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ORIGINAL PAPER

High performance column liquid chromatographic analysis of selected carboxylic acids in *Biomphalaria glabrata* patently infected with *Schistosoma mansoni*

Daniel R. Massa · Michael J. Chejlava · Bernard Fried · Joseph Sherma

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Abstract High-performance liquid chromatography (HPLC) was used to determine the effects of a patent *Schistosoma mansoni* infection on certain carboxylic acids in the digestive gland gonad complex (DGG) and hemolymph of *Biomphalaria glabrata*. An analysis of DGG samples was done using extraction with 50% Locke's solution, cleanup of the extract by anion exchange solid phase extraction (SPE), and ion exclusion HPLC with ultraviolet detection. Hemolymph was applied directly to the SPE column. Acetic, fumaric, malic, and pyruvic acids were detected, confirmed, and quantified at concentrations ranging from 12 to 280 ppm in the DGG and less than 124 to 8,000 μg/dl in the hemolymph. Infection with *S. mansoni* caused a significant reduction (Student's *t* test, $P<0.05$) in the concentrations of acetic, fumaric, malic, and pyruvic acids in the DGG but not the hemolymph of *B. glabrata* compared to uninfected cohort snails. The significant reduction of certain carboxylic acids in the DGG of *B. glabrata* patently infected with *S. mansoni* suggests that these acids are utilized by the sporocysts and cercariae in the snail tissue, or that the infection stimulates reduced production or increased utilization by the snail tissue.

D. R. Massa · M. J. Chejlava · J. Sherma
Department of Chemistry, Lafayette College,
Easton, PA, USA

B. Fried (✉)
Department of Biology, Lafayette College,
Kunkel Hall 204,
Easton, PA 18042, USA
e-mail: friedb@lafayette.edu

Introduction

Relatively little information is available on the pathobiochemical effects of larval *Schistosoma mansoni* infection on *Biomphalaria glabrata* snails (see review in Thompson 1997). Recent studies on the topic have used high performance thin layer chromatography (HPTLC) to examine the pathobiochemical effects of *S. mansoni* infection on the amino acid (Pachuski et al. 2002), neutral lipid (Muller et al. 2000), and carbohydrate (Jarusiewicz et al. 2006) content of *B. glabrata*. Qualitative and quantitative changes in certain analytes were observed as a function of the schistosome infection in the snail in all the aforementioned studies.

There appears to be no information available on the effects of *S. mansoni* infection on the carboxylic acid content of infected *B. glabrata* snails. Certain carboxylic acids are involved in the intermediary metabolism of the snail, as discussed in Thompson 1997. There are two reported studies on the carboxylic content of uninfected *B. glabrata* snails. One study used high performance column liquid chromatography (HPLC) to determine a possible relationship of certain carboxylic acids to susceptibility or insusceptibility of particular strains of *B. glabrata* to *S. mansoni* infection (Bezerra et al. 1997); a second study (Bezerra et al. 1999) examined the effects of snail estivation on the carboxylic content of *B. glabrata*. In this latter study, *B. glabrata* snails showed some changes in the concentrations of certain carboxylic acids after 7 and 14 days of estivation relative to the unestivated controls. Because of the absence of information on the concentrations of certain carboxylic acids in *B. glabrata* patently infected with *S. mansoni*, we used HPLC analysis to determine such effects, and the results of our study are reported herein.

Materials and methods

Snail maintenance

B. glabrata snails (NMRI strain) experimentally infected with *S. mansoni* were obtained from Dr. Fred Lewis, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, MD, USA) along with another cohort of uninfected snails (controls). The infected snails were exposed to *S. mansoni* en masse with about six miracidia per snail. These snails were maintained at 23+/−1°C in aerated cultures and fed Romaine lettuce ad libitum for 6 weeks until they developed patent *S. mansoni* infection. The uninfected cohort snails were maintained in the same manner for 6 weeks. For more details on the snail maintenance procedures, see the paper by Schneck and Fried (2005).

Sample preparation

Only *B. glabrata* releasing *S. mansoni* cercariae following standard snail isolation procedures were used for the infected samples. The DGG from each infected snail was prepared as follows: the shell was cracked lightly with a hammer, and the snail body was removed. The DGG was dissected free of the snail body and extracted in 50% Locke's solution (see Massa et al. 2007). The extract was centrifuged at 250×*g* for 15 min, and the supernatant then transferred to a centrifuge tube and maintained at −20°C until solid phase extraction (SPE) was used. A similar procedure was used to prepare the DGGs from the uninfected cohorts. A total of 65 DGGs from infected snails and 62 DGGs from the uninfected cohorts were used. All analyses were based on pools of two or three DGGs per sample.

For hemolymph preparation, each infected snail was cracked lightly with a hammer in the bottom of a 6 cm petri dish to obtain about 50 μl of hemolymph per snail; the hemolymph was allowed to drain into the dish. A pooled sample consisted of 550 μl of hemolymph, obtained from 10–12 snails. The hemolymph was collected with a pipet, placed in an Eppendorf tube and centrifuged at 70×*g* for 5 min to separate the supernatant from the hemocytes and cellular debris. The supernatant was removed with a pipet and placed directly on the SPE column. An identical procedure was used to collect hemolymph samples from the uninfected snails. A total of 37 snails were used to obtain hemolymph from the infected snails, and another 37 were used to obtain hemolymph from the control snails.

Extraction and HPLC

Carboxylic acids were extracted and determined essentially as described by Massa et al. (2007). Standards of acetic, fumaric, lactic, malic, pyruvic, and succinic acids were purchased from Sigma-Aldrich (St. Louis, MO) in the highest purity grade available. Two standard mixtures were each prepared at concentrations of 1,000, 500, 100, 10.0, and 1.00 ppm in 0.50 M sulfuric acid for preparation of the HPLC calibration curves: Mixture 1 contained pyruvic, succinic, and acetic acids, and mixture 2 malic, lactic, and fumaric acids.

SPE was carried out on Bond Elut-SAX (triethylaminopropyl bonded to silica, chloride form) anion exchange columns (100 mg, 3 ml; Varian, Palo Alto, CA) with a Baker-10 vacuum manifold (J.T. Baker, Phillipsburg, NJ) to cleanup and recover carboxylic acids from samples prepared as described above. The columns were conditioned with 1.0 ml of 0.50 M HCl, 1.0 ml of methanol, and 2.0 ml of deionized water. The extract from two or three DGGs (ca. 7 ml) or a quantity of hemolymph (3×550 μl) was applied to an SPE column with a glass pipet, followed by washing with 2.0 ml of deionized water. Carboxylic acids were eluted with 500 μl of 0.50 M sulfuric acid into a tapered glass vial inside of the manifold and then transferred to an autosampler vial.

Ion exclusion HPLC was performed using an Agilent (Wilmington, DE) 1100 series system with an autosampler; Bio-Rad Laboratories (Hercules, CA) Aminex ion exclusion HPX-87H column (300×7.8 mm); and diode array detector. Chromatograms were collected at 210 nm. Sulfuric acid (5.0 mM) was used as the mobile phase with a flow rate of 0.6 ml/min, and the injection volume of the series of concentrations of the two mixed standards and the samples was 100 μl. The identification of acids was made by matching peak retention time (R_t) values between standard and sample chromatograms, plus validation by comparing the standard and sample UV spectra automatically collected by the diode array detector during the separation. For quantitative analysis of the identified acids, calibration graphs were generated using Microsoft Excel by relating the standard concentrations of the acids to their peak areas. The calibration graph for fumaric acid was logarithmic (r=0.94), probably because of a double bond chromophore in the structure of this acid; the graphs for the other three acids were linear (r=0.99). The concentration (ppm) of each acid in snail DGGs was calculated by multiplication of the sample solution concentration interpolated from calibration curve (I, ppm) by the original sample volume (V, ml), and division of the product by the mass of the snail DGG (M, g). For hemolymph, carboxylic acid concentration (μg/dl) was calculated by multiplying I times V times 100, and division of the product by the hemolymph volume (HV, ml) that was applied to the SPE column. Significant differences between acid concentrations in infected and uninfected samples were evaluated by using Student's t test with P<0.05.

Details of these sample preparation and determination methods and their development are available in Massa et al. (2007).

Results

Acetic, fumaric, malic, and pyruvic acids were identified in the DGG and hemolymph sample chromatograms of infected and uninfected *B. glabrata* because their R_t values agreed with the corresponding standard acid peaks within ±0.2 min, and the spectra of the sample and the standard peaks matched. We could not confirm the presence of succinic and lactic acids, as reported by Bezerra et al. (1997, 1999), based on these dual requirements.

The results of our findings on the concentrations of these acids in the hemolymph and DGG of *B. glabrata* infected with *S. mansoni* and the uninfected controls are shown in Table 1.

Discussion

The order of concentration of the carboxylic acids in the hemolymph of both infected and uninfected snails was malic >acetic>pyruvic>fumaric. This order is similar to that found by Bezerra et al. (1999) in the hemolymph of the albino strain of *B. glabrata* used in their study. However, the order of concentration of the carboxylic acids in both infected and noninfected DGGs in our NMRI strain was different than that in Bezerra et al. (1999). We found the order to be acetic >malic>pyruvic>fumaric. Differences in the concentrations of these acids in the snails' DGG probably reflect differences in the *B. glabrata* strains used in the two studies.

Hemolyph was analyzed for the carboxylic acids using the same ion exchange SPE and ion exclusion-UV HPLC techniques and columns as reported by Bezerra et al. (1997, 1999) except that the HPLC mobile phase as 5.0 mM instead of 0.50 mM sulfuric acid to achieve better and more consistent analyte separations. Acids were extracted from DGGs with 50% Locke's solution before SPE because it was found that the use of 12% perchloric acid followed by neutralization with potassium hydroxide, as reported by Bezerra et al. (1997, 1999), gave us zero percent recovery of the acids from the SPE column. Bezerra et al. (1997, 1999) did not describe the details of how they identified their acid chromatographic peaks or what their quantitative calibration procedures were; we required matches in both retention times and spectra between standards and sample peaks for qualitative identification, and used calibration curves based on linear regression (logarithmic regression for fumaric acid) for quantitative analysis. The details of the development of our analytical methods are reported in Massa et al. (2007).

Bezerra et al. (1999) found a significant increase in lactic, succinic, malic, and acetic acids in estivated *B. glabrata* compared with the unestivated controls. These results contrast markedly with our finding of a significant decrease in succinic, malic, and acetic acids in *B. glabrata* infected with *S. mansoni*. Such marked differences in these acids between parasitized snails versus estivated snails probably indicate a major difference in metabolic events associated with carboxylic acids in infected *B. glabrata* versus estivated snails.

Our most interesting finding was the significant reduction of all of the carboxylic acids studied in the DGG of infected snails relative to the uninfected controls. Presumably, the reduction in these acids reflected their possible use as metabolites by the developing schistosome sporocysts and cercariae. These larval stages inhabit the intertubular spaces of the DGG and cause mechanical and lytic damage to the digestive gland cells of the hepatopancreas (Thompson 1997). The damage probably results in a leakage of the acids, which are in turn utilized by the larval schistosomes. Moreover, increased metabolic activity associated with the presence of larval trematodes may accelerate the use of the acids by host cells in the digestive gland as well. As documented in several studies (see review in Thompson 1997), the site of active intermediary metabolism in these snails is associated with the mitochondria of the digestive gland cells.

Table 1 Carboxylic acid concentrations in the digestive gland–gonad complex (DGG) and hemolymph of uninfected *B. glabrata* and those infected with *S. mansoni*

	Control DGG		Infected DGG		Control hemolymph		Infected hemolymph	
Acid	Sample size	ppm (μg/g±SE)	Sample size	ppm (μg/g±SE)	Sample size	Conc. (μg/dl±SE)	Sample size	Conc. (μg/dl±SE)
Acetic	15	280±40	14	*60±10	2	2,000±1,000	1	2,100
Fumaric	17	9.9±0.6	19	*7.9±0.3	3	124±1	3	126±1
Malic	16	170±40	16	*50±10	3	9,000±1,000	3	8,000±1,000
Pyruvic	14	22±5	16	*12±2	3	800±100	3	1,000±100

Two or three DGGs per sample were used for analysis, and sample size ranged from 14 to 19; hemolymph samples were pooled for analysis; a pool consisted of the hemolymph from 10 to 12 snails

SE, standard error

*A significant reduction in the concentration of carboxylic acids in the infected DGG relative to the controls (Student's *t* test, $P<0.05$)

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