Available online at www.sciencedirect.com
SCIENCE DIRECT
Parasitology International 53 (2004) 287–291
www.elsevier.com/locate/parint

Anthelmintic efficacy of genistein, the active principle of *Flemingia vestita* (Fabaceae): alterations in the free amino acid pool and ammonia levels in the fluke, *Fasciolopsis buski*

Pradip Kumar Kar, Veena Tandon*, Nirmalendu Saha

Department of Zoology, North Eastern Hill University, Shillong – 793022, India

Received 16 June 2003; accepted 22 April 2004
Available online 24 June 2004

Abstract

The crude root-peel extract of *Flemingia vestita*, its active principle genistein and the reference flukicide oxyclozanide were tested against *Fasciolopsis buski*, the giant intestinal trematode. The amino acid composition of *F. buski* was demonstrated using HPLC and it was observed that the free amino acid (FAA) pool of the control worm consisted of aspartate, threonine, serine, glutamic acid, glutamine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, lysine, histidine, arginine, phosphoserine, taurine, citrulline, ornithine, β-alanine, and γ-amino butyric acid (GABA). Of the amino acids detected valine was found to be the maximum in quantitative analysis. In qualitative analysis the FAA pool of the parasites under various treatments remained same as that of the control; however, quantitatively the level of various FAAs in the parasite was significantly affected. The treated parasites showed a marked decrease in the levels of arginine, ornithine, tyrosine, leucine, isoleucine, valine, alanine, glycine, proline, serine, threonine, and taurine following treatment with 20 mg/ml of crude peel extract, 0.5 mg/ml of genistein and 20 mg/ml of the reference drug, though an increase in the levels of glutamic acid, glutamine, phosphoserine, citrulline and GABA was noticeable. Enhanced levels of GABA and citrulline under the influence of genistein may be implicated in alterations of nitric oxide release and consequent neurological change (e.g. paralysis) in the parasite. Ammonia in the tissue homogenate as well as in the incubation medium showed a quantitative increase compared to the controls after treatment with the various test materials. The ammonia level increased by 40.7%, 66.4% and 18.16% in treatments with *F. vestita*, genistein and oxyclozanide, respectively, at the mentioned dosages. The changes in the levels of the amino acids and nitrogen components post treatment suggest that the amino acid metabolism in the parasite may have been altered under the influence of the test materials.

Keywords: Trematode; Anthelmintic; FAA pool; Ammonia; Amino acid; High performance liquid chromatography; Arginine; Citrulline; γ-Amino butyric acid

1. Introduction

Amino acids of late have emerged as potential targets for anthelmintic drug development, as they are a major constituent of all biological materials. The latest drug designs have exploited the differences between the pathways of amino acid metabolism in helminth parasites and their mammalian hosts. Scanty information is available regarding the amino acid metabolism in helminths. There are published reports on the free amino acid (FAA) pool of several cestode species [1–10]. Alanine is suggested to be the major free amino acid in *Hymenolepis diminuta* [11,12]. Evidence indicates that the FAAs may be involved in osmotic regulation [12,13]. Most of the helminths studied to date have been found to excrete significant amounts of nitrogen in the form of amino acids, peptides or proteins. The excretion of amino acids can provide a means of detoxifying ammonia that is known to have a neurotoxic effect and also to cause various neurological disorders [14,15], of which paralysis of the worm may be one such manifestation. Earlier studies on anthelmintic efficacy of *Flemingia vestita*-derived phytochemicals suggested that quantitative alterations in FAA pool of cestodes could be attributed to genistein treatment [16]. In view of the functional significance of FAAs in protein metabolism of worm parasites, we studied and report herein the changes in FAAs and tissue ammonia in the giant intestinal fluke, *Fasciolopsis buski* following exposure in

* Corresponding author. Tel.: +91-364-2722312; fax: +91-364-2550300.
E-mail address: tandonveena@hotmail.com (V. Tandon).

1383-5769/$ - see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2004.04.001

vitro to the plant test materials derived from the peel of the tuberous roots of *F. vestita*.

2. Materials and methods

2.1. Parasites and test materials

The mature trematodes, *F. buski*, were collected from the intestine of swine (*Sus scrofa*) from the local abattoirs in 0.9% phosphate buffered saline (PBS, pH 7–7.3). They were incubated at 37 ± 1 °C with crude root-peel extract of *F. vestita* 20 mg/ml, genistein 0.5 mg/ml, and the reference drug oxyclozanide at 20 mg/ml concentration (all prepared in 0.9% PBS with 1% DMSO). The aforementioned concentrations were chosen since at these dosages, as standardized in a previous study, the paralytic effect of the worm was attained within a shorter time frame as compared to lower concentrations; the time taken for onset of paralysis following treatment with the mentioned concentrations of the crude peel extract, genistein and oxyclozanide was determined as 0.6–0.8 h, 2.5–3 h and 0.5–6 h, respectively [17,18]. Three replicates for each set of incubation medium were used. On attaining the paralytic state after treatment the trematodes were further processed for histochemical and biochemical studies along with one set of control specimens (maintained in 1% DMSO in PBS).

2.2. Ammonia estimation in the incubation medium

Concentration of ammonia in the effluent (in the incubation medium) was measured enzymatically based on the procedure of Kun and Kearney [19]. Ammonia present in the incubation medium is converted completely to glutamate by the enzyme glutamate dehydrogenase (GDH) in the presence of α-ketoglutarate and NADH. The amount of NADH oxidized is taken as equivalent to the amount of ammonia present in the sample. The reaction mixture in a final volume of 1 ml contained 66 μmol Tris–HCl buffer (pH 8), 5 μmol α-ketoglutarate, 0.2 μmol EDTA, 1 μmol ADP, 0.4 μmol NADH, 10 units of GDH and 0.2 ml of incubation medium, which was incubated for 30 min at 37 °C. A blank was also prepared wherein the incubation medium was replaced with 0.2 ml of water. The difference in O.D. value obtained between the blank and the culture medium (i.e. the effluent of both control and treated parasites) at 340 nm was used to calculate the concentration of ammonia present in the effluent taking 6.22×10^6 as the molar extinction coefficient for NADH.

2.3. High performance liquid chromatography (HPLC) for amino acid analysis and ammonia

The tissue homogenate (10% w/v) was prepared in distilled water at 2 ± 1 °C. Protein was precipitated out by adding 5% perchloric acid (PCA, prepared in 0.4 N lithium citrate) in the ratio of 1:1, followed by centrifugation at 10 000 × *g* for 20 min at 2 ± 1 °C to pellet out the precipitated protein. The clear supernatant was then filtered through Whatman microfilter (0.45 μm pore size) and the pH of the filtrate was adjusted to 2.2 by adding a known volume of 0.2 N lithium hydroxide. All the processed samples were preserved in deep freeze at −20 °C until used for amino acid analysis. All the analyses were completed within 2–3 days after processing the sample.

The total FAA in the tissue was analyzed with a Shimadzu HPLC (model LC4A) with a post column derivatization method using *O*-phthaldehyde (OPA) reagent as a fluorescent dye, following method of Fujiwara et al. [20] with certain modifications [21]. A strong cation-exchange column (Shim-Pack ISC–07 Li, 10 cm long) was used for separation of FAAs. The detector (Shimadzu RF-535 fluorescent detector) was set at an excitation of 365 nm and an emission of 455 nm, and coupled to a data integrator (Shimadzu CR6A) for quantification of the eluted peak areas. The eluting mobile phase was a gradient of buffer A (0.16 N lithium citrate containing 7% methyl cellusolve, pH 2.5) and buffer B (0.32 N lithium citrate containing 0.62% of boric acid, pH 10.0), starting with 100% mobile phase A; the flow rate was 0.4 ml per min at 0 to 53 min, followed by 0.3 ml per min until the end of the run; the column temperature was 40 °C at 0–40 min, and 50 °C thereafter to 240 min. In the first 40 min the linear gradient progressed to 4% mobile phase B, followed by a linear increase to 10% in 93 min, 30% in 106.7 min, changed to 40% in 106.7 min and was held there until 123 min. The gradient was then increased linearly to 53% mobile phase B in 135 min and held there until 170 min, and finally increased linearly to 100% mobile phase B from 170 to 190 min and held there until 240 min. After gradient elution was complete the column was re-equilibrated for 15 min with mobile phase C (0.2 N lithium hydroxide) before subsequent injections. Hypochloride reagent for on-line oxidation was prepared by adding 0.4 ml of the commercial sodium hypochloride solution to 1000 ml of the buffer solution (pH 10) containing sodium carbonate (0.384 M), boric acid (0.216 M) and potassium sulfate (0.108 M). The fluorescence reagent was prepared by adding 2.0 g OPA (dissolved in 14 ml of ethanol), 4 ml of 10% aqueous Brij 35, and 2 ml 2-mercaptoethanol to 980 ml of the above alkaline buffer. The mixture of standard physiological FAAs (Sigma) containing 38 amino acids and some amino compounds was also eluted under identical conditions as mentioned above for identification and quantification of amino acids.

3. Results and discussion

As depicted in Table 1, the FAA pool of the control worm consisted of protein amino acids: aspartate (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glutamine (Gln), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu),

Table 1
Levels of different amino acids and ammonia (nmol/g wet wt*) in *F. buski*

Amino acid	Control	*F. vestita* crude extract (20 mg/ml)	Genistein (0.5 mg/ml)	Oxyclozanide (20 mg/ml)
Asp	162 ± 12	117 ± 8 (−27.8)	102 ± 5 (−37.03)	162 ± 11 (0)
Thr	251 ± 17	169 ± 14 (−32.7)	162 ± 11 (−35.5)	217 ± 19 (−13.5)
Ser	742 ± 56	649 ± 33 (−12.5)	511 ± 27 (−31.13)	688 ± 56 (−7.3)
Glu	528 ± 32	628 ± 47 (+19)	794 ± 81 (+50.4)	605 ± 48 (+14.6)
Pro	635 ± 39	518 ± 57 (−18.4)	416 ± 47 (−34.5)	554 ± 38 (−12.8)
Gly	4215 ± 412	2813 ± 157 (−33.3)	2147 ± 182 (−49)	3824 ± 245 (−9.3)
Ala	5124 ± 467	3822 ± 320 (−25.4)	3205 ± 272 (−37.5)	4267 ± 321 (−16.7)
Val	6812 ± 523	4471 ± 350 (−34.4)	2361 ± 165 (−65.3)	5138 ± 389 (−24.6)
Met	234 ± 19	238 ± 15 (+1.7)	269 ± 18 (+15)	169 ± 11 (−28)
Ile	418 ± 38	365 ± 25 (−12.7)	318 ± 24 (−24)	388 ± 22 (−7.2)
Leu	839 ± 62	639 ± 37 (−24)	762 ± 44 (−9.2)	714 ± 57 (−15)
Tyr	691 ± 59	457 ± 39 (−34)	419 ± 38 (−39.4)	653 ± 48 (−5.5)
Lys	374 ± 31	394 ± 42 (+5.35)	361 ± 29 (−3.5)	366 ± 31 (−2.1)
Gln	124 ± 11	154 ± 12 (+24.2)	239 ± 17 (+92.7)	189 ± 11 (+52.4)
His	327 ± 37	265 ± 18 (−19)	362 ± 22 (+10.7)	291 ± 17 (−11)
Arg	474 ± 41	315 ± 37 (−33.5)	217 ± 14 (−54.2)	281 ± 25 (−40.7)
Phser	205 ± 15	238 ± 22 (+16)	219 ± 16 (+6.8)	347 ± 28 (+69.3)
Tau	1568 ± 118	1324 ± 98 (−15.6)	1432 ± 117 (−8.7)	1561 ± 106 (−0.45)
Cit	15 ± 2	39 ± 4 (+160)	79 ± 8 (+426.7)	35 ± 2 (+133.33)
β-ala	719 ± 42	662 ± 48 (−8)	934 ± 88 (+30)	689 ± 38 (−4.2)
GABA	229 ± 17	255 ± 21 (+11.4)	355 ± 25 (+55)	241 ± 24 (+5.24)
Orn	531 ± 33	381 ± 24 (−28.25)	462 ± 29 (−13)	490 ± 37 (−7.7)
Total FAA	**25 217**	**18 913 (−25)**	**16 126 (−36)**	**21 869 (−13.3)**
Ammonia	435 ± 35	612 (+40.7)	724 ± 49 (+66.4)	514 ± 36 (+18.16)

Percentage increase (+) or decrease (−) of the FAA levels and also ammonia is given in parentheses.
* Values are expressed as mean ± S.E.M. ($n=3$).

tyrosine (Tyr), lysine (Lys), histidine (His) and arginine (Arg) and non-protein amino acids: phosphoserine (Phser), taurine (Tau), citrulline (Cit), β-alanine (β-Ala), GABA and ornithine (Orn), along with ammonia. The quantitative analysis data has also been depicted in Table 1. Out of the amino acids detected, Val was found to be at the maximum level, followed by Ala, Gly, Tau, Leu, Ser, β-Ala, and Tyr, respectively, in a decreasing order. Other amino acids detected were at comparatively lower levels. A low level of ammonia was also detectable.

Of the 22 amino acids detected in the FAA pool of *F. buski* Thr, Asp, Ser, Glu, Pro, Gly, Ala, Val, Met, Ile, Leu, Tyr, Lys, Gln, His, Arg, represent common or standard amino acids; besides, Phser, Tau, Cit, β-Ala, GABA and Orn are non-protein amino acids, some of which could appear as metabolic intermediates, or neurotransmitters as reported in cestodes [16] [22–24]. While Val, Ala and Gly were found to be predominantly present in the parasite, Tau and Leu also constituted a major part of the FAA pool. The size of the total FAA pool in the trematode was high (approx. 2.52 m mol/100 g fresh wt.) as in the case in most cestodes [16]. The high levels of FAAs may be significant in more than one way: (i) as a means of detoxifying ammonia; (ii) in having a role in osmotic balance; and/or (iii) as an alternate source of energy supply [25].

In qualitative analysis the FAA pool of the fluke under various treatments remained same as that of the control. However, quantitatively the level of the various FAAs and ammonia in the parasite was significantly affected by the treatment with various test materials, and also with the reference drug oxyclozanide (Table 1). The treated parasites showed a marked decrease in the levels of Arg, Tyr, Leu, Ile, Val, Ala, Gly, Pro, Ser, Thr, Orn and Tau, though an increase in the levels of Glu, Gln, Cit, Phser and GABA was noticeable. While the Lys levels showed a marginal increase in *F. vestita*-treated parasites, a slight decline in its level was observed in respect of other treatments. Asp level did not exhibit a decline but Met did, in response to oxyclozanide treatment. Further, in contrast to other treatments, β-Ala and His showed an increase following genistein treatment. The ammonia level also increased by 380.5% and 55.88% in the incubation media containing genistein and the reference drug, respectively (Table 2).

Table 2
Rate of excretion of ammonia (μmol/g/h) by *F. buski* in in vitro treatment

Treatment (mg/ml)	Ammonia in the incubation medium (μmol/g/h)	% Increase (after treatment)
Control (in 0.9% PBS)	9.27 ± 3.4	
Genistein (0.5)	44.54 ± 5.82[a]	380.5
Oxyclozanide (10)	14.45 ± 0.81[b]	55.88

Values are expressed as mean ± S.E.M. ($n=3$).
[a,b]: P value significant at <0.001 and <0.05 levels, respectively.

[22] Von Brand Th. Biochemistry of parasites, 2nd ed. New York, London: Academic Press; 1973.
[23] Gupta SP, Gupta RC. Free amino acid composition of *Opisthorchis pedicellata* (Trematoda) from the gall bladder of fresh water fish, *Rita rita* (Ham.). Z Parasitenkd 1977;54:83–7.
[24] Eriksson KS, Maule AG, Halton DW, Panula PAJ, Shaw C. GABA in the nervous system of parasitic flatworms. Parasitology 1995;110: 339–46.
[25] Il'yasov IN. Amino acid composition of *Raillietina tetragona* (Molin, 1858) and *R. echinobothrida* (Megnin, 1881). Trudy Nauchno–Issledovatel; Skogo Veterinarnogo Instituta Tadzhikskoy SSR 1978;8:75–9.
[26] Kar PK, Tandon V, Saha N. Anthelmintic efficacy of *Flemingia vestita*: genistein-induced effect on the activity of nitric oxide synthase and nitric oxide in the trematode parasite, *Fasciolopsis buski*. Parasitol Int 2002;51:249–57.
[27] Zhang H, Lee CH, Li S, Choi MH, Hong ST. Lethal effect of ammonia on metacercariae of *Clonorchis sinensis*. Parasitol Res 2003; 90(5):421–2.
[28] Barrett J. Amino acid metabolism in Helminths. In: Baker JS, Muller M, editors. London, New York, Tokyo: Academic Press; 1991. p. 39–105.
[29] Fairbairn D, Wertheim G, Harpur RP, Schiller EL. Biochemistry of normal and irradiated strains of *Hymenolepis diminuta*. Exp Parasitol 1961;11:248–63.
[30] Webster LA, Wilson RA. The chemical composition of protonephridial canal fluid from the cestode *Hymenolepis diminuta*. Comp Biochem Physiol 1970;35:201–9.
[31] Lutz PL, Siddiqi AH. Non-protein nitrogenous composition of the protonephridial fluid of the trematode *Fasciola gigantica*. Comp Biochem Physiol A 1971;40:453–7.
[32] Zavras ET, Roberts LS. Developmental physiology of cestodes: characterization of putative crowding factors in *Hymenolepis diminuta*. J Parasitol 1984;70:937–44.
[33] Sheers M, Campbell J, Beames DJ, Edwards SR, Moore RJ, Montague PE. Fasciolicidal potential of proline analogues and proline biosynthesis inhibitors. Int J Parasitol 1982;12:47–52.
[34] Vanaja D, Rao BR. Effects of flukicides on the transamination of *Fischoederius cobboldi*. Comp Physiol Ecol 1992;17(1):16–29.