STUDY ON GLUTATHIONE S-TRANSFERASE INHIBITION ASSAY BY TRICLABENDAZOLE ON *FASCIOLA* spp.

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Abstract- Glutathione S-transferase (GST) represents the major class of detoxification enzymes from helminth parasites such as *Fasciola hepatica* and *F. gigantica* and it is a candidate for chemotherapeutic and vaccine design. Therefore, GST enzyme of *Fasciola* spp. could be a target for evaluation of drugs such as triclabendazole ($C_{14}H_9Cl_3N_2OS$). For this purpose, GST enzymes were purified from *Fasciola* spp. and sheep liver tissue by glutathione affinity chromatography using a wash-batch method and subsequently their SDS-PAGE pattern was detected. Afterward, GST specific activity levels were assayed in the whole extract and purified solutions spectrophotometrically at 30°C with reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzen (CDNB) substrate. Finally, GST inhibition assay was investigated in the solutions by powder and bolus forms of triclabendazole. GST fraction as a 26 kDa (MW) band was obtained on sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). The level of GST specific activity in purified solutions was detected 18.14 µmol/min/mg proteins for Fasciola hepatica, 35.04 for *F. gigantica* and 37.84 µmol/min/mg protein for liver tissue. Comparison of the effect of powder and bolus of triclabendazole in solutions revealed inhibition concentration (IC_{50}) 8.36 and 9.05 µg/ml for *Fasciola hepatica* GSTs and 7.20 and 10.80 for *F. gigantica* GSTs and 8.65 and 9.70 µg/ml for liver tissue GSTs, respectively. These findings suggest the possibility of selective inhibition of *Fasciola* spp. GSTs by triclabendazole in vitro and use of these results for understanding of its effect in vivo and qualification of manufacturing bolus form of drug in comparison with original powder.

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Key words: Glutathione S-transferase, *Fasciola*, triclabendazole,

INTRODUCTION

Man may be infected with *F. hepatica* by ingestion of contaminated vegetables such as watercress, and triclabendazole can be administered in a single dose as the drug of choice for the treatment of fascioliasis (1). Glutathione (GSH) transferase is one of the major detoxification systems found in helminths including *F. hepatica* (2). Glutathione S-transferases (GST) are found in high levels in *F. hepatica* parasite and the level of this enzyme is approximately 4% of the total soluble protein (3, 4). Such high levels of GST infer an important role for these enzymes in helminth homeostasis and survival and this may be related to the naked tegument of helminth parasites and their potential exposure to a wide range of xenobiotics. Apart from reaction from their endogenous metabolism, GSTs of helminth

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parasite may protect against exogenous free radical damage or xenobiotics as a result of immune effector mechanisms from the host directed at the parasite (5). Glutathione transferase activity has been detected in cestodes, digeneas and nematodes. Significant higher activity has been found in intestinal cestodes and digeneas, compared with parasitic nematodes (6).

Previous studies have been revealed inhibition assay of hexachlorophene on GSTs from *F. hepatica* (7, 8) and characterization of purified GSTs by 2 - dimension electrophoresis (9). GSTs inhibition assay has been described in the cytosol of protoscolexes by triclabendazole from sheep hydatid cysts in Iran (10). In the present study we have reported the isolation, SDS-PAGE pattern, specific activity assay and specific activity inhibition assay of purified GSTs by triclabendazole from *Fasciola* spp. and sheep liver tissue.

MATERIALS AND METHODS

Fasciola spp. extract solution preparation

Fasciola spp. were obtained from liver of sheep slaughtered at a local abattoir (Puryaye vali, Tehran, Iran). They were washed 3-4 times with washing buffer [phosphate buffer saline (PBS): 0.17 M NaCL, 3.3 mM KCL, 9.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2]. *Fasciola* spp. in 3 volumes of homogenizing buffer [10 mM EDTA, 2 mM PMSF, 0.15 M NaCL, 50 mM Tris (pH=7.5) containing 0.5% V/V Triton X-100 (Sigma)] were homogenized in a glass homogenizer. Then suspension was centrifuged (12000g for 30 min at 4°C) and supernatant was stored at -80°C (7).

Liver extract solution preparation

Sheep liver sample was obtained at a local abattoir and washed 3-4 times with washing buffer. Then it was homogenized with 3 volumes of homogenizing buffer in a glass homogenizer, the suspension was centrifuged (12000g for 30 min at 4°C) and supernatant stored at -80°C (7).

Purified GSTs solution preparation

GSTs were purified from *Fasciola* spp. and sheep liver extract solution as enzyme pool by a glutathione-affinity matrix [glutathione-agarose (Sigma, G4510)] using a wash-batch method. Glutathione-agarose was provided as a lyophilized powder stabilized with lactose. Approximately 70 mg of powder swells to 1 ml of gel. Two hundred µl of glutathione- agarose gel (14 mg of powder in 200 µl of deionized water) was prepared in a microtube Eppendorf. Typically 90 to 95% swelling occurs within 30 min at room temperature, but it may require overnight at 2 to 8°C for 100% swelling occurring. After swelling, the agarose beads washed thoroughly with 10 volumes of deionized water or equilibration buffer (PBS 10 mM pH 7.4 containing 50 mM NaCl) by centrifugation at speed (200-500g) for 10 sec in a bench microcentrifuge at 4°C to remove the lactose present in the lyophilized product (11). The extract, 750 µl (2-3 mg protein), was mixed with the gel for 30 min at 4°C and centrifuged at 1000 g speed for 10 sec in a bench microcentrifuge at 4°C. The supernatant was removed and the gel matrix washed with 20 gel volumes of PBS-T (PBS 10 mM, pH 7.4 containing 50 mM NaCl and 1% Triton X-100) by centrifuge at 1000 g speed for 10 sec at 4°C. Bound GST enzymes were eluted by washing the gels with elution buffer (50 mM Tris-HCL pH 9.6 buffer containing 5 mM reduced glutathione) 3 times, 500 µl each and mixed each elution step gently, then centrifuged at 1000 g speed for 10 sec at 4°C and accumulated the supernatant into the clean tubes and stored at -80°C (8, 12).

Protein assay in the solutions

The amount of protein in the extract solutions and purified solutions of *Fasciola* spp. and sheep liver were estimated by the method of Bradford using purified bovine serum albumin as the standard (13).

SDS-PAGE of solutions

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as was reported previously (14). Briefly about 20 µg of homogenated solutions, purified GSTs from sheep liver tissue or *Fasciola* spp. were mixed with sample buffer and were run on 12.5% acrylamide gels. Obtained protein bands were stained with Coomassie blue R-250.

GSTs specific activity assay in the solutions

GSTs activity was assayed spectrophotometrically at 30°C with 1-chloro-2, 4-dinitrobenzene (CDNB) as the standard second substrate and reduced glutathione (GSH) (4, 7, 8, 10) at 340 nm. Switch on spectrophotometer (CECIL 9000) and set water bath temperature to 30°C. *Fasciola* spp. and liver extract solutions with purified solutions were removed from -80°C freezer and allowed to thaw on ice. CDNB 100 mM from 4°C and GSH 100 mM from -20°C freezer were removed and allowed reach to at room temperature, then incubated at 30°C in water bath. For each assay one ml of assay cocktail (980 µl PBS pH 6.5, 10 µl of 100 mM CDNB and 10 µl of 100 mM GSH), was prepared then 100 µl of the cocktail was removed and its remaining (900 µl) was placed into 1.5 ml plastic cuvet. To zero spectrophotometer was used 1 ml of distilled water and to the blank cuvet added 100 µl PBS to 900 µl of cocktail (because the substrates for the GST can react with glutathione non-enzymatically but at slower rates than the enzyme-catalyzed reaction) and measured absorbance at 340 nm for 5 min. To the test cuvet was added 100 µl of sample to 900 µl cocktail and mixed and measured absorbance at 340 nm for various times in 5 min period. For calculation, the Δ340/min for the blank reaction was subtracted from the Δ340/min for each sample reaction. The molar extinction of CDNB was 0.0096 μM^{-1}/cm (15, 16).

Effect of triclabendazole on GSTs activity in the whole extract and purified solutions

In the present study, GST inhibition enzyme assay was investigated in the extract and purified solutions of *Fasciola* spp. and sheep liver by powder and bolus forms of triclabendazole (RAZAK Co.). One mM triclabendazole solution was prepared by 3.59 mg of powder or 10.48 mg of bolus in 10 ml of ethanol. Inhibition of GST specific activity was measured as a IC_{50}, which is defined as the concentration of antihelminth at which 50% of enzyme specific activity is inhibited. This was determined by measuring GSTs specific activity using reduced glutathione and CDNB in the present of different concentration of powder and bolus triclabendazole solutions (3.6, 7.2, 10.8, 14.4, 18, 21.6, 25.2, 36, 43.2, 54 µg/ml) (7, 8, 10).

RESULTS

SDS-PAGE pattern of GSTs in solutions is shown in Figure 1. SDS-PAGE pattern revealed one band protein with 26 kDa that showed purified GST enzymes in the purified solution of *Fasciola* spp.; sheep liver tissue showed two bonds ranging 26-28 kDa. The level of GSTs activity and GSTs specific activity in solutions were detected, and the results presented in Table 1.

The effects of triclabendazole (powder and bolus) on GST specific activity of extract and purified solutions are determined and the results are shown in Figs 2-5. Finally, the inhibitor concentration for remaining specific activity of samples GSTs is calculated graphically and is presented in Table 2. (GST remaining specific activity based on powder of triclabendazole in the purified solution of *Fasciola gigantica* , 31.06, 12.23, 0.94, 0.13 µg/ml and based on bolus of triclabendazole in purified solution of *Fasciola gigantica*, 32.47, 18.82, 1.88, 0.8 µg/ml were obtained; These results are not showed in figures).

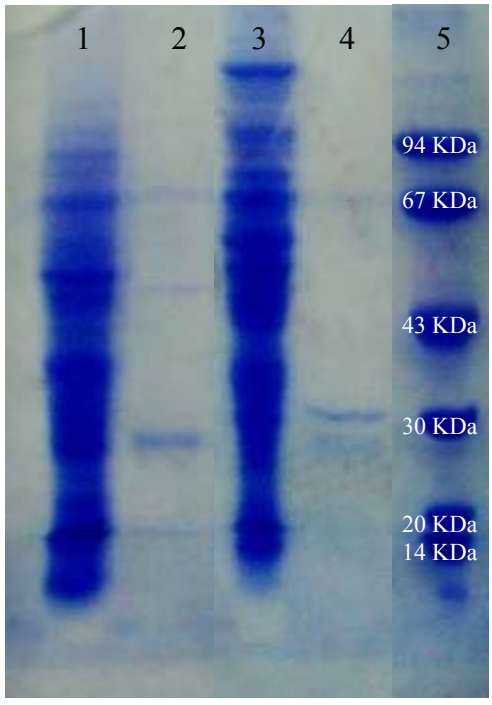

Fig. 1. SDS-PAGE pattern of extract and purified GSTs solutions of *Fasciola hepatica* (lane 1 and 2, respectively); extract and purified GSTs solutions of sheep liver tissue (lane 3 and 4, respectively) and standard marker (lane 5).

GST inhibition assay by triclabendazole

Table 1. GST activity and GSTs specific activity of extract solutions and purified solutions from sheep liver tissue and *Fasciola* spp.

Samples	GSTs activity (U/ml)	GSTs specific activity (µmol/min/mg protein)
Homogenized solutions		
Fasciola hepatica	25.48	766.31
Liver tissue	14.75	446.97
Purified solutions		
Fasciola hepatica	0.17	18.14
Liver tissue	0.17	37.84
Fasciola gigantica *	0.07	35.04

*GST enzyme activity assay was not carried out in the homogenized solution of *F. gigantica*.

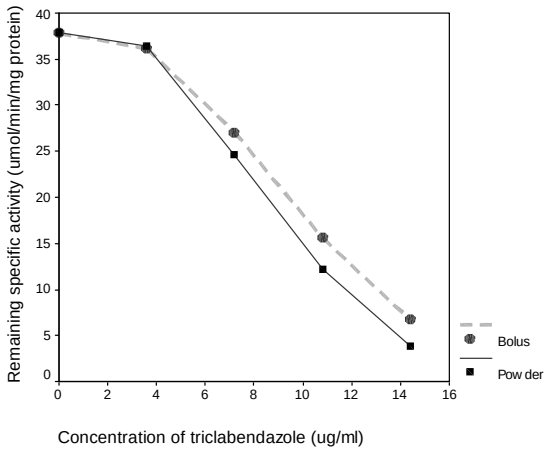

Fig. 3. GST remaining specific activity based on triclabendazole concentration in the purified solution of sheep liver tissue.

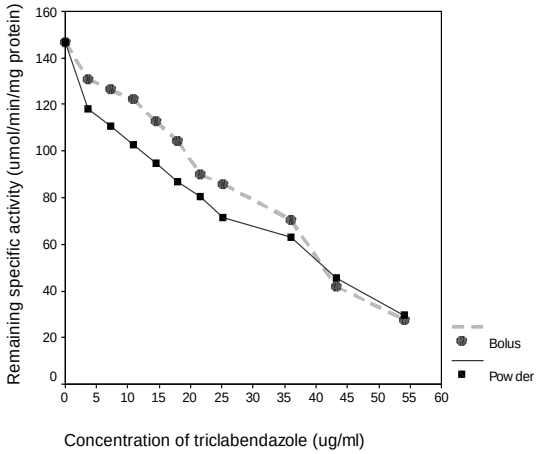

Fig. 2. GSTs remaining specific activity based on triclabendazole concentration in the extract solution of sheep liver tissue.

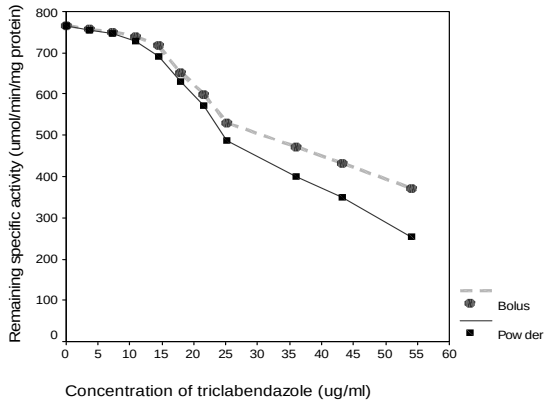

Fig. 4. GST remaining specific activity based on triclabendazole concentration in the extract solution of *Fasciola hepatica*.

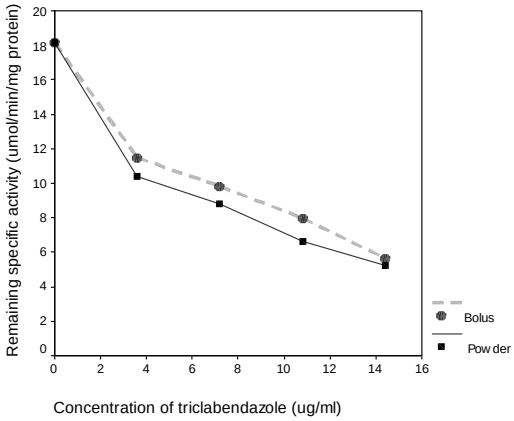

Fig. 5. GST remaining specific activity based on triclabendazole concentration (powder and bolus) in the purified solution of *Fasciola hepatica*.

Table 2. Inhibition concentrations of 50% of GSTs specific activity (IC_{50}) of *Fasciola* spp. and sheep liver tissue by triclabendazole

	IC50	
Solutions	**Powder**	**Bolus**
Fasciola hepatica		
Homogenized	40.07	49.54
Purified	8.36	9.05
Liver tissue		
Homogenized	40.95	49.66
Purified	8.65	9.7
Fasciola gigantica		
Purified	7.2	10.8

DISCUSSION

Previous studies showed that GSTs participated in detoxifying the exogenous toxins. The Fasciola GSTs, like other cytosolic GSTs of helminthes, may be involved in catalyzing the conjugation of glutathione to electrophilic compounds (5). SDS-PAGE of the present study pattern revealed one band protein with 26 kDa that showed purified GST enzymes in the purified solution of *Fasciola* spp.; however, sheep liver tissue showed two bonds ranging 26-28 kDa because some isoenzymes of GSTs were present in the liver. GST protein fraction migrating as a 26 kDa band on SDS-PAGE, has been isolated by affinity chromatography on glutathione-agarose from a soluble extract of protoscoleces of *Echinococcus granulosus* parasite from sheep in Iran (10). The results showed that GSTs activity in extract solutions was greater than purified solutions because there were some enzymes and proteins in the homogenate solution that could affect on the reaction. In addition, the purification process could be the cause of reduction of GSTs activity in solution. Comparison of the effect of triclabendazole on GST specific activity revealed that IC_{50} for bolus was greater than powder in both samples. This phenomenon may be due to this fact that the amount of triclabendazole itself in bolus is less than that of issued on its box or may be due to existence of supplementary materials in bolus, so the effectiveness of powder to bolus is a reasonable fact. Comparison of the effect of triclabendazole bolus and powder revealed that activities of both GSTs were suppressed but the difference between of inhibition assay was not significant. The general inhibition of *Fasciola* spp. and liver tissue GSTs in the microgram range (as judged by IC_{50} value) by triclabendazole may help explaining the mode of action of this chemotherapeutic agents in vitro. Further studies should focus on the elucidation of the role of GST activity in the *Fasciola* defense mechanisms against oxidative and toxic damage and efficacy of the other antihelminths on GST activity.

In brief, these findings suggest the possibility of the selective inhibition of the *Fasciola* GSTs in vitro and use of these results for understanding of molecular mechanism of triclabendazole *in vivo*.

Conflict of interests

We have no conflict of interests.

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