



***In vitro* Studies on the Effect of Parasitosis on Amino Acid Profile of *Bulinus* species**

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Authors' contributions

This work was carried out in collaboration among all authors. Authors VUO and GNI designed the study. Author VUO performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VUO, GNI and EUA managed the analyses of the study. Author VUO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Freshwater snails are intermediate hosts of some trematode diseases including Schistosomiasis. This study was designed to study the interactions between *Bulinus* species and the larval stages of *Schistosoma haematobium* specifically the amino acid levels of the infected freshwater snails. *Bulinus* species were collected from their natural habitats, reared in the laboratory, and exposed to the larval stage of *Schistosoma haematobium*. Using the method described in Association of Official Analytical Chemists in determining Amino acid profile the amino acid profile of both the infected and non-infected *Bulinus* species was determined. Histidine, valine, methionine, phenylalanine, lysine levels of the *Bulinus* species decreased amongst the infected starved snails than in the control. The amino acids profile decreased significantly across the different challenges that the snails *Bulinus* were exposed to. This study has opened research windows for the control of urinary schistosomiasis through the control of the intermediate hosts using some biochemical approach.

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1. INTRODUCTION

Many freshwater snails are vectors of diseases of human and livestock, since they are the intermediate hosts of a number of helminth diseases caused by trematodes [1]. The presence of such snail species in a locality is usually a bio-indicator of the transmission potential of such trematode parasitic diseases in the locality [2]. Many species of freshwater snails belong to a class of highly infective fluke of veterinary importance. The fluke cause debilitating illness in millions of animals. The infections are widespread and prevalent in areas where the snail intermediate host breed in water bodies contaminated by faeces or urine of infected animals [3]. Animals acquire these infections through repeated grazing on contaminated vegetation, humans on the other hand acquire these infection through repeated contact with water during fishing, farming, washing, bathing and recreational activities [4]. The snails are considered to be intermediate host [5] because snails harbour the asexual stage of the parasite while vertebrates harbour the sexual stage of the parasites. *Bulinus* species are intermediate host of urinary schistosomiasis, there is little or no information on the interactions between larval stages of *Schistosoma haematobium* and the *Bulinus* species.

2. METHODS

2.1 Snail Rearing

The snail samples were reared in plastic container for period of 56 days (14 weeks) in the Advanced Laboratory of the Department of Zoology, Federal University of Agriculture, Makurdi in 2017. The snails were reared based on the measures of successful snail rearing as provided by Evelyn and Haseeb [6]. The snails were fed with lettuce and cabbage. Five snails were kept in each container to avoid overcrowding and the aquaria were constantly cleaned to avoid other organisms.

2.2 Hatching the Eggs and Infecting the Snail

Juveniles were used for the work. Juvenile snails were kept in a Petri dish and samples containing

the ova of *Schistosoma haematobium* were introduced into the each petri dish via dechlorinated water. The snails were grouped into six- control group, infected group, infected starved (this group were infected kept in petri dish containing water but with no lettuce), infected and aestivate (this group was allowed to aestivate by not keeping them dry). Each Petri dish was exposed to artificial light (200watts of electric bulb) to induce hatching of the eggs. Miracidia swam out of eggs that hatched successfully and penetrate the snail where they are known to have biochemical attraction [7]. Six weeks after the introduction of the ova of the *Schistosoma haematobium*, when the ova should have undergone several developments as recorded by Centre for Disease Control [8], the snails were exposed to artificial light (200 watts of electric bulb) to induce cercaria shedding. All the above processes were monitored using a hand lens.

3. AMINO ACID ANALYSIS

3.1 Determination of Amino Acid Profile

The Amino Acid profile in the known sample was determined using methods described by Benitez [9] and Association of Official Analytical Chemists [10] the known sample was dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-Sample Amino Acid Analyzer (TSM).

3.2 Defatting Sample

The sample was defatted using chloroform/methanol mixture of ratio 2:1. About 4 g of the sample was put in extraction thimble and extracted for 15 hours in soxhlet extraction apparatus.

3.3 Determination of Nitrogen

A small amount (200 mg) of ground sample was weighed, wrapped in whatman filter paper (No. 1) and put in the Kjeldahl digestion flask. Concentrated sulphuric acid (10 ml) was added. Catalyst mixture (0.5 g) containing sodium sulphate (Na_2SO_4), copper sulphate (CuSO_4) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion.

Four pieces of anti-bumping granules were added.

The flask was then put in Kjeldhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100 ml in standard volumetric flask. Aliquot (10 ml) of the diluted solution with 10 ml of 45% sodium hydroxide was put into the Markham distillation apparatus and distilled into 10 ml of 2% boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70 ml of distillate was collected.

The distillate was then titrated with standardize 0.01 N hydrochloric acid to grey coloured Percentage

$$\text{Nitrogen} = (a-b) \times 0.01 \times 14 \times V \times 100$$

W x C

Where,

- a. = Titre value of the digested sample
- b. = Titre value of blank sample
- v. = Volume after dilution (100 ml)
- W. = Weight of dried sample (mg)
- C. = Aliquot of the sample used (10 ml)
- D = Nitrogen constant i

3.4 Hydrolysis of the Sample

A known weight of the defatted sample was weighed into glass ampoule. Seven milliliters (7 ml) of 6NHCl was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e.g methionine and cystine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105°C ± 5°C for 22 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humans. It should be noted that tryptophan is destroyed by 6N HCl during hydrolysis. The filtrate was then evaporated to dryness at 40°C under vacuum in a rotary evaporator. The residue was dissolved with 5 ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

3.5 Loading of the Hydrolysate into TSM Analyzer

The amount loaded was between 5 to 10 microlitres. This was dispensed into the cartridge

of the analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of the analysis lasted for 76 minutes.

3.6 Method of Calculating Amino Acid Values from the Chromatogram Peaks

An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids and the corresponding amino acid concentration in g/100 g of protein.

Alternatively, the net height of each peak produced by the chart recorder of TSM (each representing an Amino) was measured. The half-height of the peak on the chart was found and width of the peak on the half height was accurately measured and recorded. Approximately area of each peak was then obtained by multiplying the height with the width at half-height.

The norleucine equivalent (NE) for each amino acid in the standard mixture was calculated using the formula:

$$NE = \frac{\text{Area of Norleucine Peak}}{\text{Area of each amino acid}}$$

A constant S was calculated for each amino acid in the standard mixture:

Where,

$$S_{\text{std}} = NE_{\text{std}} \times \text{Molecular weight} \times \mu\text{MAA}_{\text{std}}$$

Finally, the amount of each amino acid present in the sample was calculated in g/16 gN or g/100 g protein using the following formula:

$$\text{Concentration (g/100 g protein)} = \frac{NH \times W@NH/2}{S_{\text{std}} \times C}$$

Where,

$$C = \text{Dilution} \times 16 \div NH \times W (\text{nleu})$$

$$\text{Sample Wt (g)} \times N\% \times 10 \times \text{Vol. loaded.}$$

Where,

- NH = Net height
- W = Width @ half height
- nleu = Norleucine

3.7 Statistical Analysis

Data collected was subjected to ANOVA to determine differences in mean while LSD was used to separate mean differences. Chi square test was used to determine the associations in water snail abundance while students t-test and correlation were used to determine differences in snail growth and shell length and correlation coefficients.

4. RESULTS AND DISCUSSION

There was a slight decrease in the histidine level in the starved snail than in the control snail, a sharp fall in the infected starved snail after the rise noticed in the uninfected aestivated (Table 1). There were significant differences in the valine levels as shown in Fig. 2 and Table 1. Methionine, phenylalanine, lysine and aspartic acid levels had a high significant differences as shown in Table 2. Also isoleucine and threonine levels varied significantly (Table 1).

Proline, glycine, arginine, alanine, glutamic acid, serine and cystine levels were all significantly different with the repeated challenges that the snails were exposed to as shown in Table 2.

4.1 Amino Acids

The amino acid profile of the *Bulinus* species can be altered in others to either eliminate the freshwater snail which is the intermediate host of the parasite *Schistosoma haematobium* or by making it difficult for the larval stage of the *S. haematobium* to survive in the *Bulinus* species.

It was observed that, the compositions of amino acid varied in all the repeated challenges measured with the control. These variations may be accorded to the activities of the parasite inside the intermediate host. Reduction in parameters may suggest increased usage of such parameter due to the presence of the parasite. An increase in parameters may suggest reduction in the usage of such parameter. These findings differ from the work done by Robert and Denis [11] where it was recorded that the parasite *Echinostoma revolutum* depletes the nutrient availability of its intermediate host *Lymnaea aelodes* snails.

The values obtained for Amino acids like Lysine, Histidine and Isoleucine were higher than 0.42, 0.20 and 0.25 reported for periwinkle by Ogungbenle and Omowole [12]. Though the value of Lysine obtained for *Bulinus globosus* in

this work is low compared to 0.82 reported for land snails (*Achatina achatina*) by Adeyeye and Afolabi [13] but there was observed increase of lysine in infected *Bulinus* species than in none infected. Lysine is one of the major components of muscle protein; the increase may be as a result of increase in protein level of infected *Bulinus* species. Valine levels did not differ between non-infected *Bulinus* species and infected *Bulinus* species, rather there was different in valine level for starved and infected *Bulinus* species. Valine aids in muscle development, relating this with the weekly weight gain, there was also no observable different between non-infected and infected *Bulinus* species but great difference exists between starved and infected *Bulinus* species. Muscle degradation is one of the functions of methionine and the significant difference observed in methionine level could be attributed to its function. Therefore the decrease in its level across the challenges the snails were exposed to could be linked to over usage of the methionine to suppress the effect of parasitosis on the snail. There was also reduction in phenylalanine level comparing non infected and infected snails. This could be linked to the fact that phenylalanine maintains healthy nervous system. Thus, it could be that the acid was used to maintain nervous system.

4.2 Isoleucine

The level was high in aestivated snails than those exposed to other challenges, however the increase in the level of isoleucine did not show any statistical significant. The values obtained for non essential Amino acids like proline, glycine and cystine are comparable with 0.39, 0.51 and 0.05 recorded for land snails (*Achatina achatina*) by Adeyeye and Afolabi [13]. The values for these amino acids are also comparable with 0.30, 0.51 and 0.11 recorded for periwinkle by Ogungbenle and Omowole [12].

The proline level increased in infected *Bulinus* species but decreased in starved *Bulinus* species. This could be linked to the role it plays in muscles regeneration. It therefore suggested that the increase in proline level was to aid in regenerating cells damaged by infection. Also the level of glycine increased in infected *Bulinus* species compared to non-infected, the amino acid glycine helps in cell and muscle healing. It is therefore suggest that the increase in glycine was to aid in the healing/ regeneration of damaged muscles.

Table 1. Essential amino acids concentration in *Bulinus globosus* with repeated challenges

Parameters	Control ±	Starved ±	Aestivated ±	Infected ±	Infected starved	Infected aestivated
Histidine	0.52 ^a ± 0.01	0.51 ^{a b} ± 0.01	0.53 ^{a c} ± 0.01	0.52 ^{a b d} ± 0.01	0.41 ^{e e} ± 0.01	0.49 ^{f c} ± 0.01
Valine	0.61 ^a ± 0.01	0.48 ^b ± 0.01	0.53 ^c ± 0.00	0.62 ^{a d} ± 0.01	0.49 ^e ± 0.02	0.45 ^f ± 0.00
Methionine	0.16 ^a ± 0.01	0.12 ^b ± 0.01	0.14 ^c ± 0.01	0.17 ^a ± 0.00	0.13 ^{e b c} ± 0.00	0.14 ^f ± 0.00
Phenylalanine	0.68 ^a ± 0.01	0.31 ^b ± 0.01	0.28 ^c ± 0.00	0.70 ^d ± 0.00	0.36 ^e ± 0.01	0.38 ^f ± 0.01
Lysine	0.69 ^a ± 0.00	0.71 ^{a b} ± 0.02	0.58 ^c ± 0.01	0.74 ^d ± 0.01	0.78 ^e ± 0.00	0.62 ^f ± 0.01
Aspartic acid	0.69 ± 0.01	-	-	0.73 ± 0.00	-	-
Isoleucine	0.48 ^a ± 0.00	0.63 ^b ± 0.00	0.71 ^c ± 0.01	0.38 ^d ± 0.02	0.52 ^e ± 0.01	0.52 ^{f d} ± 0.02
Threonine	0.21 ^a ± 0.01	0.44 ^b ± 0.01	0.47 ^c ± 0.01	0.19 ^d ± 0.01	0.32 ^e ± 0.00	0.31 ^{f d} ± 0.00

Table 2. Non essential amino – acids in *Bulinus globosus* with repeated challenges

Parameters	Control ±	Starved ±	Aestivated ±	Infected ±	Infected starved	Infected aestivated
Proline	0.32 ^a ± 0.02	0.21 ^b ± 0.01	0.32 ^c ± 0.01	0.38 ^d ± 0.00	0.29 ^{d c} ± 0.02	0.49 ^{f c} ± 0.01
Glycine	0.44 ^a ± 0.01	0.31 ^b ± 0.01	0.40 ^c ± 0.01	0.54 ^d ± 0.02	0.34 ^e ± 0.01	0.43 ^f ± 0.01
Arginine	0.46 ^a ± 0.01	0.42 ^b ± 0.01	0.46 ^a ± 0.00	0.45 ^a ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Alanine	0.42 ^a ± 0.02	0.18 ^b ± 0.01	0.29 ^c ± 0.00	0.38 ^d ± 0.02	0.19 ^e ± 0.01	0.25 ^f ± 0.02
Tyrosine	0.24 ^a ± 0.01	0.27 ^b ± 0.01	0.30 ^c ± 0.00	0.20 ^d ± 0.01	0.22 ^e ± 0.00	0.25 ^f ± 0.01
Serine	0.28 ^a ± 0.01	0.19 ^b ± 0.01	0.15 ^c ± 0.01-	0.23 ^d ± 0.02	0.16 ^e ± 0.01	0.11 ^f ± 0.01
Glutamic acid	1.46 ^a ± 0.01	1.45 ^a ± 0.02	1.46 ^a ± 0.02	1.44 ^a ± 0.00	1.32 ^d ± 0.01	1.40 ^a ± 0.10
Ceystine	0.07 ^a ± 0.01	0.01 ^b ± 0.01	0.04 ^c ± 0.01	0.06 ^d ± 0.01	0.02 ^e ± 0.00	0.02 ^d ± 0.00

Alanine levels decreased in infected *Bulinus* compared to none infected, one of the functions of alanine is removal of toxins from the body, so the decrease could be linked to over usage of the amino acid in infected *Bulinus* species. The level of serine also decreased in infected *Bulinus* species than in non-infected species this could be linked to its function in brain proteins, participate immune system protein synthesis and also in the stimulation of the growth muscle.

4.3 Cystine

The level of this amino acid decreased significantly in infected *Bulinus* species and the decrease was very high in starved *Bulinus* species. One of its functions is that it aids in removal of toxins and formation of skin.

It has been reported that cercaria respond and are attracted to free fatty acids, Arginine even at a very low concentration and some other amino acids like methionine and histidine in skin surfaces [14]. This could be one of the reasons why some changes were recorded in the level of amino acids in this work.

5. CONCLUSION

The result of this study has opened another approach towards the control of schistosomiasis through biochemical approach.

6. RECOMMENDATION

It is recommended that further studies be carried out biochemically to the control of Schistosomiasis.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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