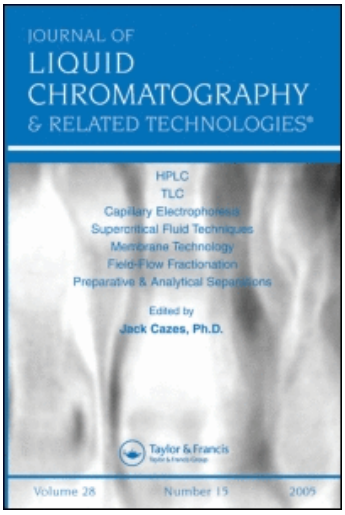

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Thin Layer Chromatographic Analysis of Free Pool Amino Acids in Cercariae, Rediae, Encysted Metacercariae, and Excysted Metacercariae of *Echinostoma caproni*

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ABSTRACT

Thin layer chromatography (TLC) was used to determine the free pool amino acid content of four larval stages (rediae, cercariae, encysted metacercariae, and excysted metacercariae) of the medically important digenetic trematode, *Echinostoma caproni.* These larval stages were obtained from experimentally infected *Biomphalaria glabrata* snails. Larvae were pooled, extracted in ethanol, and their free pool amino acids separated using four types of layers with different separation mechanisms. Zones were detected with ninhydrin spray and quantified by densitometry. Qualitative analysis revealed the presence of valine,

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leucine, lysine, histidine, and alanine in rediae; histidine in cercariae; histidine and alanine in encysted metacercariae; and histidine, alanine, and leucine in excysted metacercariae. Quantitative analysis showed that rediae contained 0.76 ± 0.20 ng of lysine per organism and excysted metacercariae contained 0.16 ± 0.05 ng of leucine per organism.

Key Words: Thin layer chromatography; Amino acids; *Echinostoma caproni*; Trematoda; Rediae; Cercariae; Encysted metacercariae; Excysted metacercariae.

INTRODUCTION

Barrett[1] reported that there is little information available on the physiology and biochemistry of echinostomes, a group of gastrointestinal parasitic flatworms of importance in human and veterinary medicine and in wildlife disease. Barrett[2] also noted that the role of free pool amino acids in the biology of the larval and adult stages of echinostomes and other digeneans is not well understood. A review of previous studies on this topic[2] has revealed that relatively few amino acids comprise the total free pool in adult and larval digeneans. In most digeneans, these amino acids are alanine, glycine, and proline. Pachuski et al.[3] analyzed the free pool amino acids in *Echinostoma caproni* adults, but similar information on free pool amino acids in the larval stages of *E. caproni* are not available.

The purpose of this study was to use thin layer chromatography (TLC) to examine, qualitatively and quantitatively, the free pool amino acids in the following larval stages of *E. caproni*: cercariae, rediae, encysted metacercariae, and excysted metacercariae. While the function of free pool amino acids in digeneans is unknown, information on these acids in different larval stages may provide a better future understanding of echinostome physiology.

EXPERIMENTAL

Sample Preparation

Biomphalaria glabrata snails were infected with miracidia of *E. caproni* as described by Idris and Fried,[4] to propagate cercariae, rediae, and encysted metacercariae. To obtain cercariae, snails were isolated individually at room temperature (23°C) in Stender dishes, each containing 4 mL of artificial spring water (ASW), prepared as described by Ulmer.[5] Cercariae were collected with a Pasteur pipet within 1 h post emergence, divided into three samples of

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1000 cercariae each in 2–10 mL of ASW in a 15 mL centrifuge tube, stored at 4°C overnight to inhibit mobility, and extracted as described below within 24 h of release from the snails. To obtain rediae and encysted metacercariae, five adult *B. glabrata* snails that had been exposed to *E. caproni* miracidia 8 weeks previously were necropsied. Rediae were removed from the digestive gland-gonad (DGG) complex and immediately divided into three samples of 600 rediae in 2–10 mL of Locke's (1 : 1) solution (NaCl, 4.5 g L^{-1}; KCl, 0.2 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}; $NaHCO_3$, 0.1 g L^{-1}) per 15 mL centrifuge tube. Rediae were extracted as described below, within 1 h of removal from the snail. Cysts were removed from the pericardial–kidney region and stored in Locke's (1 : 1) solution at 4°C and divided into three samples of 1000 cysts in 2–10 mL of Locke's (1 : 1) solution per 15 mL centrifuge tube. Cysts were maintained in Locke's (1 : 1) solution at 4°C until they were used within 2 weeks after removal from snails.

Encysted metacercariae were excysted in an Earle's alkaline solution (pH 7.8) containing 1% trypsin and 1% bile salts for 1 h at 41°C. This excystation medium was prepared as described by Fried and Roth.[6] The excysted metacercariae were then washed three times in Locke's (1 : 1) solution and separated into three samples, each with 1000 excysted metacercariae in 2–10 mL of Locke's (1 : 1) per 15 mL centrifuge tube. The excysted metacercariae were extracted within 1 h after excystation.

Prior to the extraction of all samples, the liquid overlay, ranging in volume from 2 to 10 mL, was removed from above each sample of cercariae, rediae, encysted metacercariae, and excysted metacercariae using a Pasteur pipet, and 1 mL of 70% ethanol was added. Extraction of amino acids from each sample was aided by vortexing for 10 min as described by Wagner et al.[7] Samples were then stored for 24 h at 4°C and centrifuged for 30 min at high speed. The supernatant of each sample was quantitatively transferred to separate 4 mL glass vials, and blown down to dryness with a stream of air at 50°C. All samples were reconstituted in 100 μL of 70% ethanol prior to TLC analysis.

Thin Layer Chromatography

Standards of 19 amino acids (alanine, arginine, asparagine, aspartic acid, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) purchased from Sigma (St. Louis, MO), were prepared as 0.100 μg μL^{-1} solutions in 70% ethanol. Qualitative analysis of the amino acids in the four stages of the parasitic life cycle was done on four different layers: 20 × 10 cm silica gel 60 Å HPTLC plates with a preadsorbent sample application zone and 19 channels (LHPKDF #4806-711; Whatman, Clifton,

NJ); 20 × 10 cm HPTLC cellulose F plates (Art. 15036; EM Science, Gibbstown, NJ, an affiliate of Merck KGaA, Darmstadt, Germany); 20 × 20 cm strong acid cation-exchange sheets (Art. 806013; POLYGRAM® IONEX-25 SA-Na; Macherey-Nagel, Easton, PA); and 20 × 20 cm chemically bonded reversed phase plates with preadsorbant zone ($LKC_{18}F$ #4800-820; Whatman). Quantitative analysis was performed on the reversed phase layer for lysine and on the silica gel layer for leucine. All layers were pre-cleaned by development with dichloromethane–methanol (1 : 1) and air dried before use.

Samples and standards in both qualitative and quantitative analysis were applied with a 10 μL digital microdispenser (Drummond, Broomall, PA). Standards used for qualitative identification of sample zones were applied in 10 μL aliquots. Standards used for quantitative analysis were applied in 1–12 μL aliquots (0.100–1.20 μg) to bracket the amounts of amino acids in the samples. *Echinostoma caproni* samples were applied in 20 μL aliquots using a light stream of air between application of small volume increments to keep the initial zones as small as possible.

For all analyses, applied samples were allowed to air dry, and TLC plates were developed in a twin trough TLC chamber (Camag, Wilmington, NC) containing a saturation pad (Analtech, Newark, DE) using *n*-propanol–0.5 M NaCl (4 : 6) for the reversed phase layer, pH 3.3 citrate buffer for the ion exchange layer, and *n*-butanol–acetic acid–water (3 : 1 : 1) for the other two layers. The ion-exchange sheet was supported by a 20 × 20 cm glass sheet during development to prevent bending. Development times ranged from 1.5 to 3 h. Developed plates were air dried, sprayed heavily with ninhydrin reagent (0.3 g ninhydrin solid and 3 mL glacial acetic acid in 100 mL *n*-butanol) for detection, air dried for 30 min, and heated for 10 min at 110°C on a Camag plate heater as described by Pachuski et al.[3]

Quantitative analysis of amino acids was conducted by measuring the densities of sample and standard zones using a Camag TLC Scanner II in the visible mode (tungsten light source) at 495 nm for histidine and 610 nm for all other amino acids, with settings of slit width 3, slit length 7, and scan speed 4 mm s^{-1}. The weights of analytes in the sample zones were interpolated based on linear regression calibration curves, created automatically by CATS-3 software relating scan areas to weights for standard zones. The interpolated weights were divided by the number of organisms to determine the weight per organism.

RESULTS AND DISCUSSION

Amino acids were identified by comparison of an unknown zone with the migration of standards. An amino acid was considered positively identified in a sample if its migration distance matched a standard on at least two layers.

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Valine, leucine, lysine, histidine, and alanine were identified in rediae; histidine was identified in cercariae; histidine and alanine were identified in encysted metacercariae; and histidine, alanine, and leucine were identified in excysted metacercariae. Other zones were also found, but could not be positively identified on at least two layers.

Only those zones that were clearly separated and positively identified could be quantified. The number of amino acids that could be quantified was also limited by the number of organisms in each larval stage that were available for the experiment. The number of organisms available limited the maximum total volume of the extracted sample, thus limiting the number of plates that could be spotted. Table 1 lists the quantitative data for lysine ($R_f = 0.69$) in rediae and leucine ($R_f = 0.61$) in excysted metacercariae.

Previous studies, as reviewed by Barrett,[2] have revealed alanine, glycine, and proline as the most common major free pool amino acids in digeneans. Alanine was identified in this study in the rediae, as well as in the excysted and encysted metacercariae. In a study of the adult stage of *E. caproni*, Pachuski et al.[3] identified histine, proline, alanine, leucine, and valine. With the exception of proline, all of these amino acids were identified in the rediae, and all of the amino acids identified in the larval stages were also found in the adult, except lysine. As with this study, Pachuski et al.[3] found unidentified ninhydrin-positive zones and suggested that unknown zones may be non-protein amino acids.

Cheng[8] suggested differences in free pool amino acids between larval stages of trematodes may be due to the ability of rediae to actively ingest host cells, while sporocysts can only obtain amino acids by absorption through their tegumental surface. In our study, it was evident that rediae of *E. caproni* contained more amino acids than the other larval stages. Presumably, some of

Table 1. Weight (ng/organism) of amino acids in *rediae* and excysted *metacercariae* of *E. caproni.*

	Lysine	Leucine
Rediae samples		
1	0.92	
2	1.0	
3	0.35	
Excysted *metacercariae* samples		
1		<0.10
2		0.26
3		0.12
Mean	0.76	0.16
Standard error	0.20	0.050

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these amino acids were obtained by ingesting host tissue from the snail DGG. While the exact function of the amino acids in these larval stages is unknown, further study of the quality and quantity of free pool amino acids in larval echinostomes and other digeneans should lead to a better future understanding of the importance of free pool amino acids in the biochemistry and physiology of these organisms.

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