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Glutamate Dehydrogenase, Alanine- and Aspartate Aminotransferase of E. Tenella (Apicomplexa, Coccidia) Oocysts and Effect of Amino Acids' Alkyl Derivatives on their Activities

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Abstract

It was found that E. tenella oocysts possessed glutamate dehydrogenase and also aspartate- and alanine aminotransferrase activities. N-amido propylglycine inhibited the enzymes' activities in the parasite's oocytes. The authors of the presented paper believe that the enzymes mentioned above are of significant importance for metabolic processes in coccidia and can be target for anticoccidial chemotherapeutical preparations.

Key words: chicks, Eimeria, N-amido propylglycine, N-vinyl chlorine methionine, N-amido propylglycine.

INTRODUCTION

The diseases caused by coccidian intracellular parasites, coccidioses (eimeriosis, toxoplasmosis, isosporosis, neosporosis), are widespread diseases of humans, wild- and domestic animals. Of those parasitoses the eimerioses were believed to be the most dangerous ones for animals especially for young individuals. The economic loss from eimeriosis is pooled of mortality and growth- and development retardation of affected poultry and also of reduction of their body weight, egg laying and deterioration of the meat quality [1, 5, 21].

Chemical preparations were believed to be the most effective means for control of animal eimerioses. However, after enduring use of those eimeria adapted to them and became resistant, this character was inherited by subsequent generations [8, 9, 12]. Apart from that, many of those preparations are toxic substances. They affected the host metabolism [23, 24], suppressed immunity [10, 16] and accumulated in the products obtained from the animals. To escape such negative consequences new, more effective and ecologically safe methods were sought for. Anticoccidial vaccines were created [22]; various food additives of microbiological and plant origin were tested [19].

Previously we found that alkyl derivatives of amino acids used for the treatment of eimerioses, positively influenced biochemical characters of the macroorganism, reduced the chicken mortality and impeded the sporulation process of oocysts in vitro [3, 4, 18, 27].

For coccidia *Eimeria tenella* and *E. acervulina* the protein antigen compositions and metabolites participating in the amino acid metabolism were studied [25, 26]. But till now there were no data on the enzymes glutamate dehydrogenase and aspartate- and alanine aminotransferrase actively participating in the amino acid metabo lism. This information not only is necessary for understanding the parasite metabolism but also for ascertainment effects of such preparations as amino acid derivatives on the metabolism of eimeria.

The objective of our research was the study of the activities of the enzymes glutamate dehydrogenase and aspartate- and alanine aminotransferrase in the *E. tenella* oocysts released from the experimentally infected chicks and those treated with alkyl derivatives of amino acids.

MATERIALS AND METHODS

The parasites for our experiments were multiplied on 15 chicks of 20 days age. The White Plymouth Rock eimeria free chicks were infected with oocysts of a laboratory strain of *E. tenella* obtained from the Laboratory of the Biochemichal Aspects of Host-Parasite Relationships, Institute of Zoology, Azerbaijan National Academy of Sciences. LD_{50} of the *E. tenella* strain used was 20-30 oocysts for a bird in 20 days old chicks. The chicks were infected by inoculation of oocysts into their craws. First oocysts were released with the chicks' feces on the 7th day after infection.

Tap water was added to the feces mass collected on the 7-10th days after infection. The suspension was mixed and then centrifuged at 3000 rpm for 5 minutes. The supernatant was carefully removed and the sediment was resuspended in supersaturated sodium chlorate solution (the ratio was 10:1) and then centrifuged again for 5 minutes. Again, the supernatant was removed and the sediment with oocysts was rinsed thrice with distilled water. The oocysts purified this way from feces and concomitant impurities were then left in the 2% solution of potassium bichromate at 29^0 C for oocyst sporulation.

Fifty 20 days old chicks were infected with the purified *E. tenella* oocysts. The dosage for infection was 20,000 oocysts per bird. After the infection all the chicks were divided into 5 groups, 10 chicks in a group. The first group served as the control (infected) group. The chicks of other groups on the 3rd day after infection were treated with N-amido propyl methionine, N-vynil chlorine methionine, N-amido propyl glycine (products of the *REOR* firm, Baku, Azerbaijan) and β-alanine (*Reonal*,

Hungary), respectively. Solutions were prepared from those preparations by solving 5 g of each of the preparations in 1 liter of tap water. The solutions were given to the chicks for drinking without limitation for 10 days. On the 7-10th days after infection feces were collected from infected and treated birds. The oocysts released from the chicks were first treated by the procedure describe above and then prepared for subsequent biochemical study by the method developed previously by one of the paper's authors [15]. The suspension of sporulated oocysts in potassium bichromate solution was centrifuged at the 5000 rpm for 5 min. The supernatant was removed and distilled water was added to the sediment in which it was resuspended and then the suspension was centrifuged again. This procedure was repeated thrice. Then sucrose solution of the density 1.050-1.085 g/cm^3 was added to the sediment (the sediment : solution ratio was 1:10) and the suspension was centrifuged at 1000 rpm for 15 minutes. Then the suspension of the oocysts in sucrose was collected in tubes, diluted with distilled water (1:10) and centrifuged, the supernatant was removed. The procedure was repeated thrice. Then the suspension was passed through filter No 2 and the oocysts arrested on the filter's surface were rinsed with penicillin solution (400 units per 1 liter of water). The oocysts removed from the filter surface were then resuspended in distilled water, at first with cationites CU-1 (the particle diameter 0.3-2.0 mm) and then with anionites AN-1 (the particle diameter 0.3-2.0 mm). The suspension was left for 10 minutes for sedimentation of the exchange resins. Then the suspension was placed in sterile dividing funnel. After sedimentation of the oocysts the supernatant was removed and the oocysts were used for biochemical study. The purity of the oocyst suspension was tested under microscope and by biuret reaction. The tests did not detect any contaminations including proteins.

To determine the enzyme activities in oocysts 8 million oocysts or more were used. The oocyst suspension in amount of 0.01 ml was taken with graded micropipette and placed on a slide. The oocysts number was then counted under microscope. This procedure was carried out for 5 times and the mean was calculated. Then the suspension volume for getting required amount of the oocysts was calculated.

The oocysts were destroyed by the ultra sound dispergator UDZN-1 (Russia) at the frequency 22 kHz, power 300 wt. Sonication of the oocysts for subsequent definition of glutamate dehydrogenase was carried in the presence of 5 ml of 0.25 M sucrose solution with 0.1 mM EDTA and for definition of transaminases' - in the unalloyed sucrose solution. Sonication of the materials was carried out at 4^{0}C for 3 minutes. The cell disintegration degree was checked with optical microscope.

The activity definition of the amino transferrases was carried out by Osadchaya's method [20]. For definition of the aspartate amino transferrase activity 0.5 ml of the substrate solution was put in a tube. Then 0.1 ml of the oocyst suspension was added and the tube was placed in thermostat for 1 hour at the 37^0. Then 0.5 ml of 0.2% solution of diphenylhydrazine was added and the sample was left for 20 minutes at the room temperature. Then 5 ml of the 0.4 N NaOH solution was added and the sample was left for coloration development. The optical density was measured at the spectrophotometer SF-26 (Russia) at the wave length 530 nm with the control sample that contained all components of the test sample except oocyst homogenate. Instead, the latter 0.1 ml of distilled water was added. The control sample was incubated at the same conditions as the tested one.

To define the alanine amino transferrase activity 0.5 ml of substrate solution was put in a tube and then 0.1 ml of the homogenate was added. The tube was placed in thermostat for 30 minutes at the 37^0 C. The subsequent course of the analysis was the same as for definition of the aspartate amino transferrase activity. The calculation of the enzyme activity was accomplished by the calibration graph delineated for pyruvate.

The glutamate dehydrogenase activity was determined by L.I. Zakharova method [28]. The incubation medium was prepared immediately before carrying out the enzyme reaction. The following ingredients were used: 0.6 ml of 0.25 M tris- HCl + 0.3 ml 10^{-3} M EDTA solution + 1.7 ml H_2O + 0.1 ml $18x10^{-4}$ M NADP. Into the spectrophotometer cuvette was poured 2.7 ml of the incubation medium and 0.1 ml of the tested oocyst homogenate was added. The reaction commenced by adding 0.2 ml of 0.75 M glutamate solution to the mixture. The optical density was measured after 15 second intervals during 1.5-2 minutes.

RESULTS

It was found that E. tenella oocysts possessed alanine amino transferrase-, aspartate amino transferrase and glutamate dehydrogenase activity. The activity of aspartate amino transferrase in the oocysts was higher than that of alanine amino transferrase (Table 1).

It is obvious from the data of Table 2 that when the chicks were treated with N-amido propyl methionine, N-vinyl chlorine methionine or β-alanine, the alanine aminotransferrase activity in the *E. tenella*'s released by them was the same as in oocysts released by the untreated infected ones. However, when the chicks were treated with N-amido propyl glycine the enzyme activity reduced as compared with the oocysts released from the untreated infected birds. N-amido propyl glycine also caused reduction of the aspartate amino transferrase activity in the oocysts released from the treated chicks. Other amino acid derivatives did not cause any change of that enzyme activity in oocysts when their hosts were treated.

Table 1. Glutamate dehydrogenase, alanine- and aspartate aminotransferrase activivities in oocysts of *E. tenella*

Glutamate dehydrogenase *	Alanine aminotransferrase **	Aspartate amino transferrase**
16.4±1.4	0.49±0.03	0.71±0.02

*M ± m in mkM NADP on protein of 8 million oocysts

**M ± m в mkM pyruvate 8 million oocysts per hour

Table 2. Glutamate dehydrogenase, alanine- and aspartate aminotransferrase activities in *E. tenella* oocysts released from infected and treated chicks.

Enzyme activities	Oocysts released by chicks				
	Infected	Treated			
		N-amido propyl methionine	N- vynil chlorine methionine	N-amido propyl glycine	β-alanine
Alanine amino transferrase [a]	0,49±0.03	0.39±0.02	0.40±0.03	0.36±0.03 [c]	0.44±0.02
Aspartate amino transferrase [a]	0.71±0.02	0.64±0.02	0.65±0.03	0.59±0.02 [d]	0.67±0.03
Glutamate dehydrogenase [b]	16.4±1.4	11.5±1.1	12.9±1.2	8.7±1.3 [d]	14.5±1.2

[a] M±m in mcM pyruvate 8 million oocysts per hour (n =5)
[b] M ± m in NADP/min on protein of 8 million oocysts (n=5)
[c] Enzyme activity differ at P≤ 0.05
[d] Enzyme activity differ at P≤ 0.02

Similarly, the glutamate dehydrogenase activity in the oocysts released from the chicks treated with N-amido propyl glycine was lower than in the oocysts released from the infected chicks. Other amino derivatives used by us did not affect significantly the activity of this enzyme in oocysts.

It can be seen from the data considered above that the most significant reduction of alanine amino transferrase, aspartate amino transferrase and glutamate dehydrogenase activities took place in the oocysts obtained from the chicks treated with N-amido propyl glycine. In the oocysts obtained from the chicks treated with other amino acid derivatives some reduction of the glutamate dehydrogenase activity was only took place and it was not significant.

DISCUSSION

E. tenella is an intracellular parasite and the endogenous cycle of its development occurs in the bird's cecum. To the 7th day after infection oocysts (zygotes) release with fecal mass. Further development (sporulation) occurs in the environment when optimal temperature and humidity conditions are available there and thus the oocysts become invasive. In birds infected with the oocysts of this species endogenous cycle in intestine is accompanied with severe pathogenic process. In this process the cecal appendices are being affected and big hemorrhagic ulcers appear. It results in severe hemorrhagic diarrhea and eventually in significant loss of birds [17].

In the chicks treated with N-amido propyl methionine, N-vynil chlorine methionine and β-alanine the invasion though had some clinical symptoms of the typical eimeriosis. But in contrast to the control chicks it occured mildly and percent of the survived chicks increased. In the chicks treated with N-amido propyl glycine the eimeriosis occured significantly milder. It was characterized by somewhat milder pathological condition. The cecal appendices were covered with lots of hemorrhage ulcers at the sites of the parasite development but those were small, dot-like ones.

Our previous study showed that in the livers of chicks infected with E. tenella the aspartate- and alanine amino transferrase activities increased. The glutamate dehydrogenase activity reduced in cytoplasmic fraction of liver and did not change in mitochondria. All the amino acid derivatives mentioned above normalized to some extent the activities of the amino transferrases and glutamate dehydrogenase in the livers of the infected chicks [1, 2]. The preparations also positively affected the liver proteins and blood of the infected chicks and increased amount of their immuno globulins [6, 27].

It is clear from the data mentioned above that the healing effect of the amino acid alkyl derivatives manifested in fast restoration of the host biochemical characters, increase of survival, mass gain of the invased birds and reduction of the parasite reproduction [4]. The amino acid alkyl derivatives negatively affected the parasite metabolism, reduced the activities of the enzymes participating in the amino acid metabolism such as glutamate dehydrogenase, aspartate- and alanine amino transferrase and thus to some extent impeded the amino acid metabolism.

Currently, it is difficult to understand the way the amino acid alkyl derivatives affected the parasite development and reproduction. Presumably, the amino acid derivatives affected principally the early stages of the parasite development in the chick intestines. Those were the 1st and 2nd generations of merogony (asexual reproduction) that originate gamonts. In the treated birds during merogony (days 2-5 of invasion) some amount of merozoits and meronts perished and the having survived ones became to some extent less viable and their ability to originate normal macro- and microgamets reduced. It resulted in reduction of normal gamete amount and therefore the process of fertilization and zygote (oocyst) formation became less effective. Thus, during the patent period of invasion (day 7-20 after infection) the infected chicks released with 2 g of fecal mass 17330270 oocysts and those treated with N-amido propyl glycine, N-amido propyl methionine and N-vynil chorine methionine - 13593112, 15207015, 15753526 and 16712752, respectively. In the control group the chick loss was 54% and in the groups of chicks treated with the amino acid derivatives – 8.9%, 22%, 40% and 45%, respectively.

The proteins of the eimerian oocytes like those of their host consist of ordinary 20 amino acids. Apart from the amino acids contained in proteins there are some free amino acids and their metabolites such as taurine, phosphoethanolamine, hydroxiproline, sarcosine, cysteic acid, phosphoserine, gamma-amino butyric acid and methylhistidine-3 in those [25]. The presence of many different amino acids, their precursors and metabolites in eimerian oocysts indicates that coccidia in spite of their parasitic mode of life retain most pathways of the amino acid metabolism. It was confirmed by aminotransferrases - and glutamate dehydrogenase activities presence and also by presence of their substrates: alanine, glutamate and aspartate in the oocysts.

The data obtained by us demonstrated that by the study of eimerian enzyme systems and effects of some chemical preparations on those the efficiency of anticoccidial preparation could be estimated and such a study would facilitate goal-oriented search for effective and ecologically pure preparations. One of the preparations tested by us, N-amido propyl glycine inhibited the activities of the parasite enzymes mentioned above and facilitated high level of their activities in the host liver. It enabled us to conclude that N-amino propyl glycine possessed the antieimerial activity [7]. After wide scale tests in farms it might be recommended for treatment and prevention of animal eimerioses.

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