WIADOMOŚCI PARAZYTOLOGICZNE
T. 50 (3) 2004: 503–507

THE ACTIVITY OF HYDROLASES OF LARVAL STAGES OF *ANISAKIS SIMPLEX* (NEMATODA)

ELŻBIETA ŁOPIEŃSKA-BIERNAT[1], KRYSTYNA ŻÓŁTOWSKA[1] AND JERZY ROKICKI[2]

[1]Katedra Biochemii, Wydział Biologii, UWM, ul. Oczapowskiego 1A, 10-579 Olsztyn;
[2]Katedra Zoologii Bezkręgowców, UG, al. Piłsudskiego 46, 81-378 Gdynia

ABSTRACT. Activity of hydrolases during the third and fourth larval stage of *Anisakis simplex* was identified by applying the API ZYM test method. In *A. simplex* larvae the activity of phosphatases was high, particularly that of acid phosphatase (40 nmol/mg-^{1}). Among esterases lack of activity of lipase (C_{14}) is worth noticing while the activity of esterases (C_4) and (C_8) was high. The activity of those later two enzymes was higher in L_3 larvae than in L_4 larvae. The highest activity in the subclass of glucosidases was recorded for ß-fucosidase and N-acetyl-ß-glucosaminidase. A higher activity in L_3 larvae than in L_4 larvae was recorded for: ß-glucuronidase and N-acetyl-ß-glucosaminidase (2-fold) and ß-fucosidase (3-fold). Differently the activity of ß-galactosidase and ß-glucosidase was higher in L_4 larvae than in L_3 larvae. The tests did not show activity of α-galactosidase, ß-glucosidase and α-mannosidase on both larval forms.

Key words: API ZYM, esterases, glycosidases, larval stages of *Anisakis simplex*, peptidases, proteases.

INTRODUCTION

Anisakis simplex is a parasite of the alimentary system. The third and fourth larval stages develop in the alimentary system of not only their final hosts but also the man as the opportunistic host.

The biochemical studies on *A. simplex* are fragmentary. They covered the proteolytic enzymes and aminoacids profile of proteins. Little is known on the lipids and their metabolism in *A. simplex*. It was only established that long chain fatty acids that were isolated from fats originating from various organs of that nematode dominated in them (Kawai 1968 after Smith and Wootten 1978). The available literature contains little information on transformation of saccharides in L_3 larvae of that parasite. It is known that in L_3 larvae of *A. simplex* the glycolytic sequence, elements of Krebs cycle are present and that the system of succinic dehydrogenase system is active (Smith and Wootten 1978). The presented study used the API ZYM test to confirm the presence and to determine the half-quantitative activity of hydrolases

that are important in utilization of reserve materials and for the process of host penetration by L_3 larvae and the following developmental forms of *A. simplex*, L_4 larvae. That comparison is interesting as the literature lacks entirely any reference to the physiology of the L_4 larvae. Both larval forms that were the subject of this study, in the natural conditions live in different environments. The change of the living conditions occurs during the transfer of L_3 larvae from a heterothermal organism, such as crustaceans, cephalopods or fish to a endothermal organism – a mammal, where L_4 larvae develop.

MATERIALS AND METHODS

The material for the study consisted of third stage (L_3) and fourth stage (L_4) larvae of *A. simplex*. Stage three larvae of *A. simplex* were isolated from fresh Baltic herring (*Harengus clupea*). Fourth stage larvae originated from own cultures *in vitro* run according to the modified Grabda method (1976). The cultures were maintained at 37°C in the atmosphere with 5% CO_2 in the incubator by WTB Binder type CB 150. After 5 days of culturing L_4 larvae of *A. simplex* were obtained that grew further in the culture until day 12 (reaching the average body length of 40 mm).

Extracts for examination were prepared by weighting 100 mg of L_3 or L_4 larvae. They were homogenized in a glass homogenizer with 1ml 0,65% NaCl. The homogenate was centrifuged at 800 × g for 15 min. at 4°C. In the supernatant obtained protein content was measured according to Bradford (1976). 50 µl of supernatant containing 100 µg of protein was placed in the capsules of API ZYM test by bioMeriéux. Next the manufacturer's instructions were followed. In total 19 hydrolases listed in Table 1 were tested. Their activity was defined according to the 5-point scale where 0 represented no activity and 5 the maximum activity (> 40 nmol). The presented results represent mean values for four repetitions for the larvae of each development stage of the nematode.

RESULTS AND DISCUSSION

API ZYM test showed activity of 12 out of the 19 examined hydrolases in the extracts of L_3 and L_4 (Table 1). On the basis of the test results no qualitative differences in the profile of enzymes present in both stages of the parasite were found. Their extracts were active on the same 12 substrates. It should be stressed, however, that L_3 and L_4 differed in the level of activity of enzymes belonging to individual subclasses. Extracts obtained from L_3 larvae showed a definitely higher activity of esterases and peptidases as well as that of two glycosidases – N-acetyl-ß-glucosaminidase and fucosidase than those obtained from L_4 larvae (Table 1). Those observations, as we believe, should be linked to the processes of host tissues penetration by L_3 larvae. Similar experiments carried out on larvae of the same stage by

Ruitenberg and Loendersloot (1971a, b) applying the histochemical technique showed presence of at least 18 enzymes, including 12 oxidoreductases. Those authors found weak activity of non-specific esterase and strong activity of acid phosphatase, while there was no activity of alkaline phosphatase. That last finding is contrary to the results of the test applied in this study that showed the activity of alkaline phosphatase in *A. simplex*, although it was definitely lower than that of acid phosphatase (Table 1).

Table 1. The activity of hydrolases of larval stages of *Anisakis simplex* (nmol/mg^{-1})

	No	Enzyme	Substrate	Activity L_3	Activity L_4
esterases	1	Alkaine phosphatase	2-naphtyl phosphate	30.0	5.0
	2	Acid phosphatase	2-naphtyl phosphate	> 40.0	40.0
	3	Naphtol-AS-BI-phosphohydrolase	Naphtol- AS-BI-phosphate	30.0	20.0
	4	Esterase (C4)	2-naphtyl butyrate	30.0	20.0
	5	Lipase esterase (C8)	2-naphtyl caprylate	20.0	10.0
	6	Lipase (C14)	2-naphtyl myristate	0	0
glycosidases	7	α-galactosidase	6-Br-2-naphtyl-α–D-galactopyranoside	0	0
	8	β-galactosidase	2-naphtyl-β-D-galactopyranoside	5.0	10.0
	9	β-glucuronidase	Naphtol-AS-BI-β-D-glucuronide	5.0	2.5
	10	α-glucosidase	2-naphtyl-α-D-glucopyranoside	2.5	10.0
	11	β-glucosidase	6-Br-2-naphtyl-β-D-glucopyranoside	0	0
	12	N-acetyl-β-glucosaminidase	1-naphtyl-N-acetyl-β-D-glucosaminide	20.0	10.0
	13	α-mannosidase	6-Br-2-naphtyl-α-D-mannopyranoside	0	0
	14	α-fucosidase	2-naphtyl-α-L-fucopyranoside	30.0	10.0
proteases, peptidases	15	Leucine arylamidase	L-leucyl-2-naphtylamide	30.0	10.0
	16	Valine arylamidase	L-valinyl-2-naphtylamide	10.0	2.5
	17	Cystine arylamidase	L-cystyl-2-naphtylamide	0	0
	18	Trypsin	N-benzyl-DL-arginine-2-naphtylamide	0	0
	19	Chymotrypsin	N-glutaryl-phenylalanine-2-nanaphtylamide	0	0

Triacylglycerol lipases are enzymes active in the majority of nematodes that are parasites of the alimentary system of animals (Lee and Atkinson 1976). Absence of a lipase hydrolyzing the esters of longer chain fatty acids in both larvae of *A. simplex* was surprising (Table 1). On the other hand, esterases hydrolyzing esters of short chain fatty acids (C_4 and C_8) were highly active. Almost 2-fold higher activity of those enzymes in younger larvae than in the older form was recorded. A similar phenomenon was observed in *Steinernema affinis* and *S. feltiae* as well as *Heterorhabditis zaelandica* nematodes (Żółtowska and Łopieńska 2003). It may be that in case of *A. simplex*, similar to other parasitic nematodes, short chain fatty acids, products of relatively anaerobic transformation of sugars, are built into lipids (Soprunov 1978).

The set of enzymes digesting carbohydrates in nematodes depends mainly on the

character of their food. For example, *Turbatrix aceti*, feeding on bacteria and fungi, produces chitinase and cellulase while there is no chitinase in *Ditylenchus dispaci*, a parasite of higher plants (von Brand 1973). It was established that the same types of glycosidases that are present in the brush border and pancreatic and intestinal juices of the pig or humans are also present in their parasite *A. suum* (Żółtowska 1991). It was proven on the example of α-amylase that those are own enzymes of the nematode and not those of the host (Żółtowska 1992). In case of *Nippostronylus brasiliensis* ß-glucuronidase, ß-galactosidase, α-mannosidase and ß-N-acetylamino-desaxyglucosidase were higly active while in *Trichuris* sp. ß-glucuronidase and ß-galactosidase had a lower activity (von Brand 1973). As opposed to the above-mentioned nematodes, both larval stages of *A. simplex* showed no activity of α-mannosidase, α-galactosidase and ß-glucosidase in API ZYM test (Table 1). Among the present glycosidases, α-fucosidase, N-acetyl-ß-glucosaminidase and ß-glucuronidase had the highest activity in both cases. Activity of those enzymes was 2-3-times higher in L_3 larvae than in L_4 larvae. As the sugars that belong to membrane polysaccharides and glycoproteins are the substrates for those enzymes, their activity seems to be related to the processes of host tissues penetration. On the other hand, the activity of typical digestive enzymes such as ß-galactosidase and α-glucosidase was, respectively, 2- and 4-times higher in L_4 larvae than in L_3 larvae (Table 1). The high activity of those enzymes is an expression of adaptation in sugars metabolism enzymes to the type of food found by nematodes in the alimentary system of mammals taking place in L_4 larvae.

Among peptidases, leucine and valine arylamidases had a couple of times higher activity in L_3 larvae than in L_4 larvae (Table 1). On the other hand, in the test conditions negative results were obtained for trypsin and chymotrypsin. In case of trypsin that result is contrary to the data in literature. Skanari and McKerrow (1990) as well as Morris and Sakanari (1994) showed presence of serine protease (trypsin-like) in L_3 larvae of *A. simplex*. Also in our study applying the classical Anson method, activity of alkaline proteases in *A. simplex* larvae was found (0.37 μmol/mg for L_3 and 0.70 μmol/mg for L_4). The results obtained were presented during the European Parasitology Multicolloquium in Poznań (Żółtowska et al. 2000). The contradiction between the above results may be superficial as different substrates were used in those studies for measurement of enzymatic activity. In API ZYM test the substrate was specific for trypsin only while in the above quoted study less specific substrates were used allowing determination of the general activity of alkaline proteases. In case of both larval stages of the parasite the activity of acid proteases was identified, also by applying the Anson method. It was 0.50 μmol/mg in L_3 larvae and 1.20 μmol/mg in L_4 larvae. The activity of both types of proteases was 2-times higher in L_4 larvae of *A. simplex* than in L_3 larvae. The optimum pH values for proteolytic enzymes from L_3 larvae of *A. simplex* were pH 3.0 for acid proteases and pH 8.4 for alkaline proteases (Żółtowska et al. 2000).

We believe that the differences in the activity levels of hydrolases between L_3 and L_4 larvae could be considered as an expression of L_4 larvae adaptation to life in the alimentary system environment of a mammal, more differentiated in acidity and nutrients than that of a fish.

REFERENCES

Bradford J. 1976. A rapid sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Grabda J. 1976. Studies on the life cycle and morphogenesis of *Anisakis simplex* (Rudolphi, 1809) (Nematoda: Anisakidae) cultured *in vitro*. *Acta Ichtyologica et Piscatoria* 6: 119-140.

Lee D.L., Atkinson H.J. 1976. Physiology of Nematodes. 2nd ed. The Macmillan Press LTD, London.

Morris S.R., Sakanari J.A. 1994. Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. *Journal of Biological Chemistry* 269: 27650-27656.

Ruitenberg E.J., Loendersloot H.J. 1971a. Histochemical properties of the excretory organ of *Anisakis* sp. larva. *Journal of Parasitology* 57: 1149-1150.

Ruitenberg E.J., Loendersloot H.J. 1971b. Enzymhistochemische onderzoek van *Anisakis* sp. larven. *Tijdschrift voor Diergeneeskunde* 96: 247-260.

Sakanari J.A., McKerrow J.H. 1990. Identification of secreted neutral proteases from *Anisakis simplex*. *Journal of Parasitology* 76: 625-630.

Soprunov F.F. 1978. Biochemie der Helminthen. I. Der Energiehaushalt der Helminthen. Parasitologische Schriftenreihe. VEB Gustav Fischer Verlag, Jena.

Smith J.W., Wootten R. 1978. Anisakis and anisakiasis. *Advances in Parasitology* 16: 93-163.

Von Brand T. 1973. Biochemistry of Parasites. Academic Press. N. Y, London.

Żółtowska K. 1991. Distribution of enzymes hydrolyzing carbohydrates in the alimentary tract of *Ascaris suum*. *Acta Parasitologica Polonica* 36: 93-96.

Żółtowska K. 1992. Investigation on orgini of α–amylase present in *Ascaris suum* intestine using immunodiffusion test. *Acta Parasitologica Polonica* 37: 205-207.

Żółtowska K., Łopieńska E. 2003. The activity of hydrolases of entomopathogenic nematodes. *Wiadomości Parazytologiczne* 49: 375-379.

Żółtowska K., Łopieńska E., Dmitryjuk M. 2000. Activity of lipolytic and proteolytic enzymes in extracts from stage III larvae of *Anisakis simplex*. *Acta Parasitologica* 45: 185.

Zaakceptowano do druku 30 maja 2004