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Activity of selected hydrolases in excretion-secretion products and extracts of adult *Contracaecum rudolphii*[1]

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ABSTRACT. **Background**. Enzymes contained in excretion-secretion (ES) products of parasites released to the environment play multiple roles: they facilitate hatching and moulting of larvae, enable a parasite to migrate within tissues, inhibit blood coagulation, defend the parasite from host's immunological response, and enhance feeding and nutrition. The aim of the study was to determine hydrolase activity in ES products and extracts from adult *Contracaecum rudolphii*.

Materials and methods. Adult nematodes were isolated from intestines of black cormorants nesting at Kąty Rybackie (the Vistula Lagoon). Nematode batches of 30 individuals each were placed in 5 ml portions of antibiotic-enriched physiological salt solution and incubated for 24 hours at 37°C. After incubation, the solutions containing ES products were collected and dialysed for 24 h at 4°C against distilled water. Extracts were obtained by homogenising the nematodes with the physiological salt solution (0.9% NaCl). The homogenate was centrifuged for 10 minutes at 3000xg. Enzyme activities were assayed in the supernatant. The enzymatic activity in ES products and homogenates was determined with the API ZYM kit (Bio Merieux S.A., Lyon, France). Hydrolase activities were expressed in volumetric units (nmol) of the hydrolysed substrate.

Results. The nematode ES products showed 10 hydrolases to be active. The highest activity was that of esterases, except for lipase the activity of which was not detected. Among glucosidases, the highest activity was shown by α-glucosidase, much lower activities being typical of ß-galactosidase and N-acetyl-ß-glucosaminidase. The remaining glucosidases proved inactive. Among proteases, leucine arylamidase and valine arylamidase were found to be active only.
The nematode extracts revealed activities of 15 hydrolases. The highest activity was typical of esterases. Among glucosidases, the highest activity was typical of α-glucosidase, ß-glucuronidase and N-acetyl-ß-glucosaminidase. Activities of the remaining glucosidases were much lower. No activity of α-galactosidase was detected. Among proteolytic enzymes, leucine arylamidase proved the most active, while activities of valine arylamidase and chymotrypsin were much lower. The remaining proteases revealed no detectable activity.

Conclusions. The ES products of adult *C. rudolphii* were found to contain active hydrolases which may damage the epithelium lining the host's alimentary tract. Activities of almost all glucosidases in the parasite's extracts suggests that, like in most nematodes, the parasite's main energy source is derived from carbohydrate metabolism.

Key words: black cormorant, *Contracaecum rudolphii*, enzymes, hydrolase.

Introduction

The nematode *Contracaecum rudolphii* life cycle involves piscivorous birds as the definitive hosts. In Europe, these are mainly cormorants (*Phalacrocorax carbo*, *Ph. aristotelis*) and Anatinae ducks (*Mergus merganser* and *M. serrator*). The adult parasites and the larvae which moult for the last time in the definitive host live in the birds' glandular stomach (proventriculus) or in the intestine [1]. The nematode prevalence in cormorants is very high and bird age-dependent [2-5]. Kuiken et al. [2], who studied cormorants at Dore Lake (Saskatchewan, Canada), found 50% prevalence in 1-week-old nestlings, the 2- and 3-week-old ones showing prevalences of 71 and 83%, respectively. Adult cormorants were infected in 100%. Histopathological changes recorded by these

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authors involved mainly ulceration and ecchymoses in the proventricular mucosa. The nestling stomachs showed few ulcers only, whereas birds older than 8 weeks suffered of very extensive pathological lesions.

Penetration enzymes contained in excretion-secretion (ES) products are among the agents that facilitate a parasite' infestation and settlement in the host's body. According to numerous authors [6-16], proteases in the ES products serve a multitude of functions. They inhibit host's blood coagulation, defend the parasite from the host's immunological response, facilitate the parasite's migration within the host by breaking down the tissue barrier, facilitate hatching and moulting of larvae, and are important in larval nutrition. A role similar to that of proteases may be played also by other enzymes present in the parasite ES products, e.g., hyaluronidase [17], leucine aminopeptidase [18-21], acetylcholinesterase [22, 23], and glucosidases [24].

The available literature contains no accounts of chemical composition and function of ES products of the nematode *Contracaecum rudolphii*. Hence, this study was aimed at finding out if the ES products and extracts of the parasite contain hydrolytic enzymes.

Materials and methods

Adult nematodes were isolated from the alimentary tract of black cormorants nesting at Kąty Rybackie (Vistula Lagoon). The nematodes, rinsed in a solution of antibiotics (100 U penicillin/ml; 100 µl streptomycin/ml; 100 U nystatin/ml), were placed in batches of 30 individuals, in 5 ml portions of antibiotic-enriched physiological salt solution and incubated for 24 h at 37°C. Following incubation, the solutions containing ES products were collected and dialysed, in dialysing bags (Sigma 12 kD), for 24 h at 4°C against distilled water. Extracts were obtained by homogenising the nematodes in a glass homogeniser with physiological salt solution (0.9% NaCl). The homogenate was centrifuged for 10 min at 3000xg. Enzyme activity assays were carried out in the supernatant. Activity of the enzymes contained in the ES product and homogenates was determined with the aid of the API ZYM test kit (Bio Merieux S.A., Lyon, France). The kit contains substrates that make it possible to analyse activities of 19 hydrolases (Table 1). Portions (65 µl) of the solution to be tested were placed in wells containing appropriate substrates and incubated for 4 h at 37°C. After the incubation, a drop of the ZYM A and ZYM B reagent (Bio Merieux S.A., Lyon, France) each was added to every well. Under illumination, the reagents produce a colour reaction with compounds formed by the enzymes. Activity of the hydrolases was expressed in volumetric units (nmol) of the hydrolysed substrate with respect to colour reaction intensity in 5 stage scale: 1- five , 2- ten, 3-twenty, 4-thirty, 5-forty and more nanomoles.

Results

Data on hydrolase activities in the *C. rudolphii* extracts and ES products are summarised in Table 1.

The ES products showed 10 hydrolases to be active. The highest activities were those of esterases. Activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase amounted to 40 nmol each. Lower values were produced by lipase esterase (30 nmol) and esterase (20 nmol). Among glucosidases, the highest activity (20 nmol) was that of α-glucosidase, a much lower activity (5 nmol) being typical of ß-galactosidase and N-acetyl-ß-glucosaminidase. Activity of no other glucosidase was detected.

Among proteases, it was only two aminopeptidases that were active: leucine arylamidase (30 nmol) and valine arylamidase (5 nmol).

The extracts revealed 15 hydrolases to be active. The highest activities were those shown by esterases. Activities of both acid phosphatase and naphthol-AS-BI-phosphohydrolase were 40 nmol. Lower activities were produced by alkaline phosphatase (30 nmol), esterase (20 nmol), and lipase esterase (10 nmol). No lipase activity was detected.

Among glucosidases, the highest activity (40 nmol) was that of α-glucosidase (40 nmol). Slightly lower were the activities of ß-glucuronidase and N-acetyl-ß-glucosaminidase (30 nmol). Activity of α-fucosidase was 10 nmol, while ß-galactosidase, ß-glucosidase, and α-mannosidase hydrolysed 5 nmol substrate each. No α-galactosidase activity was detected.

Among proteases, the highest activity was that of leucine arylamidase. Much lower activities were recorded for valine arylamidase (10 nmol) and chymotrypsin (5 nmol). No other protease was found to be active.

Discussion

The literature search produced no data on the presence of hydrolases in ES products and extracts

Table 1. Activity of hydrolases in extracts and ES products of *Contracaecum rudolphii*

Item No.	Enzyme	Classification	Substrate	pH	Activity in nmoles of hydrolysed substrate ES	Extract
ESTERASES	1 Alkaline phosphatase	3.1.3.1	2-naphtyl phosphate	8.5	5	30
	2 Acid phosphatase	3.1.3.2	2-naphthyl phosphate	5.4	40	40
	3 Naphthol-AS-BI-phosphohydrolase	3.1.3.31	Naphthol-AS-BI-phosphate	5.4	40	40
	4 Esterase (C 4)	3.1.1.6	2-naphthyl butyrate	6.5	20	20
	5 Esterase lipase (C 8)	3.1.1.3	2-naphthyl caprylate	7.5	30	10
	6 Lipase (C 14)	3.1.1.3	2-naphthyl myristate	7.5	0	0
GLUCOSIDASES	7 α-galactosidase	3.2.1.22	6-Br-2-naphtyl-αD -galactopyranoside	5.4	0	0
	8 ß-galactosidase	3.2.1.23	2-naphthyl-ßD-galactopyranoside	5.4	5	5
	9 ß-glucuronidase	3.2.1.31	Naphthol-AS-BI-(D-glucoronide	5.4	0	30
	10 α–glucosidase	3.2.1.20	2-naphthyl-αD-glucopyranoside	5.4	20	40
	11 ß-glucosidase	3.2.1.21	6-Br-2-naphthyl-αD-glucopyranoside	5.4	0	5
	12 N-acetyl-α-glucosaminidase	3.2.1.50	1-naphthyl-N-acetyl-αD -glucosaminide	5.4	5	30
	13 α-mannosidase	3.2.1.24	6-Br-2-naphthyl-αD-mannopyranoside	5.4	0	5
	14 α-fucosidase	3.2.1.51	2-naphthyl-αL-fucopyranoside	5.4	0	10
PROTEASES	15 Leucine arylamidase	3.4.11.14	L-leucyl-2-naphthylamide	7.5	30	40
	16 Valine arylamidase	3.4.11.14	L-valyl-2-naphthylamide	7.5	5	10
	17 Cystine arylamidase	3.4.11.14	L-cystyl-2-naphthylamide	7.5	0	0
	18 Trypsin	3.4.4.4	N-benzoyl-DL-arginine-2-naphthylamide	8.5	0	0
	19 α- chymotrypsin	3.4.4.5	N-glutaryl-phenylalanine -2-naphthylamide	7.5	0	5

of *Contracaecum rudolphii*. Numerous parasites showed their ES products to contain, in addition to proteases, active hyaluronidase, leucine aminopeptidase, acetylcholinesterase, and many glucosidases, [7, 10, 17, 19-25]. In the opinion of the authors referred to, by decomposing the tissue and hormonal barrier, the enzymes allow the larvae to migrate and settle in the definitive host's body. In addition, the enzymes play a very important role in hatching and moulting of the larvae and in their feeding. The ES products tested in this study failed to show active endoproteases. On the other hand, leucine aminopeptidase as well as esterases and glucosidases proved to be highly active. According to Irwin et al. [24], penetration of parasites into and migration within a host's body is facilitated, in addition to proteases, by glucosidases responsible for breaking down certain glucoproteins (mucins) produced, as secretions or integral membrane proteins, by epithelial cells of the alimentary and respiratory systems. In the ES products of *Fasciola hepatica* they determined activities of 8 glucosidases (ß-galactosidase, ß-glucosidase, α-fucosidase, α-galactosidase, α-mannosidase, ß-N-acetylhexosaminidase, ß-glucuronidase, and neuraminidase). In their opinion the enzymes make it possible for the parasite to feed on glucoproteins contained in the host's blood and tissues. No papers on *C. rudolphii* development in the definitive host and on the feeding modes of adults and larvae could be found in the available literature. Histopathological lesions observed by Kuiken et al. [2] in stomachs of infested cormorants could have been produced by enzymes contained in the parasite's ES products. The high activity of the ES product esterase may be considered as evidence of the parasite's high metabolic activity.

The adult nematode extracts revealed most of the enzymes tested to be active. Highly active were most of the glycolytic enzymes, associated with carbohydrate metabolism. According to Barrett [26] carbohydrate metabolism is the major energy source for most parasites. Some of the glycolytic enzymes (ß-galactosidase, α-glucosidase) are typically hydrolytic ones. Highly active glucosidases were also detected in extracts of larvae and adults of other parasitic nematodes [27-29].

The most active esterases were phosphatases and phosphohydrolases. These enzymes are important in metabolic regulation. Alkaline phosphatase is involved in active cell membrane transport. Acid phosphatase (a lyzosome marker) provides indirect information on intracellular digestion. In many parasites, the presence of phosphatases is regarded as an indication of sites responsible for secretory and

excretory activity and for nutrient absorption. High activities of both alkaline and acid phosphatases were detected in the cuticle, subcuticular cells, and parenchyma of tapeworms [30, 31]. The authors quoted found activities of those enzymes to be clearly dependent on the development stage of the tapeworm proglotids, a higher activity being typical of mature proglotids. In most parasitic nematodes, activity of acid phosphatase in the cuticle is high and correlated with glucose absorption *via* the body wall [32]. In the present study, no lipase activity was detected, while both esterase and esterase lipase, the enzymes that hydrolyse lipids with shorter carbon chains, were active.

The *C. rudolphii* extracts tested in this study provided no indication of trypsin activity, activity of chymotrypsin being very low. Polzer and Taraszewski [33] identified proteases in extracts of larvae and adults of *Anguillicola crassus*. Proteases from different developmental stages differed in their substrate affinities. Whereas enzymes in extracts of stage II larvae were most active in azocol, those in stage III larva extracts showed the highest activity against keratin; proteases present in extracts from adult individuals hydrolysed haemoglobin only. According to them, larval enzymes are used to penetrate a host, while those contained in the adults are involved in hydrolysis. Proteases were found to be present also in extracts and ES products of larvae and adults of *T. spiralis* [12, 34, 35]. According to Criado-Fornelio et al. [34], the proteases present only in the parasite extracts, are digestive enzymes, while those proteases that occur in extracts and ES products or only in ES products are enzymes associated with the moulting process and start to be synthesised after decapsulation; they make it possible for the larvae to penetrate into the host's enterocytes. Moczoń and Wranicz [12] and Ros-Moreno et al. [35] found proteases from larvae and adult *T. spiralis* showing a higher preference towards natural substrates than to synthetic ones. Proteases of the muscle-stage larvae prefer structural proteins (collagen), while those of the newborn larvae and adults are mainly active in decomposing haemoglobin, fibrinogen, and immunoglobulin G. In the present study, activity of proteases was determined using synthetic substrates which contain peptide chains much shorter than those found in natural substrates. This study revealed a high activity of leucine aminopeptidase. According to Jennings and van der Lande [36], exopeptidases are the only enzymes capable of hydrolysing proteins in leeches and other invertebrates, for which reason the process is much slower than in vertebrates. The high activity of leucine aminopeptidase is perhaps compensatory for the lack of endopeptidase activity.

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