), the absorbance was read at 850 nm against blank reagent. The activity is expressed as µmol/min through the value of the extinction coefficient of the colored complex (4-hydroxylphenyl pyruvat) as 0.41 l x $mmol^{-1}$ x mm^{-1}. For the determination of the kinetic parameters of TAT from control and infected mice, the initial velocities of the reactions were measured as (ΔA/s) with KG or Tyr as a substrate at 430 and 850 nm, respectively. Lineweaver–Burk plots were used to determine the kinetic parameters of the enzyme (Segel, 1976). The inhibition constant (K_i) value was determined by both Lineweaver–Burk and Dixon plots (Segel, 1976; Dixon & Webb, 1979).

Purification of TAT

The purification of hepatic TAT has been applied to liver tissues of control and infected mice as previously described at 4°C (Canellakis & Cohen, 1956). Mouse livers were homogenized in 5 volumes of cold 10 mM Tris HCl buffer, pH 7.4 containing 0.15 M KCl and 1 mM EDTA. After centrifugation at 14,000 rpm for 20 min, the supernatant was subjected to purification by different chromatographic techniques on DEAE-Cellulose and Sepdadex G-200 as previously described (Valeriote *et al*., 1969).

SDS-PAGE

The purified enzyme from the liver of normal mice and infected mice by the above mentioned steps was subjected to SDS polyacrylamide gel (12%, w/v) in 25 mM Tris/ Glycine buffer, pH 8.3 and he protein bands were stained by Commassie Brilliant Blue R-250 (Schagger, 2006).

***In vitro* treatment of TAT by schistosomicidal drugs**

The *in vitro* investigations were performed as previously described (Balbaa & Bassiouny, 2006). The initial velocities and kinetic parameters of purified TAT from liver of *Schistosoma*-infected mouse and control were determined in the absence and presence of different concentrations of PZQ or MZD (20–100 mg/ml) for the two substrates, KG and Tyr. Both PZQ and MZD were solubilized in ethanol.

Protein assay

Protein concentration was determined using bovine serum albumin as a standard (Layane, 1957).

Statistical analyses

Data analyses involved estimation of means, standard errors (SE) and probabilities (p values) for each of the groups. Student's t test was used to determine statistical differences among the groups. Statistical significance was identified at $p<0.05$. All experiments were run on three occasions for reproducibility and all assays were done in triplicate.

RESULTS

Effect of *Schistosoma* infection on TAT

The activities of hepatic TAT showed a significant change ($p<0.05$) in the infected mice when compared to the control. The effect of different periods of *Schistosoma*-infection on the specific activity of the investigated enzymes showed that hepatic TAT is clearly increased when the infection interval is increased. Hepatic TAT showed a significant elevation of its specific activity at 2, 4, 6, 8 and 10 weeks post infection with *Schistosoma mansoni*. The specific activity of TAT is slightly increased after 2 weeks post infection, after which the increase becomes directly proportional with infection period after 4, 6, 8, and 10 weeks ($4.55\pm0.57\times10^6$, $7.57\pm0.84\times10^6$, $14.85\pm2.12\times10^6$ and $19.75\pm2.19\times10^6$, respectively) compared to control group ($2.4\pm0.34\times10^6$, $p<0.05$) (Table 1). The comparison with hepatic aminotransferases showed a significant elevation of the specific activity of ALT after 2, 4, 6, 8 and 10 weeks post *Schistosoma*-infection ($17.7\pm1.9\times10^3$, $31.4\pm3.8\times10^3$, $45\pm4.5\times10^3$, $145\pm20.7\times10^3$ and $166.8\pm18.4\times10^3$, respectively) compared to control group ($5.9\pm0.7\times10^3$) with $p<0.05$. The same pattern of elevation for the hepatic AST specific activity after the same post infection periods of schistosomiasis ($9.8\pm0.7\times10^3$, $13.7\pm1.5\times10^3$, $24.2\pm3.4\times10^3$, $54.4\pm6.0\times10^3$, and $72.1\pm9.0\times10^3$), respectively compared to control group ($5.02\pm0.5\times10^3$, $p<0.05$) (Table 1). TAT was purified from the mouse liver by different steps. TAT from the control and infected mice was purified by about 31.3 and 12 folds, respectively starting with the crude homogenate and continuing to the final step. The post-gel filtration fraction retained approximately 8 and 12% of the original total activity (yield) for the enzyme from the control and infected mice, respectively. The purified TAT from the liver of the normal mouse showed a homogeneity by SDS-PAGE as illustrated in Fig. 1A & B. The observed molecular weight of the TAT band that is calculated from the relative electrophoretic mobility is about 100 KDa.

Table 1. Specific activity of crude hepatic TAT, ALT and AST during different intervals of *Schistosoma*-infection

Infection interval (weeks)	Specific activity of TAT (µmol/min/mg protein) x 10^6	Specific activities of AST and ALT (µmol/min/mg protein) x 10^3	
		ALT	AST
Non	2.40 ± 0.34[a]	5.9 ± 0.7[a]	5.02 ± 0.5[a]
2	3.02 ± 0.38[ab]	17.7 ± 1.9[ab]	9.80 ± 0.7[ab]
4	4.55 ± 0.57[b]	31.4 ± 3.8[b]	13.70 ± 1.5[b]
6	7.57 ± 0.84[c]	45.0 ± 4.5[b]	24.20 ± 3.4[c]
8	14.85 ± 2.12[d]	145.0 ± 20.7[c]	54.40 ± 6.0[d]
10	19.75 ± 2.19[e]	166.8 ± 18.4[d]	72.10 ± 9.0[e]

Values are expressed as means ± SE of mice groups. Superscripts of different letters in the same column (a, b, c, d and e) are significantly different at $p<0.05$.

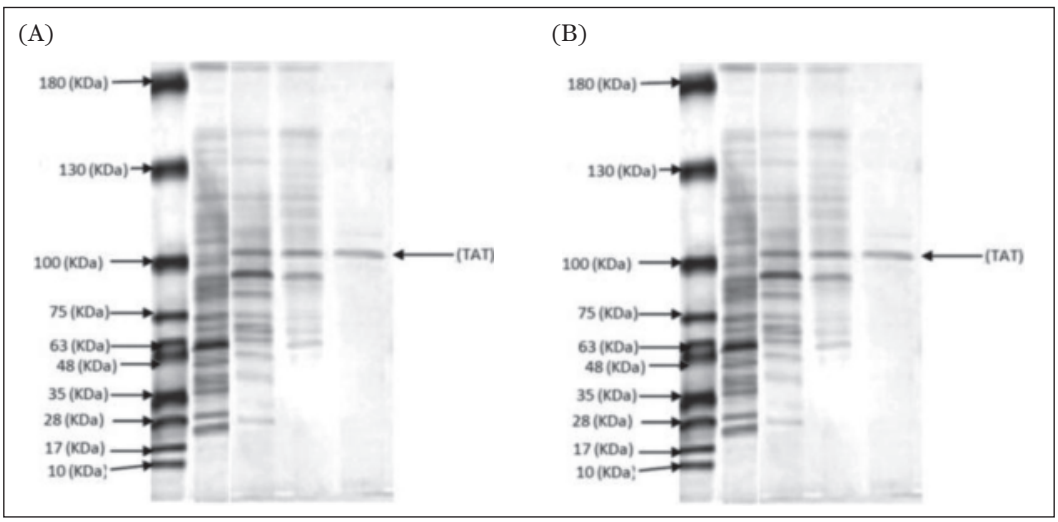

Figure 1. SDS-PAGE pattern of hepatic TAT purification steps from control (A) and *Schistosoma*-infected (B) mice. From left to right: Lane 1, protein markers; Lane 2, crude homogenate; Lane 3, post ammonium sulfate precipitation; Lane 4, post DEAE-cellulose; Lane 5, post Sephadex G-200. Arrows indicate the observed standard protein.

Kinetic studies

The observed K_m values of the enzyme towards KG are 4.76 mM, and towards Tyr they are 4.55 mM both in control and infected cases, respectively (Table 2). The observed values of V_{max} are 7.58 and 21.94x10^6 µmol/min/mg protein towards KG (2.9-fold increase of K_{cat} value), while they are 12.82 and 29.41x10^6 µmol/min/mg protein towards Tyr in control and infected cases (2.3-fold increase of K_{cat} value), respectively. Thus, the kinetic parameters of the purified enzyme from control and infected mouse showed a marked elevation was noticed in the V_{max}, K_{cat} and hence the efficiency ratio K_{cat}/K_m values of the enzyme from the infected mouse compared to control (Table 2).

Effect of some schistosomicidal drugs on TAT

The effect of some schistosomicidal drugs on the kinetic parameters of purified hepatic TAT from the infected mice compared to

Table 2. The kinetic parameters of TAT from control and *Schistosoma*-infected mice towards KG and Tyr

Parameter	Control		Infection	
	KG	Tyr	KG	Tyr
K_m *	4.76	4.55	4.76	4.55
V_{max} #	7.58 x 10^6	12.82 x 10^6	21.94 x 10^6	29.41 x 10^6
K_{cat} **	583.1	986.2	1687.7	2262.3
K_{cat} / K_m	122.5	216.7	354.6	497.2

* Expressed in mM.
\# Expressed in µmol/min/mg protein.
** Expressed in min^{-1}.

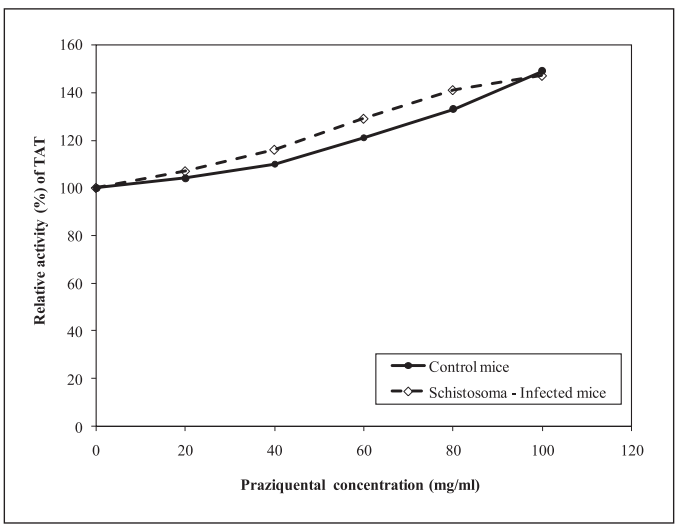

Figure 2. The effect of different praziquental concentrations on the relative activity of hepatic TAT purified from control and *Schistosoma* – infected mice. The data is an average of three kinetic runs.

control one was studied. The effect of PZQ of different concentrations (20 - 100 mg/ml) on hepatic TAT is shown in Fig. 2. The relative activity was found to be 149 and 151% for the enzyme from control and infected mice, respectively at the highest PZQ concentration (100 mg), i.e. it activates hepatic purified TAT from infected mice slightly more than that of control one (Fig. 2). On the other hand, according to Lineweaver-Burk plot, the *in vitro* effect of different MZD concentrations (20–100 mg) yielded a competitive inhibition pattern in which the lines intersected at the Y-axis at varied concentrations of KG and fixed Tyr as well as at varying concentrations of Tyr and fixed KG The effect of *Schistosoma* infection appears to be real at a higher MZD concentration (100 mg) as it decreases the apparent K_m value from 6.5 mM to 4.2 mM. Moreover, *Schistosoma* infection shows the same effect on the V_{max} value of TAT at all MZD concentrations. V_{max} values were found to be 33.3 and 45.4 µmole/min/mg of the enzyme from control and infected mice, respectively towards KG as a substrate, and 12.5 and 20 µmole/min/mg towards Tyr as a substrate. MZD is an inhibitor of hepatic TAT from the *Schistosoma*-infected mouse

compared to control with a K_i value of 75 mg/ml and 80 for control and infected mice, respectively calculated from Dixon plot.

DISCUSSION

Since it is worthy to perform a biochemical and molecular screening of schistosomiasis, TAT was purified from the liver of *Schistosoma*-infected mice and subjected to further investigation. A highly purified TAT was obtained from rat liver in which the enzyme had been induced by glucocorticoid hormone (Hayashi *et al.*, 1967). Also, TAT from human liver was purified 2200-fold by successive chromatography on DEAE – cellulose (Andersson & Pispa, 1982).

However, the purified TAT had the same electroporetic homogeneity as the control, but its specific activity was higher at 199 than that of the control at 75 µmole/min/mg protein, respectively. The illustrated value is very much higher than those reported for the enzyme from *Crithidia fasciculata* (97.4 units / mg protein) (Rege, 1987) and lower than that reported by others (Hargrove & Granner, 1980) for the enzyme from rat liver (616 units / mg protein). Although the values of specific activity varied according to the source and the methods of separation and purification, it was reported that the specific activity of TAT purified from rat liver stabilized by tetracycline is 2640.9 units / mg protein (Hannah & Sahib, 1975). TAT of both control and infected mice was purified about 31.3 and 12 fold with a yield 8% and 12%, respectively. The results obtained in our study at the DEAE – cellulose step were similar to those previously reported (Rita *et al.*, 1975).

In fact, the hepatic cytosolic TAT consists of four subunits, and four pyridoxal–phosphate groups, suggesting the possibility of four active subunits (Rosenberg & Litwack, 1970). In addition, other reports have suggested the ability of the coenzyme, pyridoxal-phosphate, to protect the enzyme from the effects of agents, which are known to alter the conformation of certain proteins (Holten & Kenney, 1967). On the other hand, the existence of two subunits of TAT with different or identical molecular weight was reported (Belarbi *et al.*, 1977; Hargrove & Granner, 1980; Lee *et al.*, 1979; Donner *et al.*, 1978).

In the present study, the results showed that the purified hepatic TAT from both control and *Schistosoma*–infected mice has a non native molecular weight of about 100 KD using the relative mobility curve of the standard protein marker mixture against their molecular weight in KD. However, the molecular weight of TAT varies according to its source as well as its purification method. A preparation of TAT from the liver of rats previously treated with the synthetic glucocorticosteroids (Valeriote *et al.*, 1969) of about 115 KD, whereas the apparent subunit molecular mass of purified TAT is 58 KD (Prabhu & Hudson, 2010). Also, it was reported that the molecular mass of the native enzyme determined by gel filtration on sephacryl S-200 is 91 KD, which suggests that the native is a dimer made up of two similar subunits (Montemartini *et al.*, 1993). Taken together, the detected band on SDS- PAGE represents one subunit structure and the enzyme may be a homodimeric form.

The kinetic studies of purified hepatic TAT of control and *Schistosoma*–infected mice indicate a change in the catalytic properties of TAT due to *Schistosoma* infection, which is shown by the elevation of its specific activity in Table 1. It was reported that the K_m value of TAT towards Tyr is 1.67 and 1.43 mM towards KG (Kenney, 1962), whereas it ranges from 0.2 – 5.0 mM towards Tyr (Mehere *et al.*, 2010). The variation in K_m value is dependent on the source of TAT and the degree of purification. However, the change in the catalytic activity of the enzyme probably occurs with a change in the structure of TAT and this change occurs slowly during the infection. The apparent continuous change in the activity during the infection represented by the ratio of changed and unchanged enzyme, a ratio that will slowly increase and give the appearance that there is an overall change in its catalytic activity. This may be related to some specific changes in some structural domains of the enzyme. Moreover, k_{cat} / k_m ratio is also higher for the enzyme from infected mice. Taken together, the catalytic change of this enzyme

in *Schistosoma*-infection involves a higher efficiency and specificity of the enzyme.

PZQ was found in our results to activate slightly TAT purified from the liver of both control and *Schistosoma*–infected mice. This mode of action of the slight activation may be related to PZQ structure as a pyrazino-isoquinoline derivative (Patra *et al.*, 2012). Moreover, other studies on the enzyme activity showed that it is promoted by carbon tetrachloride (Sato & Maruyama, 1974) and pyrimidine analogues (Rothrock *et al.*, 1983). PZQ works exclusively against adult worms and repeated treatment or a prolonged course has been used (Bethlem *et al.*, 1997). We suggest that it is a weak inhibitor in our study because the rate of decomposition is increasing with the increase of the pH value (Suleiman *et al.*, 2004) of the TAT reaction. Hence, PZQ may be used to control *Schistosoma* infection, but not recommended for controlling TAT level due to its slight effect.

In fact, TAT inhibition is correlated well with the ability to form a complex with pyridoxal cofactor compound, which inhibit enzyme activity. Therefore, the suggested mechanism may involve the mediation of Tyr since amino acids combine with pyridoxal-5-phosphate to form Schiff bases (Matsuo, 1957). In addition, this inhibitory effect may be related to the catalytic changes of the enzyme either and / or structural properties (Balbaa *et al.*, 2010). The decrease in the apparent K_m value of the hepatic purified TAT from *Schistosoma*–infected mice as well as the control one at different MZD concentrations is attributed to a diminishing change of the affinity of TAT due to inhibition. Also, the competitive inhibition of TAT by MZD suggests that that the main bulk of the drug has a structural similarity with the corresponding substrate of the enzyme. It was reported that rat hepatic TAT was inhibited *in vivo* by L-tryptophan (Cihak *et al.*, 1973) and regulated by etanol administration (Donohue Jr *et al.*, 1998).

In conclusion, there is a change in the catalytic properties of hepatic TAT due to infection as confirmed by the elevation of its specific activity and the increase of K_{cat}/ K_m ratio. This is more supported by the *in vitro* effect of the schistosomicidal drugs, which may be activators or inhibitors.

Conflict of interest

This research work was supported in aid by Alexandria University, Egypt. The authors report no conflict of interest.

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