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Research Article

THE ACTIVITY OF SELECTED HYDROLASES IN EXCRETION-SECRETION PRODUCTS AND EXTRACTS FROM LARVAE AND MATURE SPECIMENS OF *CYSTIDICOLA FARIONIS*.

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Abstract

The results of the study indicated that there are differences in the activity of hydrolases depending on the development stage of the parasite and the season of the year. Twelve hydrolases were confirmed to be active in excretion-secretion (ES) products of larvae collected in fall, while nine were active in spring. The activity of hydrolase from the extracts of fall samples was most often higher than in spring. Eight active hydrolases were confirmed in mature specimens both in spring and fall, and they exhibited a lower activity level than the ES products of larvae. However, the activity of enzymes was higher in mature specimens than in larvae in the extracts from both spring and fall samples.

INTRODUCTION

The nematode *Cystidicola farionis* is a parasite which occurs in the swim bladders of salmonids. The rate at which fish are infested with this parasite depends on many factors including fish age, feeding patterns and season (Valtonen 1978, Giaever *et al.* 1991, Knudsen *et al.* 2002). According to Giaever *et al.* 1991, the degree to which these fish are infested largely depends on how and where they feed. Salmonids from age group 5+ mainly inhabit littoral areas where *Amphipoda*, the intermediary host of this parasite, occur. Younger fish feed primarily in profundal and pelagic areas where these crustaceans do not occur, thus the rate of infestation in them is much lower. Giaever *et al.* 1991 also observed significant seasonal differences in infestation intensity related to the availability of the intermediary host. Increased fish mortality has been observed in fall (Black and Lankester 1980, Black 1984, Giaever *et al.* 1991, Knudsen *et al.* 2002). This parasite is a highly pathogenic species (Moravec 1994). Salmonids belong to the open-bladder group (physostomous), thus they are subject to infestation by this parasite throughout their lives. The long life cycle of this parasite, its highly intense infestation rate and the absence of host immunity to re-infestation, lead to anemia and swim bladder inflammation. The latter can result in the total destruction of the bladder walls, problems with filling and with hydrostatic and respiratory functions (Lankester and Smith 1980, Black 1984, Willers *et al.* 1991). Cusack and Cone 1986 emphasized the role of *C. farionis* as a transmitter of many bacterial diseases.

In addition to the mechanical impact of parasite on the host's body, they also excrete toxins, commonly known as excretion-secretion (ES) products. No publications were found in the literature regarding the chemical composition or the function of these products in the nematode *C. farionis*. However, many publications focused on the ES products of other internal parasites were found, including Hinck and Ivey 1976, Matthews 1984, Knox and Kennedy 1988, Sakanari and McKerrow 1990, Morris and Sakanari 1994, Moczoń and Wranicz 1999, Moczoń 1999, Kotomski and Wędrychowicz 2001 and Sajid and McKerrow 2002. Papers were also found which focused on external parasites such as ticks and mites Sarcoptidae (Buczek and Madoń 1998, Nisbet and Billingsley 2000, Kenyon and Knox 2002). According to these authors, the proteases included in these products have various functions. They block blood coagulation in the host, defend the parasite from the host's immunological response and facilitate internal tissue migration by destroying tissue barriers. They also facilitate larvae hatching and molting and play an important role in their feeding. Other enzymes contained in the ES products of parasites can also

play similar roles to that of proteolysis, including hyaluronidase (Hotez *et al.* 1994), leucine aminopeptidase (Lee 1962, Rogers 1982, Rhoads *et al.* 1997, Rhoads and Fetterer 1998) and acetylcholinesterase (Opperman and Chang 1992, Lee 1996).

The aim of this work was to verify if the ES products and extracts from larvae and mature specimens of *C. farionis* contained hydrolytic enzymes.

MATERIALS AND METHODS

Larvae and mature specimens of *C. farionis* were obtained from the swim bladders of European smelt (*Osmerus eperlanus*) caught in the Baltic Sea in spring (March-April) and fall (September-October). The ES products from the larvae and mature specimens were obtained in the same way. After they were rinsed in an antibiotic solution (penicillin – 100U/ml, streptomycin - 100µl/ml, nystatin 100j. m/ml), 30 specimens were placed in 2ml PBS with the addition of penicillin, streptomycin and nystatin and then incubated at a temperature of 37⁰ C for 48 hours. Following this, the solutions containing the ES products were collected and dialyzed for 24 hours in distilled water at a temperature of 4⁰C.

Extracts of larvae and mature specimens were obtained by homogenizing them with a salt solution (0.9% NaCl). The homogenates were centrifuged for 10 minutes at 3000 x g. The supernatant was used in further analyses.

The API ZYM test by Bio Mérieux was used to determine the enzymatic activity of the ES products and homogenates. This test has substrates which permit the activity of 19 hydrolases to be determined. (Table 1). Sixty-five µl of the studied solution was placed in depressions with the substrates and then incubated for four hours at a temperature of 37⁰C. The results were read according to the instructions from the manufacturer. The activity of hydrolases was determined in nanomoles (nmol) of the hydrolyzed substrate.

The proteolytic activity in the ES products and the homogenates were assayed using the Anson method (1939) with a substrate of hemoglobin denatured with 2% urea and dissolved in an appropriate buffer solution. The following buffers were used in the assays: 0.066M Sørensen buffer at a pH of 7.8; 0.2M borate buffer at a pH of 7.0; 9.0 and a 0.1M citric acid-sodium citrate buffer at a pH of 2.0 and 4.0. The reaction was conducted for 30 minutes at a temperature of 37⁰C. The results were expressed in Anson milliunits (mUA) and recalculated to a milligram of protein assayed using the Bradford method (1976). The assays were repeated three times for each sample and the standard deviation was determined.

Table 1

Activity of enzymes from ES and extract of larvae and adult *Cystidicola farionis*.

	ENZYME	Classification	SUBSTRATE	pH	Activity in nmoles of hydrolysed substrate							
					Larvae				Adult			
					ES		Extract		ES		Extract	
					S	A	S	A	S	A	S	A
1	Alkaline phosphatase	3.1.3.1	2-naphthyl phosphate	8.5	0	20	5	30	5	0	10	40
2	Esterase (C 4)	3.1.1.6	2-naphthyl butyrate	6.5	5	30	0	30	5	5	10	30
3	Esterase lipase (C 8)	3.1.1.3	2 - naphthyl caprylate	7.5	5	30	0	20	5	5	5	30
4	Lipase (C 14)	3.1.1.3	2- naphthyl myristate	"	0	10	0	5	0	0	0	5
5	Leucine arylamidase	3.4.11.14	L-leucyl-2-naphthylamide	"	5	30	10	40	5	5	10	40
6	Valine arylamidase	3.4.11.14	L-valyl-2-naphthylamide	"	5	5	0	20	0	5	5	20
7	Cystine arylamidase	3.4.11.14	L-cystyl-2-naphthylamide	"	0	0	0	0	0	0	0	5
8	Trypsin	3.4.4.4	N-benzoyl-DL-arginine-2-naphthylamide	8.5	0	0	0	0	0	0	0	5
9	α - chymotrypsin	3.4.4.5	N- glutaryl-phenylalanine-2-naphthylamide	7.5	0	0	0	0	0	0	0	5
10	Acid phosphatase	3.1.3.2	2- naphthyl phosphate	5.4	40	40	30	40	20	40	40	40
11	Naphthol-AS-BI-phosphohydrolase	3.1.3.31	Naphthol-AS-BI-phosphate	"	30	40	40	40	40	20	40	40
12	α -galactosidase	3.2.1.22	6-Br -2- naphthyl- α D-galactopyranoside	"	0	0	0	0	0	0	0	5
13	β - galactosidase	3.2.1.23	2-naphthyl- β D-galactopyranoside	"	0	0	0	5	0	0	0	10
14	β - glucuronidase	3.2.1.31	Naphthol-AS-BI- β D-	"	0	0	0	5	0	0	0	5
15	α -glucosidase	3.2.1.20	2-naphthyl- α D-glucopyranoside	"	5	5	5	20	0	0	5	30
16	β -glucosidase	3.2.1.21	6-Br-2-naphthyl- β D-glucopyranoside	"	0	5	0	10	5	0	5	20
17	N-acetyl- β -glucosaminidase	3.2.1.50	1-naphthyl-N-acetyl- β D-glucosaminide	"	5	20	30	30	20	10	30	40
18	α -mannosidase	3.2.1.24	6-Br-2-naphthyl- α D-mannopyranoside	"	0	0	0	10	0	0	0	5
19	α -fucosidase	3.2.1.51	2-naphthyl- α L-fucopyranoside	"	5	10	0	30	0	5	5	30

S – Spring; A - Autumn

The gelatinolytic activity of the ES products and the extracts of larvae and mature specimens were studied using exposed and developed photographic film (Henry *et al.* 1974). Three μ l of the studied sample fluid mixed with 3 μ l of an appropriate buffer were placed on Kodak film in slide frames. The same buffers

were used as in the proteolytic activity assays. A sample of saline solution with the appropriate buffer was placed next to this as a control. These frames were closed in humid chambers and then incubated for periods of 20, 30 and 60 minutes at a temperature of 37⁰ C. Then the frames were rinsed with tap water and the film was inspected under a stereomicroscope to determine to what extent the gelatin layer had been digested. The results were then compared with those obtained for the control samples.

RESULTS

Results regarding the activity of hydrolase in the ES products and extracts of stage-III larvae and mature specimens of *C. farionis* from the both the spring and fall series are presented in Table 1.

Nine hydrolases were confirmed active in ES products from larvae from the spring series. The highest activity was observed in acid phosphatase (40 nmol) and naphthol-AS-BI-phosphohydrolase (30 nmol). The activity of the remaining hydrolases was the same at 5 nmol. The activity of 12 hydrolases was observed in the ES products from larvae obtained in fall. The highest activity was confirmed in acid phosphatase and naphthol-AS-BI-phosphohydrolase (40 nmol), while that of esterase, esterase lipase and leucine arylamidase was 30 nmol. Significantly lower activity was confirmed in alkaline phosphatase and N-acetyl- β -glucosaminidase (20 nmol). The activity of lipase and α -fucosidase was 10 nmol, while that of valine arylamidase and α - and β -glucosidase was 5 nmol.

Eight hydrolases were confirmed to be active in the ES products from mature specimens obtained in spring and fall. The highest activity in both series was noted for naphthol-AS-BI-phosphohydrolase, acid phosphatase and N-acetyl- β -glucosaminidase. The activity of the remaining five hydrolases was much lower at 5 nmol.

There were six active hydrolases in extracts from larvae obtained in spring. The highest activity was confirmed for naphthol-AS-BI-phosphohydrolase (40 nmol), acid phosphatase and N-acetyl- β -glucosaminidase (30 nmol). The activity of the other hydrolases was much lower. The activity of 15 hydrolases was confirmed in extracts from larvae obtained in fall. The highest activity was observed for leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (40 nmol), alkaline phosphatase, esterase, N-acetyl- β -glucosaminidase and α -fucosidase (30 nmol). The activity of esterase lipase, valine arylamidase and α -glucosidase was confirmed to be 50% lower. The activity of α -mannosidase, β -glucosidase was 10 nmol, while that of β -galactosidase, lipase, β -glucuronidase was 5 nmol. The activity of

11 hydrolases was confirmed in extracts obtained from mature specimens in the spring. The highest activity was confirmed for acid phosphatase, naphthol-AS-BI-phosphohydrolase (40 nmol) and N-acetyl- β -glucosaminidase (30 nmol). Four-fold lower activity was confirmed in alkaline phosphatase, esterase and leucine arylamidase. The lowest activity was obtained for esterase lipase, valine arylamidase, α and β glucosidase, as well as α -fukosidase. The activity of all 19 hydrolases was observed in extracts from mature specimens obtained in fall. The highest activity was observed for alkaline and acid phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetylo- β -glucosaminidase (40 nmol) as well as esterase, esterase lipase, α -glucosidase and α -fukosidase (30 nmol). The lowest activity was determined for lipase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, α -mannosidase and β -glucuronidase.

The results regarding the proteolytic activity of the studied samples are presented in Table 2. The highest proteolytic activity was determined in extracts of mature specimens and larvae in environments with a pH of 7.8 and 9.0. The activity of the proteases was many times lower in the acid environment (pH 2.0 and 4.0). The activity of proteases in the ES products in larvae and mature specimens was many times lower than in the extracts.

Table 2

Influence of the pH on activity of protease [in mUA] in products ES and extracts from larvae and adult *Cystidicola farionis*.

pH	ES				Extracts			
	Adult		Larvae		Adult		Larvae	
	Spring	Autumn	Spring	Autumn	Spring	Autumn	Spring	Autumn
2.0	0.04±0.01	0.45±0.10	0.02±0.02	0.28±0.06	0.39±0.14	0.46±0.9	0.03±0.03	0.02±0.02
4.0	0.81±0.55	2.23±0.14	0.76±0.27	2.11±0.40	0.65±0.10	4.23±0.23	0.88±0.03	3.38±0.16
7.8	5.11±0.38	7.13±1.50	6.62±1.40	9.86±1.47	25.32±1.47	31.68±6.56	9.46±1.75	17.56±2.51
9.0	3.08±1.10	3.52±1.00	4.21±1.58	6.57±0.89	12.75±2.46	14.93±3.11	9.22±1.81	12.19±2.54

The assays using the photographic film indicated the presence of proteases which digest gelatin both in the ES products and the extracts from larvae and mature specimens. Total digestion of the gelatin layer occurred with the samples of the ES products in a buffer of pH 7.8 and 9.0 after 60 minutes of exposure. With the samples of extracts from larvae and mature specimens, full digestion of the gelatin layer was observed after 30 minutes. Only slight gelatin digestion occurred after 30 minutes of incubation with ES products and extracts in buffers with a pH of 2.0 and 4.0. However, following 60 minutes of

incubation, slight digestion of the film surface was observed, although the gelatin layer was not completely digested.

DISCUSSION

No papers were found in the available literature which address the composition of ES products or extracts of nematodes of the genus *Cystidicola*. Żółtowska *et al.* (2001) did, however, study the activity of enzymes of carbohydrate katabolism and the glycogen level in extracts of larvae and mature specimens of this nematode. Primarily the activity of proteases were confirmed in the ES products of nematodes (Matthews 1984, Sakanari and McKerrow 1990, Morris and Sakanari 1994, Kotomski and Wędrychowicz 2001). According to these authors, these enzymes play a leading role in the migration of larvae in the body of the host. In the current study, no trypsin activity was detected by the API ZYM test in the ES products of larvae and mature specimens of *C. farionis*, although it was confirmed in extracts of mature specimens obtained in fall. All of the studied samples exhibited proteolytic activity with regard to natural substrates such as hemoglobin and gelatin. Similar results were obtained by Moczóń and Wranicz 1999 for larvae of *Trichinella spiralis*. According to them, proteases from the ES products of *T. spiralis* larvae are incapable of digesting short-chained synthetic peptides and only digest long-chained proteins. Polzer and Taraszewski 1993 confirmed the presence of proteases in extracts of larvae and mature specimens of *Anguillicola crassus*. The proteases from different stages had different substrate preferences. The enzymes from extracts of stage II larvae exhibited the highest activity on azocoll, while those in stage III did so on keratin azure, while the proteases of mature specimens digested only hemoglobin. According to the authors, enzymes from the larval stage are for penetration while those from mature specimens are for digestion. Willers 1991 described histopathological changes in the swim bladders of fish infested with *C. farionis* which might not only have been caused by the local, mechanical influence of larvae and mature specimens, but also could have been the result of the impact of the enzymes produced by this parasite. The activities of trypsin and chymotrypsin in extracts of mature specimens are connected to feeding processes. At the larval stage, this parasite might be similar to the larvae of other nematode species (Sommerville and Davey 1976) which do not absorb food and which obtain energy from accumulated stores. It follows that extracts from these larvae do not indicate the activities of either trypsin or chymotrypsin.

The activity of leucine aminopeptidase was observed in the ES products and extracts of larval stages. According to many authors, aminopeptidase plays an

important role in activating the precursors of the hormones and proenzymes of parasite larvae during hatching and molting (Rhoads *et al.* 1997). The activity of this enzyme has been confirmed in the bodies of many parasites, as well as in the medium during the hatching and molting of their larvae (Rogers 1982, Sakanari and McKerrow 1990, Niemczuk 1993). When an inhibitor of this enzyme was added to the medium it prevented *Ascaris suum* larvae from molting from stage III to IV (Rhoads *et al.* 1998). These enzymes also caused host immunoglobulin to decay on the body surface of the parasite (Auriault *et al.* 1981, Xu and Dresden 1986). The activity of leucine aminopeptidase determined in the ES products of larvae obtained in fall was six-fold higher than that of nematodes obtained in spring. A similar dependence was confirmed for the activity of this enzyme in extracts from both larvae and mature specimens. Such high differences in the activity of this enzyme can be explained by the acceleration of certain metabolic processes related to larva molting, maturation and the general preparation of the parasite for winter. Aminopeptidase also plays an important role in the feeding process of parasites by breaking down proteins into aminoacids. Thus, the relatively high activity of aminopeptidase confirmed in the extracts of larvae and mature specimens may be connected to the feeding process. Ruitenbergh and Loendersloot 1971a and b noted the highest levels of leucine aminopeptidase activity during their histochemical studies of stage-III *Anisakis simplex* in muscles and the brushborder of the small intestines, which, according to these authors, suggests it has a digestive function.

No information regarding the presence of other hydrolases in the ES products of parasites was found in the available literature. The activities of both acid and alkaline phosphatase were confirmed to be very high in the ES products and extracts of larvae and mature specimens in the current study. Phosphatase plays an important role in regulating metabolic processes with alkaline phosphatase taking part in active transport through cellular membranes and acid phosphatase (lysosome marker) indirectly providing information regarding intracellular digestion processes. In many parasites the presence of phosphatase is regarded as the indicator of the areas responsible for secretion and excretion activities and the absorption of nutritive components. High activities of both acid and alkaline phosphatase were confirmed in the cuticle, subcutaneous cells and parenchyma of tapeworms (Arme 1966, Niemczuk 1993). Both Arme and Niemczuk confirmed a clear dependence of the activity of these enzymes on the degree of maturity of tapeworm segments with greater activity observed in mature segments. Among the majority of nematodes the activity of acid phosphatase in the cuticle is high and is correlated with the absorption of glucose through the body walls (Maki and Yanagisawa 1980).

According to Skotarczak 1987, both enzymes play an important role in the metabolic processes of *A. suum* embryos, and their activity depends on metabolic intensity. The author observed the activity of these enzymes to be especially high in the early developmental stages (blastulation and gastrulation) when energy demand is very high. Simultaneously, carbohydrate use is high and glycogen surpluses are reduced to zero, which necessitates its resynthesis from fat. Glycosydase activity is also related to carbohydrate management in the larvae and mature forms of *C. farionis*. Żółtowska *et al.* 2001 confirmed a two-fold higher concentration of glucose, trehalose and glycogen in extracts from mature specimens than in those from larvae. The authors explained that these differences were due to the greater energy demand of mature specimens and the lower activity of enzymes that metabolize carbohydrates, such as glycogen phosphorylase and α -amylase among mature specimens. The same authors, however, did not confirm that such relationships existed for the other enzymes studied, i.e. glucoamylase, maltase, trehalase and trehalose phosphorylase. Nisbet and Billingsley 2000 confirmed a clear dependence between feeding patterns and glycosydase activity in dust mite extracts. Using API ZYM tests, these authors found that the extracts of free-living mites (*Acarus siro*) exhibited the highest glycosydase activity, while in extracts of dust mites which feed on blood (*Dermanyssus gallinae* and *Psoroptes ovis*) the activity of these enzymes was much lower. The authors detected especially high enzyme activity for N-acetyl- β -glucosaminidase among all the studied dust mites, which, according to the authors, occurs during molting among all invertebrates. The results obtained in the current study concur with those obtained by the researchers cited above.

The results of this study indicate that there is a distinct difference in the activity of hydrolases depending on the developmental stage of the parasite and on the season of the year. The activity of 12 hydrolases was detected in the extracts of larvae collected in the fall, while the activity of nine was confirmed in the extracts of larvae collected in the spring. Similarly, in most cases the activity of the identified hydrolases from samples collected in the fall was higher than that of those collected in the spring. Such unequivocal conclusions cannot be drawn from the results of the study of the hydrolase activity in the ES products of mature specimens. Eight hydrolases were detected in both the fall and spring samples and their activity was decidedly lower than that in the ES products from larvae. A significant difference in the number of active hydrolases and the level of their activity was noted during the current study between the extracts of larvae and mature specimens. In both the spring and fall samples, the activity of the studied enzymes was lower in the larva extracts than in those from mature specimens. The activity of all the studied hydrolases (19)

were only confirmed in the fall samples of mature specimens. That the hydrolase activity was higher in fall is due to the superior physical condition of the parasite at this time as well as to its heightened ability to cause disease in the host. This is also why the highest mortality rate of fish infested with these parasites is observed during this season (Black and Lankester 1980, Black 1985, Giaever 1991, Knudsen *et al.* 2002.)

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