A C T A I C H T H Y O L O G I C A E T P I S C A T O R I A
Vol. XXXIII, Fasc. 2 2003

ACTIVITY OF SELECTED HYDROLASES IN EXCRETION-SECRETION PRODUCTS AND HOMOGENATES FROM L_3 AND L_4 LARVAE OF *ANISAKIS SIMPLEX* (NEMATODA: ANISAKIDAE) PARASITISING HERRING

Janina *DZIEKOŃSKA-RYNKO*[1], Jerzy *ROKICKI*[2*], Zbigniew *JABŁONOWSKI*[1]

[1] *Faculty of Biology, University of Warmia and Mazury, Poland*
[2] *Division of Invertebrate Zoology, Gdańsk University, Poland*

Dziekońska-Rynko J., Rokicki J., Jabłonowski Z., 2003. Activity of selected hydrolases in excretion-secretion products and homogenates from L_3 and L_4 larvae of *Anisakis simplex* (Nematoda: Anisakidae) parasitising herring. Acta Ichthyol. Piscat. 33 (2): 125–136.

Background. Proteolytic enzymes may serve multiple functions: they may inhibit the host's blood clotting, protect the parasite from the host's immune response, facilitate parasite's migration within a tissue by decomposing the tissue barrier, enhance the hatching and moulting of larvae, and play an important role in their feeding. Learning their identity leads to a better understanding of a host-parasite system. The objective of this study was to check, using biochemical methods, if, in addition to proteases, ES products and extracts of 3rd and 4th larval stages of *Anisakis simplex* (Rudolphi, 1809) contain other hydrolases.

Material and methods. Stage-3 larvae (L_3) of *A. simplex* were removed from Baltic herring, *Clupea harengus membras* Linnaeus, 1761 caught in the Baltic Sea. Stage-4 larvae (L_4) were obtained from an L_3 stage culture kept in Eagle's medium. The solutions containing ES products were collected and dialysed at 4°C against distilled water for 24 h. Larval extracts were obtained by homogenising the larvae in a physiological saline (0.9 % NaCl) solution in a glass homogeniser. The homogenate was centrifuged for 10 min at 3000 G. The supernatant was used in enzyme activity assays. Enzymatic activity of ES products and homogenates of L_3 and L_4 larvae of *A. simplex* was determined with the API ZYM test.

Results. The excretion-secretion product of L_3 and L_4 larvae of *A. simplex* revealed activities of 10- and 11 hydrolases, respectively. Activity of esterase, esterase lipase, valine arylamidase, and N-acetyl-ß-glucosaminidase in the L_4 larvae extracts was higher than the activity of a corresponding enzyme assayed in the L_3 extracts. Only in the case of acid phosphatase, its activity in L_3 ES products was twice that of the activity found in ES products of L_4 larvae. Enzymes such as trypsin, chymotrypsin, and ß-glucosidase were not detected in extracts from L_3 larvae.

* Correspondence: Prof. dr hab. Jerzy Rokicki, Katedra Zoologii Bezkręgowców, Uniwersytet Gdański, Al. Piłsudskiego 46, 81-378 Gdynia, Poland; e-mail: *rokicki@univ.gda.pl*

Conclusion. Activities of most hydrolases in the L_4 extracts were higher than the activities of corresponding enzymes assayed in the L_3 extracts. The high activity of these enzymes found in L_4 larval extracts could be related to a different feeding mechanism of stage-4 larvae.

Key words: *Anisakis simplex*, herring worm, enzymes, hydrolase, Nematoda, fish, Baltic herring, *Clupea harengus membras.*

INTRODUCTION

When in a host's body, parasites release toxins commonly described as excretion-secretion (ES) products. The relevant literature contains very few publications on the chemical composition and function of these products. Most papers focus on proteases contained in ES products and in fluids extracted from homogenates of internal parasites (Hinck and Ivey 1976, Matthews 1982, 1984, Knox and Kennedy 1988, McKerrow 1989, Morris and Sakanari 1994, Perteguer et al. 1996, Moczoń and Wranicz 1999, Young et al. 1999, Moczoń 1999, Kotomski and Wędrychowicz 2001, Kinsella et al. 2002, Sajid and McKerrow 2002) and those of external parasites such as ticks and follicle mites (Buczek and Madoń 1998, Nisbet and Billingsley 1999, 2000, 2002, Kenyon and Knox 2002). According to the authors referred to, the proteases may serve multiple functions: they may inhibit the host's blood clotting, protect the parasite from the host's immune response, facilitate parasite's migration within a tissue by decomposing the tissue barrier, enhance the hatching and moulting of larvae, and play an important role in their feeding. A role similar to that played by proteases may be inferred also with respect to other enzymes contained in parasitic ES products; those enzymes include hyaluronidase (Hotez et al. 1994), leucine aminopeptidase (Lee 1962, Rhodes et al. 1969a, b, Rogers 1982, Rhoads and Fetterer 1998), and acetylcholinesterase (Opperman and Chang 1992, Riga et al. 1995, Lee 1996).

Histochemical studies on the excretion-secretion organ of *A. simplex* L_3 larva showed the presence of numerous enzymes, i.e. dehydrogenase, aminopeptidase, oxidoreductase, esterase, dehydrogenase, and phosphorylase (Ruitenberg and Loedersloof 1971a, b).

The majority of papers on biochemistry and physiology of larval *A. simplex* concern the L_3 larvae. No reference to studies on ES products in the parasite's L_4 larvae was available to the present authors. Łopieńska et al. (2001) compared properties of α-amylase in L_3 and L_4 extracts and found differences in intracellular location, pH optimum, and responses to effectors. The objective of this study was to check, using biochemical methods, if, in addition to proteases, ES products and extracts of larval stages L_3 and L_4 of *A. simplex* contain other hydrolases.

MATERIALS AND METHODS

Stage-3 larvae (L_3) of *A. simplex* were removed from Baltic herring, *Clupea harengus membras* Linnaeus, 1761 caught in the Baltic Sea. Stage-4 larvae (L_4) were obtained from a stage-3 culture kept in Eagle's medium (enriched with 20% bovine

serum); the pH of medium was adjusted to 2.0 at 37°C (Dziekońska-Rynko et al. 2001). The developmental advancement of the larvae was monitored daily. The lack of the boring tooth and mucron evidenced a completed moulting (Grabda 1976). The majority of L_3 larvae completed moulting attaining L_4 stage on day 6 of incubation. The ES products of L_3 and L_4 larvae were obtained in the same way. The larvae, rinsed in an antibiotic solution (penicillin: 100 U · ml^{-1}, streptomycin: 100 µl · ml^{-1}, nystatin: 100 U· ml^{-1}), were placed, 30 specimens in each batch, in 2 ml phosphate-buffered saline (PBS) and incubated at 37°C for 48 h. Subsequently, the ES products-containing solutions were collected and dialysed at 4°C against distilled water for 24 h. Dialysis tubing (Sigma) was used for dialyses. It detains proteins, exceeding12 kD.

Larval extracts were obtained by homogenising the larvae in a physiological saline solution (0.9 % NaCl) in a glass homogeniser. The homogenate was centrifuged for 10 min at 3000 G. The supernatant was used in enzyme activity assays. The protein contents in all samples tested were determined using Bradford's (1976) method. Homogenates from L_3 and L_4 larvae were standardized by dilution with the physiological salt solution.

Enzymatic activity of ES products and homogenates of L_3 and L_4 larvae of *A. simplex* was determined with the API ZYM test (Bio Mérieux SA, Lyon, France). The test kit contains substrates, which make it possible to determine activities of 19 hydrolases (Table 1). The wells containing appropriate substrates received 65-µl portions of the solution tested; the plate was incubated at 37°C for 4 h. The results were interpreted following the manufacturer's instructions. Hydrolase activities were expressed as nmol of the hydrolysed substrate.

RESULTS

Data on hydrolase activities in ES products and extracts of L_3 and L_4 larvae of *A. simplex* are summarised in Table 1.

The ES products of stage-3 larvae revealed activities of 10 hydrolases. The highest activity (40 nmol) was that shown by acid phosphatase, the activity of naphthol-AS-BI-phosphohydrolase being as low as one-fourth of it (10 nmol), while alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, trypsin, α-galactosidase, and α- and β-glucosidases showed identical activities (5 nmol). No activity of valine- and cystine arylamidases, lipase, chymotrypsin, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were detected. The ES products of L_4 showed the highest enzymatic activity to be typical of N-acetyl-β--glucosaminidase (40 nmol), acid and alkaline phosphatases, and trypsin showing lower activities (10 nmol). Esterase, cystine arylamidase, β-galactosidase, and α-fucosidase showed activity of 5 nmol each.

Table 1

Activity of enzymes from ES and extracts of stage-3 and stage4 stage larvae *Anisakis simplex*

	ENZYME	Classification	SUBSTRATE	pH	Activity in nmoles of hydrolysed substrate			
					L_3		L_4	
					ES	Extract	ES	Extract
1	Alkaline phosphatase	3.1.3.1	2-naphtyl phosphate	8. 5	5	40	10	20
2	Esterase (C 4)	3.1.1.6	2-naphthyl butyrate	6. 5	5	5	5	20
3	Esterase Lipase (C 8)	3.1.1.3	2 - naphthyl caprylate	7.5	5	5	5	10
4	Lipase (C 14)	3.1.1.3	2- naphthyl myristate	"	0	0	0	0
5	Leucine arylamidase	3.4.11.14	L-leucyl-2-naphthylamide	"	5	30	0	40
6	Valine arylamidase	3.4.11.14	L-valyl-2-naphthylamide	"	0	5	0	10
7	Cystine arylamidase	3.4.11.14	L-cystyl-2-naphthylamide	"	0	0	5	0
8	Trypsin	3.4.4.4	N-benzoyl-DL-arginine-2-naphthylamide	8. 5	5	0	10	30
9	α- chymotrypsin	3.4.4.5	N-glutaryl-phenylalanine-2-naphthylamide	7.5	0	0	0	20
10	Acid phosphatase	3.1.3.2	2- naphthyl phosphate	5.4	40	40	10	40
11	Naphthol-AS-BI-phosphohydrolase	3.1.3.31	Naphthol-AS-BI-phosphate	"	10	40	0	40
12	α -galactosidase	3.2.1.22	6-Br –2- naphtyl- αD-galactopyranoside	"	5	0	0	0
13	β- galactosidase	3.2.1.23	2-naphthyl- αD- galactopyranoside	"	0	5	5	5
14	β- glucuronidase	3.2.1.31	Naphthol-AS-BI-βD-glucoronide	"	0	5	0	10
15	α -glucosidase	3.2.1.20	2-naphthyl- αD-glucopyranoside	"	5	0	0	5
16	β-glucosidase	3.2.1.21	6-Br-2-naphthyl-βD-glucopyranoside	"	5	0	0	40
17	N-acetyl-β-glucosaminidase	3.2.1.50	1-naphthyl-N-acetyl-βD-glucosaminide	"	0	30	40	40
18	α -mannosidase	3.2.1.24	6-Br-2-naphthyl- αD-mannopyranoside	"	0	0	0	0
19	α -fucosidase	3.2.1.51	2-naphthyl- αL-fucopyranoside	"	0	5	1	5

Extracts obtained from *A. simplex* stage-3 larvae showed 11 hydrolases to be active. The highest activities (30 or 40 nmol) were those of acid- and alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase. A lower activity (5 nmol) was typical of esterase, esterase lipase, valine arylamidase, β galactosidase, β glucuronidase, and α-fucosidase. The L_4 extracts showed 15 hydrolases to be active. Similarly to the extracts of L_3 larvae, the highest activity was that of acid and alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase. In addition, these extracts showed high activities of trypsin (30 nmol), chymotrypsin (20 nmol), and β-glucosidase (40 nmol). Activity of those enzymes was not detected in extracts from L_3 larvae. Activities of most hydrolases (esterase, esterase lipase, valine arylamidase, and N-acetyl-β-glucosaminidase) in the extracts of stage-4 larvae were higher than the activity of a corresponding enzyme assayed in the stage-3 extracts; it was acid phosphatase only that the activity of which in stage-3 extracts was twice that of the activity found in L_4 larvae.

DISCUSSION

Most of the available literature on ES products in *A. simplex* stage-3 larvae focuses primarily on protease activity (Matthews 1982, 1984, Kennedy et al. 1988, Sakanari and McKerrow 1990, Morris and Sakanari 1994). Two proteases: trypsin-like serinpeptidase and aminopeptidase were found to be active. According to the authors referred to, the enzymes are crucial in larval penetration. Histochemical assays of the stage 3rd larvae excretory organ (Ruitenberg and Loendersloot 1971 a, b) showed the presence of 18 enzymes. According to those authors, the enzymes are secreted and, by digesting host's tissues, facilitate the parasite's penetration of and feeding in the host's body. The literature search failed to reveal references to studies on enzymes of the stage-4 larva excretory organ. The experiment described in this paper showed similar numbers of hydrolases (10 and 9) to be active in the ES products of the L_3 and L_4 larvae. On the other hand, more hydrolases were active in extracts of the stage-4 larvae. Most of the hydrolases (both in the ES and in the extracts) were present in all the larvae, some, however, being specific to one or the other stage.

Trypsin activity in ES products of L_4 was twice that found in stage-3 products; the stage-4 extracts showed also a high activity of trypsin and chymotrypsin. L_3 larvae of *A. simplex* do not feed (Sommerville and Davey 1976), but are nourished by reserve materials stored in the body. Perhaps this is the reason why no trypsin or chymotrypsin activity could be detected in extracts of L_3 larvae in the experiment described. The high activity of these enzymes found in the stage 4th larval extracts could be related to a different feeding mechanism of stage-4 larvae.

The ES products of L_3 larvae showed leucine aminopeptidase to be active. According to numerous authors, aminopeptidases are important as activators of

hormone-and enzyme precursors during the hatching and moulting of larvae (Rhoads et al. 1997). Leucine aminopeptidase was found to be active in numerous parasites and also during hatching and moulting of their larvae (Rogers 1982, Sakanari and McKerrow 1990, Niemczuk 1993, Rhoads et al. 1997). Aminopeptidase activity was detected in a medium containing *Ascaris suum* larvae when these were moulting from L_3 to L_4 (Rhoads et al. 1997). Protease inhibitors added to the medium stopped the *A. suum* larvae moult from L_3 to L_4 (Rhoads et al. 1998). Moreover, aminopeptidases were found to be active during the entire period of development of *Schistosoma mansoni* (cf. Auriault et al. 1981, Xu and Dresden 1986). According to those authors, aminopeptidases are extremely important during hatching and then trigger disintegration of host's immunoglobulins on the parasite body surface. In the present experiment, the activity of leucine aminopeptidase could be detected only in ES products of stage-3 larvae; the presence of the enzyme is most likely related to the larval moulting process. As they decompose proteins into peptides, aminopeptidases are also important in feeding. Activity of aminopeptidase was found in the body wall, ovaries, vulva, intestine, and body cavity fluid of mature *A. suum* (cf. Lee 1962, Rhodes et al. 1966, 1969a, b). The authors quoted found the enzyme to be most active in the intestine, for which reason they inferred the enzyme to serve a digestive function. Nisbet and Billingsley (1999, 2000) found aminopeptidases to be highly active in parasitic mite extracts, the enzymes being involved in digestive processes as well. The fairly high activity of these enzymes in larval extract, found in the experiment described in this paper, was perhaps related to feeding. The results obtained in the present experiment are consistent with those reported by Ruitenberg and Loendersloot (1971a, b) whose histochemical assays on *A. simplex* stage-3 larvae showed the highest leucine aminopeptidase activity to be characteristic of muscles and intestinal epithelium, which would be an indicative in a digestive function of the enzyme. Activity of the enzyme in stage-3 larvae's excretory organ was much lower. The present results are in agreement with data reported by the authors referred to above, as much higher aminopeptidase activity was found in the extracts of stage-3 and stage-4 larvae.

The available literature lacks publications reporting the presence of other hydrolases in parasite ES products. The present experiment showed a very high activity of acid- and alkaline phosphatases, both in the ES products and in the larval extracts (L_3 and L_4). Histochemical assays on the excretory organ of the stage-3 larvae failed to detect the presence of phosphatases (Ruitenberg and Loendersloot 1971a, b), high activity of acid phosphatase being revealed in the body wall and in the intestine. Phosphatases are important in controlling metabolic processes. Alkaline phosphatase is involved in active trans-membrane transport. Acid phosphatase, as a lysosome marker, provides indirect information on intracellular digestive processes. In numerous parasites, the presence of phosphatases is regarded as a marker for sites of secretory activity and nutrient absorption. A high activity of alkaline- and acid phosphatases was revealed in the cuticle, subcuticular cells, and the parenchyma of

cestodes (Arme 1966, Niemczuk 1993). The authors quoted found a clear relationship between activities of those enzymes and the maturity of cestode segments: mature segments showed a higher activity. Nisbet and Billingsley (1999, 2000) found alkaline- and acid phosphatases to be highly active in extracts from parasitic mites (*Psoroptes cuniculi, Psoroptes ovis, Dermanyssus gallinae*). Most parasitic nematodes examined for enzymatic activity showed a high activity of acid phosphatase in the cuticle, the activity being correlated with glucose absorption through the body wall (Maki and Yanagisawa 1980). On the other hand, *A. suum* showed a low activity of phosphatase in the cuticle and a high activity in the intestine (Van den Bossche and Borgers 1973). Skotarczak (1987) is of the opinion that the two enzymes are very important in *A. suum* embryonic metabolic processes, their activity being related to metabolic rate. She found the enzymes to be particularly active at early stages of the embryonic development (cleavage and gastrulation) when the energy demand is very high. As the parasites show a very high demand for energy at those developmental stages, carbohydrates are intensively utilised. Glycogen reserves become exhausted and glycogen has to be resynthesised from lipids. The carbohydrate metabolism of *A. simplex* is associated with glucosidase activity. In this experiment, glucosidases were more active in the stage-4 larvae, compared to those at stage 3. Żółtowska et al. (2000) and Łopieńska et al. (2001) reported a difference in distribution and properties of α-amylase between the two larval stages in question. The authors observed activity of the enzyme in stage-4 larvae to be twice as high as that in stage-3 larvae; the difference could have resulted from metabolic changes. According to the authors quoted, a switch from a poikilotherm host of the stage-3 larvae to a homoiotherm one of the stage-4 larvae should be accompanied by a rearrangement of endogenous reserve carbohydrates, and hence a change in the activity of enzymes involved in carbohydrate metabolism. The present experiment supports this inference. The extracts of stage-4 larvae showed a fairly high activity of enzymes involved in carbohydrate metabolism (β-glucuronidase and N-acetyl-β--glucosaminidase), the activity of those enzymes being much lower in the extract of stage-3 larvae. A clear relationship between glucosidase activity in mite extract and mite feeding mode was observed. Glucosidases were most active in free-living mites (*Acarus siro*), while the parasitic blood-feeding species (*Dermanyssus gallinae* and *Psoroptes ovis*) and those parasitising plants (*Tetranychus urticae*) showed a much lower activity of the enzymes. A particularly high activity in all the mites studies was typical of N-acetyl-β-glucosaminidase. According to Nisbet and Billingsley (2000), the results they obtained were caused by the fact that the mite extracts were mostly made of larval stages, the enzyme being present during moulting in all invertebrates. In the experiment described in this paper, high activity of the enzyme was observed in extracts of the two nematode larval stages studied.

Histochemical assays on the excretory organ of *A. simplex* stage-3 larvae, reported by Ruitenberg and Loendersloot (1971a, b) showed esterases to be particularly active

in the lateral part of the organ. In the central part, like in other parts of the larval body, the activity was lower. Esterases were found to be active also in the hatching fluid of *A. suum* (cf. Rogers 1958, 1963). Niemczuk (1993) found esterase in the vitellaria and eggs of the cestodes *Bothriocephalus acheilognathi* and *Khawia sinensis*, while Arme (1966) found the enzyme in the parenchyma and subcuticular cells of *Ligula intestinalis*. Non-specific esterases is a term applied to a group of enzymes that split esters of a fatty acid of a chain not longer that 8 carbon atoms, the splitting being particularly efficient in the case of 2–4 carbon atom-long chains. The group includes acetylcholinesterase present in the nematode ES products (Opperman and Chang 1992, Riga et al. 1995); the enzyme splits acetylcholine, which in turn contributes to reducing intestinal peristalsis and facilitates parasite migration and settlement (Lee and Foster 1995). Activity of the enzyme was detected also in the salivary glands of ticks (Buczek and Madoń 1998); by decomposing cholesterol esters, the enzyme enhances blood vessel permeability. In the present experiment, esterases were found to be active both in the ES products and in the extracts of stage-3 and stage-4 larvae. The activity recorded in the L_4 larvae extracts was several times higher than that in L_3 larvae, which might be suggestive of changes related to preparation of the larvae to digesting the homoiotherm host's stomach mucosa where the subsequent moult of the larvae will take place.

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Received: 25 July 2003
Accepted: 5 December 2003