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Effects of 3rd stage *Anisakis simplex* larvae on digestive tract protease activity of guinea pigs 24 and 48 hours after infection

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Summary

The duodenum content trypsin activity in the infected guinea pigs was lower than that in the control, the difference being significant and highly significant after 24 and 48 h of infection, respectively. Similarly, the pancreatic extract trypsin activity was lower in the infected guinea pigs. The difference was significant only after 48 hours. The stomach content pepsin activity was significantly lower in the infected animals after 24 hours.

Key words: *Anisakis simplex*; guinea pig; protease

Introduction

Larvae of 3rd stage of *Anisakis simplex* are capable, as incidental parasites, of penetrating the digestive system of numerous mammals (Jones *et al*., 1990; Podolska *et al*., 1997; Dziekońska-Rynko *et al*., 1997b). In experimentally infected rats, the larvae were observed to penetrate the stomach wall one hour after infection and, after 3 – 4 hours, live larvae were found in the peritoneal cavity (Young and Lowe, 1969; Gibson, 1970). Myers (1963) observed larvae in the pancreas, liver, and intestinal mesentery 8 h after experimental infection of guinea pigs. She found live larvae in the hosts' bodies 6 days after infection.
Experimental infection of small mammals, the larvae – like they behave in the human body – penetrate, *via* the digestive tract, the body cavity and other organs, thereby damaging the stomach and intestinal walls and injuring other organs, e.g., the pancreas and liver (Jones *et al*., 1990; Podolska *et al*., 1997). Experimentally infected guinea pigs 6 h after infection when most larvae were present in the digestive tract contents and wall showed a reduction in stomach secretion (Asami and Inoshita, 1967) and changes in protease activity in the stomach, duodenum, and pancreas (Dziekońska-Rynko *et al*., 1997b).

In this paper was aimed at finding out if 3rd stage larval infection of *A. simplex*, longer than 6 hours, would affect the activity of proteolytic enzymes in the guinea pig digestive tract and pancreas.

Materials and Methods

Anisakis simplex larvae were obtained from herring (*Clupea harengus membras*) caught in the Baltic Sea. Until infection, the larvae were kept in sterile 0.83 % NaCl, 24 or 48 h at 4°C. The guinea pigs of similar age were weighed an average of 300 g and divided into 4 groups 7 individuals each. In the experimental groups animals received 30 larvae each, introduced in the physiological solution *per os*. The controls (groups 1 and 3) were identically treated with pure physiological solution.
The guinea pigs were autopsied after 24 (groups 1, 2) and 48 h (groups 3, 4) post-infection or post-physiological saline application to find out if migrating *A. simplex* larvae were present in the digestive tract and other organs.
The activity of trypsin was determined in the pancreas and duodenal content extracts, pepsin activity being determined in the gastric content extract. To prepare the extracts, 1 g samples each of the duodenal and stomach contents were mixed with 5 ml physiological solution. The suspension was centrifuged for 10 min. at 3000 *g* (MPW-340). The duodenal content supernatant was used in the trypsin activity assays, the pepsin activity assays being conducted in the stomach content supernatant. The pancreatic extracts were prepared as described by Żółtowska *et al*. (1989). The pepsin and trypsin activity assays were carried out using Anson's method described by Kłyszejko-Stefanowicz (1982), with the present authors' quantitative modifications. The results, expressed in Anson units, were standardised to protein weight unit (mg), as determined using the

method of Lowry *et al.* (1951).
The data on digestive tract protease activity of the guinea pigs were subjected to statistical treatment involving Duncan's multiple range test (Ruszczyc, 1978).

Results

About 29 % of the larvae *A. simplex* were located in the guinea pigs bodies 24 hours after infection (Tab. 1). Most of the larvae were found in the stomach wall and outside the stomach as well as in the colon content. The larvae were situated in the abdominal cavity as well. About 27 % of the larvae were found after 48 hours; they were abundant in the stomach wall and in the abdomen near the stomach. Most larvae were located in the abdominal cavity, around the kidneys and the intestinal mesentery. Those observations allow us to conclude that, as time went by, the larvae penetrated internal organs farther and farther.

Table 1. Location and mean number of *Anisakis simplex* larvae in guinea pig organs

Hours post infection	24	48
No. of larvae introduced	30	30
Location and mean number of larvae in		
Stomach content	0.7	0
Small intestine content	0.3	0
Colon content	1.3	0
Stomach wall	2.6	2.3
Small intestine wall	0.4	0.6
Outside stomach	1.4	2
Abdominal cavity	2	3
Pancreas	0	0.3
Total	8.7	8.2

The duodenal content of the infected animals showed a lower trypsin activity, compared to the relevant controls (Tab. 2). The difference was statistically highly significant in those animals assayed 48 hours post-infection (group 4 vs group 3), a significant difference relative to the control being revealed for the guinea pigs 24 hours after infection (group 2 vs group 1).
A lower trypsin activity was recorded in the pancreatic extracts of the *A. simplex*-infected guinea pigs, compared to the controls (Tab. 2). The difference was significant after 48 hours from infection (group 4 vs group 3), a 24-h-long infection producing non-significant difference (group 2 vs group 1).
The gastric pepsin activitiy of the infected animals (Tab. 2) was lower than that in the control, both 24 and 48 hours after infection (group 2 vs group 1 and group 4 vs group 3). The difference was significant after 24 hours of infection and non-significant after 48 hours.

Table 2. Activity of trypsin [AU/mg protein] in duodenum and pancreas and activity of pepsin [AU/mg protein] in stomach of guinea pigs infected with 3rd stage of *Anisakis simplex* stage 3 larvae

Group No.		1	2	3	4
Infection (h)		0	24	0	48
Trypsin activity in guinea pig duodenum*	x	7.92 ± 0.99	1.90 ± 0.82	22.17 ± 2.64	13.13 ± 4.20
	v	12.52	43.43	11.89	31.99
Difference between means	1		6.02	14.25	5.21
Significance of differences between	2	+		20.27	11.23
means **	3	++	++		9.04
	4	+	++	++	
Trypsin activity in guinea pig pancreas*	x	48.67 ± 5.66	47.63 ± 4.86	41.70 ± 4.85	26.13 ± 4.82
	v	11.62	10.20	11.62	18.43
	1		1.04	6.97	22.54
Difference between means	2	-		5.93	21.5
Significance of differences between	3	-	-		15.57
means **	4	++	++	+	
Pepsin activity in guinea pig stomach*	x	8.14 ± 2.00	6.46 ± 2.36	10.11± 1.54	9.09 ± 2.88
	v	24.62	36.61	15.22	31.68
Difference between means	1		1.68	1.97	0.95
	2	+		3.65	2.63
	3	++	++		1.02
Significance of differences between means **	4	-	++	-	

* x – arithmetic mean; v – coefficient of variation; ** ++ – highly significant difference ($P < 0.01$); + – significant difference ($P < 0.05$); - non-significant difference

Discussion

Activities of pepsin in the stomach and trypsin in the duodenum and pancreas, assayed in this work, were lower 24 and 48 hours after the 3rd stage of *A. simplex* larval infection than they were in the controls. The highest numbers of larvae were found at these times located in the stomach wall, all the larvae being live and very motile. This finding is in agreement with Asami and Inoshita (1967) who observed a reduction in the gastric secretion during *A. simplex* infection to be dependent on the number of larvae penetrating the stomach mucosa. The larvae penetrating the stomach wall produce particularly copious amounts of excretory/secretory (E/S) products containing, i.a., penetration enzymes such as proteases and hyaluronidase (Matthews, 1982, 1984; Sakanari and McKerrow 1990; Hotez *et al*., 1994; Morris and Sakanari, 1994). According to these authors, the enzymes mentioned – in addition to their penetration-enhancing function – are also vasoactive and serve as immunomodulators and anticoagulants. It is commonly known that vasoactive intestinal peptide (VIP) is a neurotransmitter produced in large amounts by intestinal interneurons. The enzyme inhibits conductivity in intestinal loop cholinergic neurons, thereby reducing gut motility. The protein was detected in E/S products of such nematodes as *Ascaridia galli*, *Nippostrongylus brasiliensis*, and *Nematodirus battus* (Foster and Lee, 1996). According to Opperman and Chang (1992), E/S products of parasitic nematodes contain acetylcholinesterase, an acetylcholine-decomposing enzyme. Consequently, the enzyme affects gastric secretion. The lower activity of pepsin and trypsin, observed 24 and 48 hours after infection compared to the control could have resulted from accumulation of E/S products in the host's digestive tract. Another cause of the lower activity of the enzymes assayed could lie in their being bound by inhibitors. The literature lacks data on a pepsin inhibitor in *A. simplex* larvae, while such inhibitor was identified in *A. suum* (Keilová and Tomášek, 1972; Abu-Erreish and Peanasky, 1974a,b; Martzen *et al*., 1991), although the parasite does not settle in a pepsin-affected location. It seems, therefore, that, since the adult and larval *A. simplex* dwell most frequently in the stomach, a pepsin-affected habitat, they ought to contain an inhibitor that would protect them from the enzyme's effects. The presence of a pepsin inhibitor was indirectly evidenced by the results obtained in *in vitro* cultures kept by Dziekońska-Rynko *et al*. (1997a). Those *A. simplex* larvae maintained in animal protease-containing media survived longest in the pepsin-containing medium and reduced the pepsin activity throughout the incubation period. A pepsin inhibitor was found in *A. simplex* L_3 larvae by Morris and Sakanari (1994) and by Lu *et al*. (1998). Like the trypsin inhibitor, inhibitors of trypsin, chymotrypsin, pepsin, and carboxypeptidases A and B, identified in *A. suum*, inactivate the host's digestive enzymes, thereby protecting the parasite from digestion (Hawley *et al*., 1994; Martzen *et al*., 1985). The *A. suum* trypsin inhibitor is highly specific. It inactivates pig trypsin from the specific host, and is less effective towards human trypsin. The *A. simplex* inhibitor studied *in vitro* by Morris and Sakanari (1994) was found to reduce activities of bovine trypsin and human leukocyte elastase, but did not reduce the pig chymotrypsin activity.

The results of this study confirm observations made during *in vitro* experiments (Dziekońska-Rynko *et al*., 1997a) and those yielded by an earlier experiment involving guinea pigs examined 6 hours after infection (Dziekońska-Rynko *et al*., 1997b). The duodenal and pancreatic tryptic activity assayed in the present experiment 24 and 48 hours after infection was much lower in the experimental than in the control animals. Similar results were reported by Dziekońska-Rynko *et al*. (1997) when the enzymatic activity assays were performed 6 hours after infection. A lower pancreatic tryptic activity in *A. suum*-infected guinea pigs, compared to the control, was found by Żółtowska *et al.* (1991). *Ascaridia galli*-infected chicks showed the tryptic activity to be reduced both in the pancreas and the duodenum (Hurwitz *et al*., 1972).

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