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Effects of *Echinostoma caproni* miracidia dose on the amino acid contents of *Biomphalaria glabrata* as determined by high-performance thin-layer chromatography

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Abstract

The effects of 5, 20, and 40 miracidia dose exposures of *Echinostoma caproni* on the amino acid contents of *Biomphalaria glabrata* were studied using high performance thin-layer chromatography-densitometry. Amino acids were identified and quantified in whole bodies of exposed snails and in the uninfected matched controls at 2 and 4 weeks post-exposure. Using cellulose layers with the mobile phase 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26) and ninhydrin detection reagent [2% ninhydrin in acetone-n-butanol (1:1)], five amino acids were identified, i.e., leucine/isoleucine, valine, alanine, glycine, and ornithine, by hR_F value comparison and color differentiation. Quantitatively, there was a marked elevation in the amounts of four of these five amino acids (isoleucine/leucine, valine, alanine, and ornithine) across dose levels at 4 weeks post-infection ($P<0.05$). Elevation of the amino acid content in the high dose snail group suggested that some changes occurred in the amino acid metabolism of the snails in that group as a function of miracidia dose.

Keywords

Biomphalaria glabrata, *Echinostoma caproni*, *trematodes*, *amino acids*, *thin-layer chromatography*, *miracidia dose*

Introduction

Past studies have confirmed that parasite metabolism cannot be understood without considering its role in host-parasite integration (Faddah and El-Ansary, 1999). An investigation of the free amino acids (AAs) in *B. glabrata* infected with different doses of *E. caproni* miracidia may provide information regarding induced physiological and biochemical changes in the snail's protein metabolism. AAs are of critical importance in the energy metabolism of *B. glabrata* snails, as they provide intermediates for the Krebs cycle. For example, glutamic acid (Glu) represents the amino nitrogen pool for amino-transferase activities that were described for trematode-infected *B. glabrata* (Schnell *et al.* 1985). Certain AAs such as Glu, aspartic acid (Asp), glycine (Gly), and glutamine (Gln) have a stimulatory effect on the development of parasitic worms (Hata, 1994). The regulation of free AA levels in freshwater species is a complicated process, and the identification and concentration of free AAs in the tissues and extracellular fluid compartments of snails vary with the diet, season, temperature, reproductive and developmental states, environmental stress, and parasitism (Faddah and El-Ansary, 1999).

The use of high-performance thin-layer chromatography (HPTLC)-densitometry for the determination of free AAs in complex mixtures has been extensively investigated in the Fried-Sherma laboratory. A study by Norfolk *et al.* (1994) compared the separation of 18 AAs on high performance silica gel, cellulose, and reversed phase chemically bonded silica gel plates and made use of ninhydrin reagent for the detection and scanning densitometry for the quantification of alanine (Ala) and Asp in *B. glabrata* hemolymph and the digestive gland-gonad complex (DGG). Using the same techniques, Pachuski *et al.* (2002) determined the AA content in the DGG of *B. glabrata* snails infected with larval *S. mansoni* for eight weeks. They found the presence of histidine (His), lysine (Lys), Ala, methionine (Met), Thr, asparagine (Asn), proline (Pro), and leucine/isoleucine (Leu/Ile) in both the infected and uninfected DGGs, but only Lys showed a significant

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quantitative reduction (Student's *t*-test, $P<0.05$) in the concentration of infected snails compared to the controls.

Ponder *et al.* (2004) also looked at the free-pool AAs in the DGGs of *B. glabrata* infected with *E. caproni*. In this study, qualitative analysis revealed the presence of His, Lys, Ser, Ala, valine (Val), and Ile/Leu in all samples, and the differences in Lys and Val amounts between infected and uninfected snails were determined to be insignificant. The current study used HPTLC protocols established in earlier papers and a new ninhydrin formula reported by Qureshi *et al.* (2014). In this study, AAs were identified and quantified in four populations of *B. glabrata*, one control group and three groups infected with different doses of *E. caproni* miracidia.

Materials and Methods

Sexually immature *B. glabrata* snails were maintained in the laboratory as described by Fried *et al.* (2001). Four populations each containing 10 snails were maintained for 4 weeks at 22 to 24°C in glass jar cultures containing approximately 800 mL of artificial spring water (ASW) prepared as described by Ulmer (1970). Each population was exposed to a different dose of miracidia, specifically 5, 25, or 40 miracidia. A control population consisted of unexposed snails. Snails were maintained on a diet of boiled romaine lettuce *ad libitum* and cultures were changed every 2–3 days.

For each population, half of the number of snails present was necropsied at week 2, and the remaining at week 4 post-infection. AAs were extracted from individual whole bodies as described by Vasta *et al.* (2010). Samples were removed from the freezer and defrosted, and the blotted wet weight of each snail was recorded. Whole bodies were homogenized individually in a 7-mL glass homogenizer (Wheaton, Millville, NJ) with 1 mL ethanol-water (70:30), which was used as the solvent in all steps of the extraction. Each homogenate was then transferred to 2.0-mL microcentrifuge tubes (VWR International, West Chester, PA, USA). The glass homogenizer was washed with 500 µL solvent. Homogenates were centrifuged at 8,000 × *g* for 5 min and supernatants collected in individual glass vials. The pellets were resuspended in 500 µL solvent by vortexing and the centrifugation was repeated. The resultant supernatants were added to the corresponding vial, after which extracts were evaporated to dryness under a stream of air in a water bath (40–60°C). Residues were reconstituted at a ratio of 250 µL solvent per 30 mg snail tissue and subsequently stored at –20°C prior to HPTLC analysis.

Qualitative and quantitative HPTLC analyses were performed as described by Holman *et al.* (2011) and Vasta *et al.* (2009). Standards of the AAs Ala, Arg, Asn, citrulline (Cit), Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, phenylalanine (Phe), Pro, Ser, taurine (Tau), Thr, tryptophan (Trp), tyrosine (Tyr), and Val were obtained from Sigma (St. Louis, MO, USA). Individual stock standards of each AA were prepared in ethanol-water (70:30) at 0.100 µg μL^{-1} for AA identifications and quantitative analyses.

Silica gel and cellulose layers were tested for their potential use in the identification and quantification of AAs in *B. glabrata* snails. The HPTLC silica layers were 20 × 10 cm silica gel with a concentration zone (CZ) No. 13728-6 (EMD Millipore Corp., Billerica, MA, an affiliate of Merck, KGaA, Darmstadt, Germany), and the cellulose layers were 20 × 10 cm HPTLC cellulose F, No. 15036-6 (EMD Millipore Corp.). All layers were prewashed by development to the top with dichloromethane-methanol (1:1) and air-dried before use.

In all cases, aliquots of standard and sample solutions were applied to the layer (cellulose) or CZ (silica gel) using a Linomat IV (CAMAG, Wilmington, NC, USA) equipped with a 100 µL syringe and operated with the following settings: band length 6 mm, application rate 15 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from plate left side edge 7 mm, and distance from the plate bottom 1.0 cm. The Linomat allows automatic overspotting of individual standard solutions at the origin of a lane and therefore, the difficult task of preparing mixed standard solutions is avoided. Plates were developed with the mobile phases 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26; mobile phase A) or 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26; mobile phase B) as described by Holman *et al.* (2011) in a solvent vapor-equilibrated CAMAG HPTLC twin-trough chamber with a saturation pad (Analtech, Newark, DE, USA) for a distance of 7 cm beyond the origin. The cellulose layer with mobile phase A was chosen as the system used for the quantification of Ile/Leu, Val, Ala, Gly, and Orn.

Visualization of AAs was tested by postchromatographic derivatization with either 0.3 g ninhydrin in 100 mL *n*-butanol plus 3 mL glacial acetic acid or 2% ninhydrin in acetone-*n*-butanol (1:1) solution. The latter was chosen for its better color differentiation for the identification of AAs using cellulose plates and mobile phase A. Developed plates were air dried in a fumehood, sprayed to saturation with ninhydrin solution, and heated on a CAMAG plate heater for 10 min at 100°C.

For the quantitative analyses of Leu/Ile, Val, Ala, Gly, and Orn, HPTLC standards were spotted in 1.00, 3.00, 5.00, 7.00 µL aliquots (corresponding to 0.100 to 0.700 µg) to generate calibration curves of each. HPTLC standards of Ile, Val and Gly were overspotted on the first four lanes and HPTLC standards of Ala and Orn were overspotted on the next four lanes of the HPTLC plate. Following the standard lanes, reconstituted samples were spotted in 5.00–15.0 µL aliquots chosen so that the scan areas of sample zones would be bracketed within the scan areas of the standards used to generate the calibration curve. The limit of quantification was 0.100 µg, as determined by the lowest mass of standard applied to the layer. Band areas were measured by slit-scanning densitometry using a CAMAG TLC Scanner 3 with a tungsten light source set at 610 nm. CATS-3 software was used to generate polynomial regression calibration curves relating the peak areas of each standard zone to the weight of AA spotted. The correla-

tion coefficient (r-value) for each calibration curves was at least 0.99 in all analyses.

The following equation was used to determine the weight percent (Wt%) of AA present in each snail body:

$$Wt\% = (w*R)/(10{,}000*W)$$

Where w = weight (ng) interpolated from calibration curve, R = [reconstitution volume (µL)] / [spotted volume (µL)], and W = weight of snail body (mg). If more than one scan area from different aliquots of the same sample was bracketed within the calibration curve for a particular analyte, the area closest to the average area of the two middle standards was chosen for interpolation of the weight used for the calculation of the Wt% of the analyte.

All statistical analyses were performed using IBM® SPSS® Statistics Version 22 software. The Univariate ANOVA test was used to determine the statistical significance of quantitative data, based on mean ± standard deviation of the Wt% of AAs in the whole snail bodies, as doing multiple two-sample *t*-tests would result in an increased chance of committing a statistical type I error. A P value of<0.05 was accepted as the cutoff for the significance of difference in all statistical comparisons.

Results and Discussion

Differences in survival were observed between the control and the population infected with miracidia regardless of the doses. While no death was observed in the control population at 2 and 4 weeks, 1 and 2 deaths were observed at week 2 in snails infected with 5 and 40 miracidia, respectively, and 3 deaths were observed at week 4 in each of the same populations. There were also 2 deaths in the population infected with 25 miracidia at week 4. Thus, the total survival rates of each population at week 2 were: control – 10/10 (100%), dose 5 – 8/10 (80%), dose 25 – 10/10 (100%) and dose 40 – 6/10 (60%). The total survival rates of each population at week 4 were: control – 5/5 (100%), dose 5 – 2/4 (50%), dose 25 – 3/5 (60%) and dose 40 – 2/3 (67%). Food consumption, although not measured quantitatively, seemed consistent across all populations.

Due to a combination of its efficiency (compactness of zones), selectivity (separation of zone centers), and good color differentiation between bands, the system using cellulose HPTLC plates developed with mobile phase A was chosen for both the identification and quantification of AAs in snail samples. Five AAs were identified conclusively in the samples using this system: Val, Ala, Gly, Orn, and Ile/Leu.

When comparison of hR_F values between sample and standard bands in the four studied HPTLC systems was not sufficient to determine the identity of an AA, color differentiation was used as an additional identifier. For example, in the chosen system, the second lowest band could be either Gly or Cit based on its hR_F value. However, color differentiation allowed identification of the band as Gly (dark purple) instead of Cit (bluish purple). Similarly, in the chosen system, the lowest band could be Orn, Asn, or Asp. However, color differentiation allowed identification of the band as Orn (dark purple) instead of Asn or Asp, which are both light purple.

Another observation that was also useful when the identification of certain AAs was uncertain was that the same AAs showed up at the same time as colored bands when the plates were being heated at 100°C for 10 min. In the case of Gly and Orn, bands indicating their presence in sample chromatograms became visible on the plate at the same time as did their respective bands in the standard chromatograms. The time difference between when these spots became visible as compared to when Cit, Asn, or Asp standard spots became visible was significant enough (about 1 minute) and was observed consistently across the plates tested. This can be looked into further as a useful tool to identify AAs in complex mixtures when the use of hR_F values and color differentiation are not sufficient.

All five AAs that were identified were quantified because of their separation from other ninhydrin-positive sample components and the symmetry of the zone scans. Fig.1 shows representative chromatograms of selected standards and samples. A pink background was observed from about $hR_F = 25$ to the mobile phase front. The bands that are not labeled could not be identified conclusively. The two bands representing Leu and Ile on sample chromatograms overlapped. Therefore the Wt% for Ile/Leu were quantified together as one unresolved scan peak and calculated using the calibration curve generated by Ile standard only as a marker.

The average weight percentages of AAs for each sample group together with the main conclusions from quantitative analysis are reported in Table I. Except for Gly at week 2 from

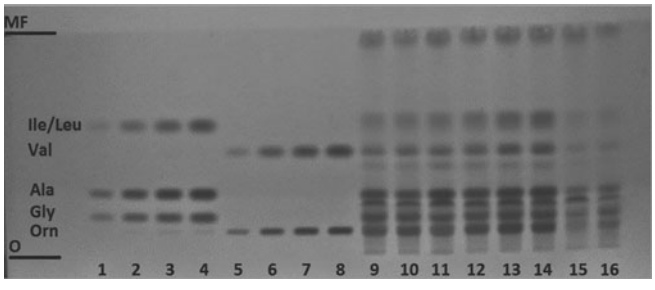

Fig. 1. Chromatograms on a cellulose HPTLC plate developed with 2-butanol-pyridine-glacial acetic acid-water (39:34:10:26) photographed in white light with a smart phone camera showing the AA profiles of *B. glabrata* snails infected with different doses of *E. caproni* miracidia at week 4. Lanes 1–4 contain Ile, Ala, and Gly standard solutions overspotted in 1.00, 3.00, 5.00, 7.00 µL aliquots, respectively. Lanes 5–8 contain Val and Orn standard solutions overspotted in 1.00, 3.00, 5.00, 7.00 µL aliquots, respectively. Lanes 9–10 contain reconstituted samples 1 and 2 infected with 5 miracidia. Lanes 11–12 contain reconstituted samples No. 1 and 2 infected with 25 miracidia. Lanes 13–14 contain reconstituted samples No. 1 and 2 infected with 40 miracidia. Lanes 15–16 contain uninfected reconstituted samples No. 1 and 2. All samples were spotted in 15.0 µL aliquots. The origin is marked with O and the mobile phase front is marked with MF

Table I. Weight percent (Mean ± Standard Deviation) of Ile/Leu, Val, Ala, Gly and Orn in whole bodies of *B. glabrata* snails in the control population and in infected sample populations at 2 and 4 weeks post-treatment

Time	AA	Uninfected[a]	Dose – 5 miracidia[b]	Dose – 25 miracidia	Dose – 40 miracidia[c]
Week 2	Ile/Leu	0.028 ± 0.004	0.029 ± 0.011	0.028 ± 0.019	0.014 ± 0.002
	Val	0.013 ± 0.002	0.016 ± 0.004	0.015 ± 0.009	0.014 ± 0.001
	Ala	0.016 ± 0.002	0.018 ± 0.002	0.015 ± 0.005	0.014 ± 0.001
	Gly	[d]	[d]	[d]	0.015 ± 0.011
	Orn	0.021 ± 0.004	0.021 ± 0.002	0.022 ± 0.011	0.032 ± 0.008
Week 4	Ile/Leu[e]	0.013 ± 0.005	0.028 ± 0.006	0.041 ± 0.001	0.061 ± 0.004
	Val[f]	0.011 ± 0.004	0.012 ± 0.002	0.013 ± 0.002	0.021 ± 0.001
	Ala[g]	0.008 ± 0.003	0.013 ± 0.001	0.018 ± 0.002	0.020 ± 0.003
	Gly	0.023 ± 0.012	0.024 ± 0.002	0.023 ± 0.002	0.028 ± 0.001
	Orn[h]	0.016 ± 0.009	0.036 ± 0.001	0.041 ± 0.008	0.044 ± 0.001

[a]For the uninfected population, there was decrease in the Wt% of Ile/Leu (P<0.05) and in the Wt% of Ala (P<0.05) between week 2 and 4.
[b]For the population infected with 5 miracidia, there was a significant decrease in the Wt% of Ala (P<0.05) and in the Wt% of Orn (P<0.05) between week 2 and 4
[c]For the population infected with 40 miracidia, there was a significant increase in the Wt% of Ile/Leu (P<0.05), in the Wt% of Val (P<0.05) and in the Wt% of Ala (P<0.05) between week 2 and 4
[d]The Wt% is below the limit of quantification of 0.100 µg as set by the lowest standard
[e]At week 4, there was a significant increase in the Wt% of Ile/Leu (P<0.05) across dose levels
[e]At week 4, there was a significant increase in the Wt% of Ile/Leu (P<0.05) across dose levels
[f]At week 4, there was a significant increase in the Wt% of Val (P<0.05) across dose levels
[g]At week 4, there was a significant increase in the Wt% of Ala (P<0.05) across dose levels
[h]At week 4, there was a significant increase in the Wt% of Orn (P<0.05) across dose levels

the uninfected population and the populations infected with 5 and 25 miracidia, which was determined to be below the limit of quantification of 0.100 µg as set by the lowest standard, all other values could be calculated.

When comparing the Wt% of Ile/Leu at week 4, there was a significant increase (P<0.05) across dose levels. The Wt% of Ile/Leu in the uninfected population was determined to be significantly lower than that in each of the other three populations respectively (P< 0.05). The Wt% in the population infected with 5 miracidia and in the population infected with 25 miracidia was each significantly lower than that in the population infected with 40 miracidia. Also at week 4, there was a significant increase in the Wt% of Val, Ala, and Orn (P<0.05) across dose levels. In the case of Ala, the Wt% in the uninfected population was determined to be significantly lower than that in the population infected with 25 miracidia and that in the population infected with 40 miracidia respectively. Similarly for Orn, the Wt% in the uninfected population was determined to be significantly lower than that in the population infected with 25 miracidia and that in the population infected with 40 miracidia respectively. Thus, there was marked elevation in the amount of Val, Ala, and Orn between the uninfected population and the infected populations at week 4.

When comparing the Wt% of the AAs in the same population between week 2 and week 4, for the uninfected population, there was a decrease in the Wt% of Ile/Leu (P<0.05) and in the Wt% of Ala (P<0.05) between week 2 and 4. For the population infected with 5 miracidia, there was a significant decrease in the Wt% of Ala (P<0.05) and in the Wt% of Orn (P<0.05) between week 2 and 4. Lastly, for the population infected with 40 miracidia, there was a significant increase in the Wt% of Ile/Leu (P<0.05), in the Wt% of Val (P<0.05) and in the Wt% of Ala (P<0.05) between weeks 2 and 4.

The results suggest interplay of both time and infection levels as responsible for affecting the amount of certain free AAs in whole bodies of snails. The study by Faddah and El-ansary (1999) found that there was marked elevation of most of the investigated AAs except Ser, Arg, and Thr in the *B. alexandarina* snails exposed to *S. mansoni* compared to the uninfected snails. According to the authors, Gly and Ala, among a few other AAs, arise from the metabolism of a variety of gluconeogenic compounds and glycogen, and all free AAs can be derived from the AAs released during peptide and protein turnover and may accumulate if catabolism is slowed. Therefore, marked elevation in the levels of Ile/Leu, Val, Ala, and Orn, as found in our study, can be a sign of the Krebs cycle impairment in *E. caproni* miracidia-infected *B. glabrata* snails at 4 weeks post-infection.

Such elevation in the AA contents of snails can also result from the removal of nutrients by the parasites post-infection. This may lead to increased synthesis of essential AAs by the snail hosts so that the levels of essential AAs in the body tissues can be held high enough for continued removal by the parasites. This is a sign of host-parasite adaptation and has been demonstrated in another study that found increased arginine synthesis by snails infected with *S. mansoni* and suggested that only by rapid arginine synthesis can the arginine level in the tissues of hemolymph of snails be held high

enough for the trematodes' large requirement for this AA during their reproductive phase (Senft, 1967).

Several recent studies on echinostomes published in Acta Parasitologica are related to the work reported herein. These studies are those of Toledo *et al*. (2014), Zimmmerman *et al*. (2014), Hunsberger *et al*. (2013) and O'Sullivan *et al*. (2013). These papers should be consulted for valuable information on the physiology and biochemistry of echinostomatid trematodes. Given results of this study and supporting findings from previous work, future research can look further at the interplay of both time (number of days/weeks post-infection) and infection levels in *E. caproni*-infected *B. glabrata* snails.

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