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Article

Effects of Different Levels of *Echinostoma caproni* Miracidial Dose on Glucose and Maltose Composition of *Biomphalaria glabrata* Snails as Determined by High Performance Thin-Layer Chromatography-Densitometry

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Abstract: The effects of 5, 25, and 40 *Echinostoma caproni* miracidia on the sugar content of young adult and mature adult *Biomphalaria glabrata* were studied using high performance thin layer chromatography (HPTLC)-densitometry. Analysis was done on the snail's digestive gland gonad complex (DGG) at two and four weeks postmiracidial exposure. The sugars were extracted from the DGG using 70% ethanol and analyzed on silica gel HPTLC plates with a preadsorbent zone using 1-butanol-glacial acetic acid-diethyl ether-deionized water (27:18:5:3) mobile phase. The separated bands were then detected using alpha-naphthol-sulfuric reagent and quantified by densitometry at 515 nm. Significant differences were found in the maltose content between two and four weeks post exposure for both age groups. Additionally, significantly lower maltose and glucose levels were observed in the high exposure groups of both ages.

Keywords: *Biomphalaria glabrata*; *Echinostoma caproni*; miracidia; digestive-gland gonad complex; DGG; glucose; maltose; sugars; thin-layer chromatography

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1. Introduction

Previous studies on *Biomphalaria glabrata* have been concerned with the maltose and glucose composition in the snail; these sugars were confirmed as the primary carbohydrates in this snail using thin layer chromatography (TLC) and high performance TLC (HPTLC) [1]. Therefore, it is important to

focus on these sugars when conducting carbohydrate analysis. Some HPTLC studies have been done on influences of the carbohydrate composition of *B. glabrata*, including salinity [2]. In the salinity study, it was found that increased salinity did not affect the carbohydrate composition of the snail's digestive gonad gland complex (DGG). However, in a study by HPTLC on the effects of temperature on carbohydrate composition, snails maintained at higher temperatures were found to have a lower maltose concentration and higher glucose concentration than those kept at lower temperatures [3].

It has also been found using HPTLC that carbohydrate composition in both the snail body and hemolymph is affected by *E. caproni* miracidial infection, with infected snails having a lower mass percentage of carbohydrate [1,4]. Although infection itself has been studied, work on the effects of the level of miracidial dose on the carbohydrate levels has not been done. Therefore, we examined the carbohydrate composition as affected by the level of *E. caproni* miracidial infection. In an HPTLC study by Hunsberger *et al.* [5], the lipid content of the snails was studied as affected by the level of miracidial infection, with five miracidia per snail being the low dose and 25 miracidia per snail being the high dose. The current research used the Hunsberger *et al.* [5] paper as a model; however, the high level dose was increased to 40 miracidia per snail. Thus, the purpose of our current study was to examine the effects of exposing snails to 0, 5, 25, and 40 miracidia on the glucose and maltose content of adult and juvenile *B. glabrata* using silica gel HPTLC-densitometry.

2. Materials and Methods

2.1. Snail Collection and Maintenance

Cultures of young adult *B. glabrata* measuring 5.0–10.0 mm in shell diameter and mature adults of *B. glabrata* measuring 10.1–15.0 mm in shell diameter were obtained from Dr. Mathew Tucker, Head, Schistosomiasis Laboratory, Biomedical Research Institute Rockville, MD, USA. All experimental and control groups were maintained, 10 per culture, in 1000 mL mason jars with approximately 800 mL of artificial spring water (ASW), as described by Ulmer [6]. Snail cultures were maintained at ambient temperature, 23 ± 1 °C, under artificial fluorescent light on a 12 h light/dark cycle. The snails were fed Romaine lettuce *ad libitum*, and the ASW in the cultures was changed every 2–3 days.

2.2. Experimental Design

Four different experimental groups, each consisting of 10 snails, were prepared for both the young adult and mature adult *B. glabrata* as follows: (A) control (unexposed), (B) low dose exposure, (C) medium dose exposure, and (D) high dose exposure. This resulted in a total of eight cultures of snails.

2.3. Snail Exposure

Thirty snails from both age groups (young adult and mature adult) were placed individually into a multiwell plate with approximately 2 mL of ASW per snail. Ten snails from each age group were then assigned to an experimental group, B, C, or D, and exposed with the corresponding number of *E. caproni* miracidia. The low dose exposure, group B, was given 5 miracidia per snail, the medium dose exposure, group C, was given 25 miracidia per snail, and the high dose exposure, group D, was given approximately 40 miracidia per snail. Thus, we obtained three different miracidial dose exposures for

both the young and mature adult snails. The snails were kept in the multiwall plates for 8 h to ensure proper miracidial exposure and then transferred into their respective mason jars.

2.4. Sample Collection and Preparation

Snails were necropsied 2 and 4 weeks after the initial date of exposure. Due to variable survival rates between the groups, there was no consistent number of snails necropsied at any time period. In order to ensure there would be enough snails alive to necropsy at 2 weeks, about one-half of the surviving snails were necropsied at 2 weeks.

Necropsy of the snails was done as described by O'Sullivan *et al.* [3]. At necropsy, the DGG was dissected from the visceral mass, and its wet weight was recorded. The visceral mass was then discarded. The sample was homogenized with 70% aqueous ethanol in a 7-mL glass homogenizer (Wheaton, Millville, NJ, USA). The homogenate was then equally distributed into four 1-mL microcentrifuge tubes and centrifuged at 8000 × g for 5 min. The supernatants were then recombined and transferred into a single glass vial and evaporated to dryness under a stream of air. The sample was then reconstituted with 70% aqueous ethanol in an 8-μL μg^{-1} ratio.

2.5. Standard Solution Preparation

The glucose and maltose standards were obtained from Sigma (St. Louis, MO, USA). The two sugars were weighed, mixed, and dissolved in 70% aqueous ethanol. Two standards were prepared, one with a 0.100 μg μL^{-1} concentration of each sugar and the second with a 0.600 μg μL^{-1} concentration of each sugar.

2.6. HPTLC Analysis

HPTLC analysis was performed on 20 × 10 cm silica gel 60 F_{254} glass plates with a preadsorbent zone (Catalog No. 13728; EMD Millipore Corp., Billerica, MA, a division of Merck KGaA, Darmstadt, Germany). Prior to use, plates were prewashed by development with dichloromethane-methanol (1:1) to the top of the plate and allowed to dry overnight in a fume hood.

Standards and reconstituted samples were applied using a Linomat IV spray-on bandwise applicator (CAMAG, Wilmington, NC) with a 100-μL syringe. The Linomat was operated using settings of 6 mm band length, 4 mm distance between bands, 7 mm distance from the side edge of the plate, 1 mm distance from the bottom of the plate and spotting rate 15 s μL^{-1}. Leaving the first lane empty, the following five lanes were spotted using the standards as follows: 3.00, 6.00, and 8.00 μL of the 0.100 μg μL^{-1} standard and 2.00 and 4.00 μL of the 0.600 μg μL^{-1} standard. The reconstituted samples were then spotted in the next two lanes with volumes of 2.00 and 4.00 μL. Four different samples were able to be analyzed on each plate.

The spotted plates were developed with approximately 50 mL of 1-butanol-glacial acetic acid-diethyl ether-deionized water (27:18:5:3) in an HPTLC twin trough chamber (CAMAG) for a distance of 8 cm (approximately 1 h). Prior to inserting the plate for development, the closed chamber was equilibrated for about 20 min with the mobile phase vapors min using a saturation pad (Analtech, Inc., Newark, DE, USA). Once developed, the plates were dried in a fume hood under a warm air stream from a hair dryer for 2 h. The plates were then sprayed using α-naphthol-sulfuric acid reagent prepared as described by Cline *et al.* [1]. The plates were then heated on a CAMAG plate heater at 110 °C; after about 10 min, the carbohydrate bands appeared as dark spots against a light pink background.

The sugar bands were scanned using a CAMAG TLC Scanner 3 with tungsten light source set to 515 nm, slit dimensions of 4.00 × 0.45 mm, and scanning speed of 20 mm s^{-1}. The winCATs software produced a polynomial calibration curve from the weights of the standards and their corresponding peak areas. The bracketed sample weights were interpolated from the calibration curve. Percentages of each sugar were calculated using Equation 1:

$$Carbohydrate\ \% = \frac{[(w)(R)(100)]}{[ng\ snail\ sample]} \quad (1)$$

where w = ng of the sample as interpolated from the calibration curve and R = reconstituted sample volume (µL) /applied sample volume (µL)].

2.7. Statistical Significance

A Univariate ANOVA analysis was performed in order to determine the effects of time and exposure on the mass percent of sugars for each age group using IBM SPSS software. The mean mass percent of each sugar was compared between the two week and four week necropsies, and between the different levels of exposure. By using a Univariate ANOVA, the accepted value could be kept at $P < 0.05$.

3. Results and Discussion

Some of our earlier studies on the determination of sugars in snails were carried out on preadsorbent silica gel TLC plates with manual standard and sample solution application [1,7]. However, a comparative study [8] of eight different precoated TLC and HPTLC stationary phases, eight mobile phases, three detection agents, manual and automated standard and sample solution application, weights of standards applied, and scanning parameters showed that the HPTLC conditions used in this paper, including automated sample and standard application onto silica gel preadsorbent silica gel plates, 1-butanol-glacial acetic acid-diethyl ether-deionized water (27:18:5:3) mobile phase, and naphthol-sulfuric acid detection reagent, are optimum. Calibration curves covering the range of 0.300 to 2.40 µg of each sugar gave correlation coefficients of at least 0.99 with polynomial regression using the WinCats software of the Scanner 3, and they were completely separated in compact bands with R_f values of 0.40 for glucose and 0.30 for maltose that were ideal for accurate and precise densitometric quantification.

The maltose and glucose analyses for both the young adult and mature adult groups yielded statistically significant results as described below in the text and tables.

3.1. Young Adult Snails

The results for the 5–10 mm (young adult) snails are summarized in Table 1. A value of $P < 0.05$ was used in order to determine the significance of our results. The analysis of maltose showed a significant increase from the two- to four-week necropsy. Comparing the results from the different miracidial exposures within the two-week necropsy, the maltose content of the five miracidia group was greater than both the 25 and 40 miracidia groups. In the four-week necropsy, the maltose content of the 40 miracidia group was less than the unexposed, five miracidia, and 25 miracidia groups. In the glucose analyses, it was found that the glucose content of the 40 miracidia group was less than the unexposed,

five miracidia, and 25 miracidia groups at the two-week necropsy and less than the unexposed and five miracidia groups at the four-week necropsy.

Table 1. Percent by mass (mean ± standard error) of the sugars studied at 2 and 4 weeks post-exposure for the 5–10 mm *B. glabrata*.

	Maltose [a]		Glucose	
Group	**2 Week Necropsy**	**4 Week Necropsy**	**2 Week Necropsy**	**4 Week Necropsy**
A	0.250 ± 0.13	0.318 ± 0.20	0.429 ± 0.30	0.485 ± 0.11
B	0.332 ± 0.065 [b]	0.603 ± 0.10	0.384 ± 0.10	0.708 ± 0.26
C	0.105 ± 0.047	0.380 ± 0.20	0.475 ± 0.24	0.428 ± 0.074
D	0.142 ± 0.039	0.136 ± 0.061 [c]	0.151 ± 0.024 [d]	0.178 ± 0.066 [e]

Groups defined as follows: A = control culture (unexposed), B = low dose culture (5 miracidia/snail), C = medium dose culture (25 miracidia/snail), D = high dose culture (40 miracidia/snail). [a] Increase in maltose content from the 2 week necropsy to the 4 week necropsy, ($P < 0.05$). [b] Maltose content of group B was greater than groups C and D, ($P < 0.05$). [c] Maltose content of group D was less than groups A, B and C, ($P < 0.05$). [d] Glucose content of group D was less than groups A, B, and C, ($P < 0.05$). [e] Glucose content of group D was less than groups A and B ($P < 0.05$).

3.2. Mature Adult Snails

The results of our findings for the 10–15 mm (mature adult) snails are summarized in Table 2. A value of $P < 0.05$ was used to determine statistical significance. The maltose analysis again showed a significant increase from the two- to the four-week necropsy. Within both the two- and four-week necropsies, the maltose and glucose content of the 40 miracidia group was significantly less than that of the unexposed, five miracidia and 25 miracidia groups.

Table 2. Percent by mass (mean ± standard error) of the sugars studied at 2 and 4 weeks post-exposure for the 10–15 mm *B. glabrata*.

	Maltose [a b]		Glucose [c]	
Group	**2 Week Necropsy**	**4 Week Necropsy**	**2 Week Necropsy**	**4 Week Necropsy**
A	0.364 ± 0.068	0.585 ± 0.092	0.465 ± 0.20	0.643 ± 0.035
B	0.324 ± 0.065	0.448 ± 0.13	0.560 ± 0.020	0.584 ± 0.048
C	0.325 ± 0.052	0.483 ± 0.11	0.616 ± 0.17	0.470 ± 0.16
D	0.0835 ± 0.017	0.140 ± 0.022	0.106 ± 0.044	0.110 ± 0.040

Groups defined as follows: A = control culture (unexposed), B = low dose culture (5 miracidia/snail), C = medium dose culture (25 miracidia/snail), D = high dose culture (40 miracidia/snail). [a] Increase in maltose content from the 2 week necropsy to the 4 week necropsy, ($P < 0.05$). [b] Maltose content of goup D was less than groups B, C and D for both the 2 and 4 week necropsies ($P < 0.05$). [c] Glucose content of group D was less than groups A, B and C for both the 2 and 4 week necropsies ($P < 0.05$).

3.3. Discussion

The results indicated relatively minor changes in the sugar content of the snails, except at the high miracidia dose. Specifically, a dramatic decline in the snail's maltose and glucose content was seen at the 40 miracidia dose for both age groups. In the study performed by Hunsberger *et al.* [5], high dose

results could not be observed because the 50 miracidia dose used in their study killed most of the snails. Therefore, the results found at the adjusted high miracidia dose in the current study are important for understanding the effects of high versus low miracidia dose. Because the snails were able to survive the duration of the study at a 40 miracidia exposure, this would be a good high dose to use for future experiments involving different *E. caproni* miracidia doses.

We also observed a significant increase in the maltose content of the snails between the two-week necropsy and the four-week necropsy for both age groups, and an increase in the glucose content between the necropsies. Similar increases were seen in the results of the Popovic *et al*. study [2]; it was suggested that higher metabolic rates could be present in the older snails after two weeks, resulting in fluctuations in the sugar content.

4. Conclusions

Maltose and glucose contents of *B. glabrata* were quantified by performing HPTLC analysis on the DGG of the snail. Statistically significant results were observed between weeks two and four for the maltose content of both age groups and for both sugars at high miracidial dose of *E. caproni*. Additionally, the low dose miracidia group showed a significantly higher maltose content than the 25 and 40 miracidia groups at two weeks in the young adult snail group.

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Author Contributions

Dolcie DeGrandchamp conducted the majority of the experiments and wrote the paper under the supervision of Dr. Bernard Fried and Dr. Joseph Sherma. Sage Hartlaub assisted in many experimental procedures throughout the duration of the study.

Conflicts of Interest

The authors declare no conflict of interest.

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