Original Article:

In vitro assay of alkaline phosphatase enzyme activity in Fasciola infected livers and Fasciola hepatica parasite

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ABSTRACT

The present work was designed to determine alkaline phosphatase (ALP) activity level in *Fasciola hepatica* parasite and Fasciola infected livers to evaluate the effects of liver infection on enzyme activity and to compare enzyme activity in liver and parasite. The sheep livers were collected and adult *Fasciola hepatica* parasites were isolated and washed with PBS buffer. Collected healthy and infected livers and parasite were homogenized and extract solutions were centrifuged and stored at -20°C. ALP enzymes activity was measured in the extract solution of samples. Proteins of the samples were measured and protein bands were detected through using Bradford and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods respectively. Independent two samples t-test was performed to determine significant difference between different groups. The mean values of the assayed ALP specific activities of infected and healthy livers and *Fasciola hepatica* parasite were estimated 0.163,0.133,0.048 U/mg protein/ml correspondingly. Gel electrophoresis (SDS-PAGE) of *Fasciola hepatica* and liver extract solution samples displayed different protein bands, including ALP enzyme. Statistical analysis did not show significant difference between enzyme's activity in infected and healthy livers ($P>0.05$). However, the liver demonstrated more than ALP activity level to parasite ($P<0.05$). The findings show that ALP enzymes activity in *Fasciola hepatica* infected livers could not be concerned as a specific pathological biomarker in fascioliasis, but meanwhile this enzyme displays interest activity in parasite.

Keywords: Alkaline phosphatase; *Fasciola hepatica*; SDS-PAGE; Protein; Liver

INTRODUCTION

Fasciola hepatica is one of the important helminthes parasites. The presence of *Fasciola hepatica* parasite in the biliary duct and gallbladder of human causes fascioliasis and liver disorders. Sheep are definitive hosts and snails are intermediate hosts[1]. Detected enzymes including glutathione S-transferase (GST), superoxide dismutase (SOD) and protease have a vital role in survival, migration and nutrition of fasciola parasite [2-4]. Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic composition and inorganic phosphate. ALP is detected in extensive variety of organisms including helminthes parasites whose inhibition might lead to paralysis and extirpation of the parasites[5]. ALP contributes to the conversion of carbohydrates to lipids and additionally, participates in the proteins and nucleic acids metabolism[6]. In humans, it exists in liver, bones, intestine, placenta, kidneys and leukocytes. Alkaline phosphatase activity can be increased in some hepatobiliary disorders. The common reasons for the ALP intensification include primary biliary cirrhosis, cholangitis, and biliary obstruction[7] .The aim of this in vitro study was to detect ALP enzyme activity level in Fasciola infected livers in order to evaluate the

effects of liver infection on ALP activity and to compare its enzyme activity with *F. hepatica* parasite.

MATERIALS AND METHODS

Protein measurement and identification of collected parasite and liver tissues

Fasciola hepatica infected and healthy livers (10 samples for each) of sheep were collected from local abattoir and transferred to Helminthology Lab - School of Public Health in Tehran University of Medical Sciences, Tehran, Iran, from 2015-2016. After dissection, 10 adult worms (Juvenile forms were excluded) of *F.hepatica* were isolated from bile ducts of naturally infected sheep livers. The samples were rinsed 3 times in PBS buffer pH 7.2 for the elimination of the remaining blood clots and host constituents. Recovered parasites and liver tissues were homogenized with 3 volumes of phosphate buffer solution (PBS) pH7.2 in Mortar and pestle. Subsequently, suspensions were centrifuged by 10000 g for 30 min at 4°C and supernatants were stored at -20°C. Protein concentration was measured by Bradford method with Bovine Serum Albumin standard solutions as duplicate in supernatant samples. The absorbance of samples was measured at 595 nm[8]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie blue staining were used to identify the protein constituents of samples. For this purpose, samples were mixed with sample buffer and were run on 10% acrylamide gels. Lastly, the gel was stained with coomassie blue G-250. To determine Molecular weights (MW) of proteins in samples, PeqGold protein marker was used (http://www.peqlab.de/wcms/de/). The MW of proteins was detected through using calculated ratio factor (Rf) of protein bands and identification of protein was performed in Expasy protein database (http://www. Expasy.org).

ALP enzyme activity assay of samples

ALP activities were assayed via using the enzyme assay kit (Ref. number 10-503; ZiestChem Diagnostic Company). The measuring results are based on the change in absorbance per unit of time. The kit package includes Diethanolamine, and P-Nitrophenylphosphate as buffer and substrate respectively. To perform the experiment, buffer and substrate reagents were mixed with each other with a ratio of 4 to 1. 1ml mixed prepared solution (reagent); 25µL extract sample of liver tissue or parasite was added to a cuvette and the absorbance of each sample was measured at 405 nm at 37°C with a spectrophotometer. The conversion of the P-Nitrophenyilphosphate to p-Nitrophenol produces yellow color which is proportionate to the level of ALP enzyme in the samples. The enzyme value was calculated according to manufacture manual. One unit enzyme activity is defined as the amount of ALP enzyme that catalyzes the transformation of one micromole of substrate per minute at 37°C.

Statistical analysis

Independent two sample t-test was performed to compare mean values of understudied groups (http://www.socscistatistics.com/tests/studenttt est/).

RESULTS

Protein concentration, total activity and specific activity of ALP for *F. hepatica*, healthy and infected livers are presented at Table 1. (Mean of 10 samples for each). Statistical analysis showed no significant differences between the activity of the enzyme in the healthy and infected ($P>0.05$). However, the liver demonstrated more than ALP activity level to parasite ($P<0.05$). Parasite samples showed protein band of ALP with MW 59kDa in SDS-PAGE gel (Fig. 1). Recognized proteins of gel electrophoresis are demonstrated in tables 2 for parasite and 3 for liver samples.

Table 1. The measured mean values of protein concentration, ALP total activity and specific activity for *F. hepatica* parasite and liver samples (10 samples for each)

Samples	Protein amounts (mg/mL)	ALP Total activity (U/mL)*	ALP Specific activity (U/mL/mg protein)
Liver (Healthy)	0.569±0.065	0.093±0.026	0.163
Liver (infected with *F. hepatica*)	0.563±0.072	0.075±0.033	0.133
F. hepatica	0.457±0.101	0.022±007	0.048

*****One unit of enzyme activity is the quantity of enzyme that catalyzes the reaction of 1µmol of substrate (ALP) per minute at 37°C

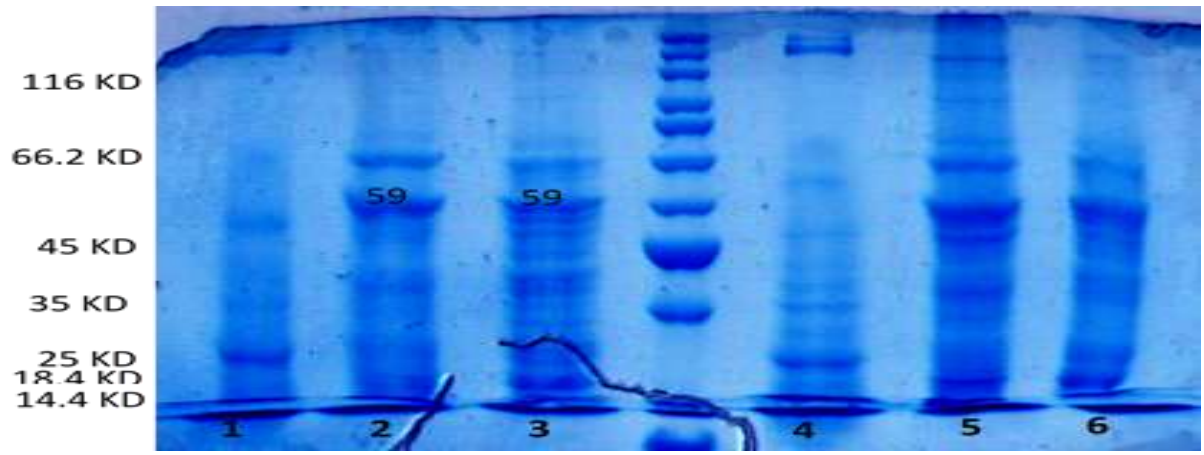

Figure 1. Detected molecular weight of proteins from extracts of samples by using SDS-PAGE analysis
The infected liver samples were presented in lane 3, 6 and healthy liver samples in lane 2, 5 and parasite samples in lane 1, 4. Protein marker is in the middle of page.

Table. 2. Recognized protein bands of parasite according to Expasy database

Bands MW (Dalton) obtained by SDS	Bands MW (Dalton) by Expasy database	Protein name in Expasy database
83564	79497	ATP-binding cassette protein
	79325	Putative cys1 protein (cys1)
73279	70753	Heat shock protein 70 (HSP70)
66654	66010	Thioredoxin-glutathione reductase (tgr)
61032	61223	Gut antigen protein (2fas1)
57391	58378	NADH dehydrogenase subunit5 (nad5)
	56464	Cytochrome oxidase subunit1 (cox1)
	56432	Cytochrome oxidase subunit1
	56395	Leucyl aminopeptidase (LAP)
	55223	Protein disulfide-isomerase (PDI gene)
51256	51584	Citrate synthase
45701	47929	Legumain like (Leg1)
	47367	NADH-ubiquinone oxidoreductase chain4 (nad4)
	47309	Cathepsin D-link aspartic protease
	47124	Cathepsin D-link aspartic protease
	47000	Phosphoglycerate kinase
	46280	Enolase (eno)
	46275	Enolase (ENO)
44133	43421	Phosphoglycerate kinase
	41545	Cytochrome b (cob)

Table 3. Recognized protein bands of liver according to Expasy database

Bands MW (Dalton) obtained by SDS-PAGE	Bands MW (Dalton) by Expasy database	Protein name in Expasy database
123919	128266	Calcium-transporting ATPase
80697	79497	ATP-binding cassette protein
78709	79325	Putative cys1 protein (cys1)
71474	70753	Heat shock protein 70 (HSP70)
67210	66010	Thioredoxin-glutathione reductase (tgr)
59926	59522	ALP
55422	56395	Leucyl aminopeptidase (LAP)
	55223	Protein disulfide-isomerase (PDI gene)
53077	53478	Galactokinase-like protein (GALK)
52115	52433	Mitochondrial acetate: sumlinate COA-transferase
	50108	Glucose-transporter

50327	50509	Alpha-tubulin (alpha-tub5)
	50505	Tubulin alpha-5 (alpha-tub5)
	50063	Alpha-tubulin (alpha-tub1)
	50557	Alpha-tubulin (alpha-tub3)
	50011	Alpha-tubulin (alpha-tub2)
	50024	Tubulin alpha-2 (alpha-tub2)
	50559	Tubulin alpha-3 (alpha-tub3)
49006	48859	Beta-tubulin (beta-tubulin-1)
	48346	Beta-tubulin (beta-tub5)
	48373	tubulin beta-5 (beta-tub5)
	48348	Beta-tubulin (beta-tub6)
	48330	Tubulin beta-6 (beta-tub6)
	48360	Alpha crystalline-containing small heat shock protein variant NtermFhHSP35a
	49801	Alpha-tubulin (alpha-tub4)
	49787	Tubulin alpha-4 (alpha-tub4)
	49861	Beta-tubulin (beta-tub1)
	49849	Beta-tubulin (beta-tub3)
	49819	Tubulin beta-3 (beta-tub3)
	49632	Beta-tubulin (beta-tub2)
	49604	Tubulin beta-2 (beta-tub2)
	49821	Beta-tubulin (beta-tub4)
	49838	Tubulin beta-4 (beta-tub4)
46933	47367	NADH-ubiquinone oxidoreductase chain (nad4)
	47309	Cathepsin D-link aspartic protease
	47124	Cathepsin D-link aspartic protease
46159	46280	Enolase (eno)
	46275	Enolase (ENO)
44133	43421	Phosphoglycreate kinase

DISCUSSION

The first step in detecting liver damage is a simple blood test (liver function test); the presence of high level of certain enzymes shows that liver is damaged because of their leak into the bloodstream[9]. This study compared alkaline phosphatase enzyme activity and total protein level in none parasitic and parasitic liver by *F. hepatica* (Table 1). Statistical analysis did not demonstrate significant differences between the amount of protein and enzyme activity in the healthy and infected liver ($p>0.05$), which might probably be due to the early stages of infection or mild contamination. Previous study showed that the level of enzyme activity in the serum of patient did not alter which confirms our findings[10]. In this study we determined the total amount of protein and ALP activity level in *F. hepatica* parasite and liver (Table 1). Statistical t-test results showed the amount of liver protein was significantly higher than parasite ($p<0.05$), which might probably be due to the difference in the amount of sample mass, although they had the same size. The study results showed that specific activity of ALP in healthy and infected liver were more than parasites. According to the role of this enzyme in the metabolism of the liver, this finding was expected [11]. From the standpoint of comparing the parasite and host biochemical compounds (Tables 2 and 3), it seems that the differences between them like *2fas1*, *Nad5,citrate synthase* and *Leg1* which are present in parasite and absent in healthy and infected liver could be used as a marker for diagnostic or vaccine manufacturing aims. Gel electrophoresis (SDS-PAGE) of *Fasciola hepatica* and sheep liver tissue extracts displayed a protein band of ALP enzyme with MW of 59kDa. ALP enzyme activities were measured in the parasite sample extracts but not detectable in the gel bands. Perhaps due to the small amount of the enzyme that is nanogram, the amount of the enzyme of protein SDS-PAGE at microgram can be detected by staining coomassie Blue [12].

CONCLUSION

The results indicate that ALP enzyme activity in *Fasciola hepatica* infected livers could not be concerned as a pathological biomarker in fascioliasis. However, this enzyme displays significant activity in parasite which is important from standpoint of host-parasite relationship.

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"The authors declare no conflict of interest"

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