Comparative Assay of Glutathione S- Transferase (GSTs) Activity of Excretory-Secretory Materials and Somatic Extract of *Fasciola spp* Parasites

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Abstract- Fascioliasis is a worldwide parasitic disease in human and domestic animals. The causative agents of fascioliasis are *Fasciola hepatica* and *Fasciola gigantica*. In the recent years, fasciola resistance to drugs has been reported in the many of publications. Fasciola spp has detoxification system including GST enzyme which may be responsible for its resistance. Therefore , the aim of the study was to assay of GST enzyme activity in fasciola parasites. *Fasciola gigantica* and *Fasciola hepatica* helminths were collected from abattoir as a live and cultured in buffer media for 4 h at 37 °C. Excretory-Secretory products were collected and stored in -80°C. *F. gigantica* and *Fasciola hepatica* were homogenized with homogenizing buffer in a glass homogenizer to prepare of somatic extract. Suspension was then centrifuged and supernatant was stored at -80°C. In order to assay the enzyme activity, excretory-secretory and somatic extracts in the form of cocktails (potassium phosphate buffer, reduced glutathione and 1-chloro-2,4-dinitrobenzene substrates) were prepared and their absorbance recorded for 5 minutes at 340 nm. The total and specific GST activity of *F. gigantica* somatic and ES products were obtained as 2916.00, 272.01 micromole/minute and 1.33, 1.70 micromole/minute/mg protein, respectively. *Fasciola hepatica* also showed 2705.00, 276.86 micromole/minute and 1.33, 1.52 micromole/minute/mg protein, respectively. These results are important for analysis of parasite survival / resistance to drugs which use for treatment of fascioliasis.

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Key words: *Fasciola*; enzymes assays; glutathione S- transferase; parasites

Introduction

The parasitic fluke, *Fasciola,* infects humans and ruminant livestock worldwide. An estimated of 2.4 million people are infected with *Fasciola* species, and a further 180 million are at risk (1). In addition, *F. hepatica* causes an estimated loss of $3 billion worldwide per annum through livestock mortality, especially in sheep, and by decreased productivity via reduction of milk and meat yields in cattle (2). In the absence of commercial vaccines, the benzimidazole derivatives including praziquantel were used for treatment of fasioliasis. However, at the present time, triclabendazole (TCBZ; Fasinex®) drug most extensively has been used against *Fasciola* spp. The most common diagnostic field method used to detect *Fasciola* infection requires counting fluke eggs in fecal samples. Juvenile *Fasciola* develop and mature within the intrahepatic bile duct and gall bladder of infected hosts, continuing to release their excretory-secretory (ES) survival products that can ultimately lead to fibrosis and calcification of host tissues.

In vitro biochemical studies have predicted that ES products of *F. hepatica* have roles in feeding behavior, detoxification of bile components, and the evasion of the immune system. In addition, in vitro proteomics support the release of several major protein superfamilies from liver fluke. For example, general phase II detoxification GST with proposed immune evasion roles were found to be secreted by *in vitro* cultured liver flukes (3,4).

In the present study, enzyme activity assay approach as drug detoxification indicators was used for analysis of GST enzyme activity of ES materials from *F. hepatica*/*Fasciola gigantica* and for comparison with the somatic extract of parasites.

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Table 1. Preparation of reagent cocktail in 2-ml cuvette for GST enzyme activity assay of reagent blank and *F. hepatica/F.gigantica* samples

Solutions	Reagent blank	Samples
PB 0.1M ,PH 6.5	1.960µl	1.960µl
GSH 100mM	20µl	20µl
CDNB 100mM	20µl	20µl

Materials and Methods

ES products collection and preparation

Live extracted *Fasciola spp* from naturally infected sheep livers on the day of slaughter were washed for a minimum of three times in PBS, pH 7.4, to remove host material. Live flukes were cultured in buffer media for 4 h at 37 °C. The ES products were clarified via centrifugation at 2000g for 15 min at 4 °C (5). The ES supernatant was concentrated by sephadex G-25 (6). A volume of 200 µl of ES products from each 1 ml supernatant was collected and stored at -80°C.

Somatic extraction preparation

Fasciola spp helminths were collected from abattoir (Ilam, , Iran). *Fasciola* species identified, based on morphological characters and were washed 3-4 times with washing buffer (PBS 7.4). Collected parasites homogenized with 3 volumes of homogenizing buffer, phosphate buffer 6.5, in a glass homogenizer, The suspension was centrifuged (10000g for 30 min at 4°C) and supernatant was stored at -80°C (7).

Enzyme activity assay

To prepare of reagent cocktail in a 2-ml cuvette, added potassium phosphate buffer on GSH (reduced glutathione) and CDNB substrates as presented in table 1. For each assay (Reagent and samples), 200 µl of mixture was removed and then the same volume of buffer or *F. hepatica/F.gigantica* ES/Somatic extract was added into reagent and sample cuvette respectively and mixed well. Meanwhile, the UV spec was set up at 340 nm to assay of GST activity. Finally the cuvette was placed into the barrel of the UV/Visible spectrophotometer and absorbance recorded for 5 minutes (7).

Statistical analysis

To detect the statistical difference between ES/Somatic GST enzyme activity samples mean of *Fasciola hepatica* and *Fasciola gigantica*, two-sample (independent) t-test was conducted by using SPSS software.

Results

Absorbances data of somatic extract and ES products (triplicate samples) are showed at the table 2 , 3, 4 and 5.

Table 2. Absorbances data of reagent blank and samples of *F. gigantica* somatic extract product

Time	Reagent Blank	Sample 1	Sample 2	Sample 3
0′.10″	0.378	0.712	0.758	0.860
1′.10″	0.382	0.781	0.818	0.924
2′.10″	0.394	0.828	0.888	0.975
3′.10″	0.403	0.880	0.923	1.038
4′.10″	0.409	1.030	0.971	1.084
Abs 340/4min	0.031	0.296	0.182	0.193
Abs 340/1min	0.007	0.074	0.045	0.048

Table 3. Absorbances data of reagent blank and samples of *F. gigantica* Excretory - Secretory materials

Time	Reagent Blank	Sample 1	Sample 2	Sample 3
0′.10″	0.572	0.609	0.588	0.586
1′.10″	0.584	0.624	0.618	0.616
2′.10″	0.601	0.645	0.639	0.630
3′.10″	0.614	0.656	0.652	0.641
4′.10″	0.629	0.672	0.662	0.655
Abs 340/4min	0.057	0.006	0.017	0.012
Abs 340/1min	0.014	0.015	0.004	0.003

Table 4. Absorbances data of reagent blank and samples of *F. hepatica* somatic extract product

Time	Reagent Blank	Sample 1	Sample 2	Sample 3
0′.10″	0.542	0.724	0.702	0.750
1′.10″	0.558	0.785	0.764	0.800
2′.10″	0.580	0.855	0.830	0.864
3′.10″	0.585	0.932	0.907	0.933
4′.10″	0.600	1.000	0.970	1.006
Abs 340/4min	0.058	0.218	0.210	0.198
Abs 340/1min	0.014	0.054	0.052	0.049

Table 5. Absorbances data of reagent blank and samples of *F. hepatica* Excretory - Secretory materials

Time	Reagent Blank	Sample 1	Sample 2	Sample 3
0′.10″	0.555	0.602	0.589	0.601
1′.10″	0.564	0.605	0.601	0.603
2′.10″	0.580	0.610	0.605	0.607
3′.10″	0.594	0.622	0.609	0.613
4′.10″	0.608	0.639	0.617	0.622
Abs 340/4min	0.053	0.016	0.005	0.032
Abs 340/1min	0.0132	0.004	0.001	0.008

The amount of protein in the solutions based on Bradford method by using the albumin solutions 10-100 µg/ml as duplicated were detected .The level of protein concentration and total GST activity and GST specific activity in solutions were calculated, and the results presented in Table 6,7.

The results of independent t-test revealed that there is no significant difference between GST activity of *F.hepatica* (M= 276, SD= 4) and *F.gigantica* (M= 272, SD= 2) conditions; t (4)=1.27, p- value > 0.05. There is also no significant difference between excretory-secretory GST activity of *F.hepatica* (M= 1.52, SD= 0.02) and *F.gigantica* (M= 1.70, SD=0.05) conditions; t (4)= 0.71, *P*- value > 0.05.

Table 6. Total GST activity and Specific GST activity of *F. gigantica* products

***F. gigantica* helminth**	**Total GST activity (micro mole /minute)**	**Protein concentration(mg)**	**Specific GST activity (micro mole /minute/ mg protein)**
Somatic extract product	2916	10.72	272.01
Excretory-Secretory materials	1.33	0.78	1.70

Table 7. Total GST activity and Specific GST activity of *F. hepatica* products

***F. hepatica* helminth**	**Total GST activity (micro mole /minute)**	**Protein concentration(mg)**	**Specific GST activity (micro mole /minute/ mg protein)**
Somatic extract product	2705	9.77	276.86
Excretory-Secretory materials	1.33	0.87	1.52

Discussion

Three phase enzyme detoxification system has been defined. The Phase I detoxification system, composed mainly of the cytochrome P450 supergene family of enzymes, is generally the first enzymatic defense against foreign compounds. Phase II conjugation reactions generally follow Phase I activation, resulting in a xenobiotic that has been transformed into a water-soluble compound that can be excreted through urine or bile. Phase III detoxification system includes antiporter activity. The antiporter is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics (8). The helminth GSTs participate in the phase II detoxification. GST enzymes detoxify of oxygen-reactive intermediates (ORI). The ORIs can come from the endogenous parasite metabolism or from the host (9). Xenobiotics are toxic substances which produced and released by the human/animal host during the course of metabolism. Liver tissue has high level of metabolic reactions, therefore *Fasciola spp* which are localize into the liver ducts may be damaged by xenobiotics attack. Endobiotics are products of metabolism pathways of parasite and may be toxic subestances for parasite. GST enzyme of *Fasciola spp* conjugate GSH to xenobiotics and endobiotics materials for neutralization in the adjacent of parasite or excretion from parasite respectively. Tegumental GST probably not secreted to out side of parasite body. In contrast, the parasite to discharge secretory GST enzyme into surrounding tissues.

Statistical analysis indicated that there is no significance difference between *Fasciola* species samples , however, the results of assay show the somatic extract enzyme activity was higher than ES materials. This is correct, because the tegument layer of *Fasciola* parasite is in connection with the hazardous environment and needs to robust detoxification system for protection of itself from xenobiotic materials.

This data is very important and expectable, because *F. hepatica* and *F. gigantica* have similar feature from survival time view point in their hosts which result to chronic infection in fascioliasis diseases.

Briefly, GST enzyme has a basic role in the protection of fasciola parasite against biochemical attack of their host and subsequently cause to aging of parasite. We can also imagine these parasites have the potential for resistance to drugs by this phenomenon. In the recent years, resistance to benzimidazole derivatives has been reported (10-12). These comparative data are very important for analysis of resistance to drugs as exogenous xenobiotics which use for treatment of fascioliasis including triclabendazole and praziquantel drugs.

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