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INFLUENCE OF *ANISAKIS SIMPLEX* STAGE III LARVAE UPON THE ACTIVITY OF PROTEASES UNDER *IN VITRO* CONDITIONS

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ABSTRACT. The larvae of *Anisakis simplex* had the largest influence upon decreasing the activity of porcine pepsin. The activity of that enzyme in tests, where the larvae were present during the entire period of incubation, was lower than in the controls. No similar trends were observed in case of the solutions with bovine and porcine trypsin. The activity of those enzymes in the solutions containing the larvae was higher than in the controls. Only the activity of porcine trypsin after 10 h of incubation was slightly lower in the experimental sample than in the control, however, during the later hours the dynamics of the activity decrease of that enzyme in the controls was higher than in the experimental samples.

The recorded activity of papain in the samples containing the larvae was higher than that in the controls during the entire time of the experiment.

Key words: *Anisakis simplex*, bovine trypsin, larvae, papain, porcine pepsin, porcine trypsin.

INTRODUCTION

Production of enzyme inhibitors is one of the mechanisms protecting the intestinal parasites against the digestive enzymes of the host. The inhibitors of proteases isolated from the muscle and membrane sack as well as from other parts of the bodies of *Ascaris suum* and *Ascaridia galli* have been characterized quite extensively (Green 1957; Peanasky and Laskowski 1960; Abu-Erreish and Peanasky 1974 a, b; Ansari et al. 1976).

Morris and Sakanari (1994) isolated an inhibitor of proteases from *A. simplex* stage III larvae. That protein inhibited the activity of bovine trypsin and elastase from human leukocytes and did not inhibit the activity of porcine chymotrypsin. Dziekońska-Rynko et al. (1997b) proved that infection of guinea pigs with *A. simplex* stage III larvae influenced a change in the activity of proteases in the alimentary tract of the infected animals.

Papers concerning the influence of various intestinal parasites upon inhibition of proteases in case of rearing those parasites *in vitro* are found in the available literature (Pappas and Read 1972 a, b; Juhasz and Matskasi 1979; Kadłubowski et al. 1992). No papers, however, were found on the influence of *A. simplex* stage III larvae upon the activity of proteases under *in vitro*

conditions. Similarly, no papers were found concerning the effect of other parasites of the activity or proteases of vegetable origin.

This study was aimed at investigating the influence of *A. simplex* stage III larvae on the activity of proteases of animal and vegetable origin in case of rearing under controlled environment, *in vitro*.

MATERIAL AND METHODS

The subject of this study were *Anisakis simplex* stage III larvae obtained from herring caught in the Baltic Sea. Prior to the experiment, the larvae were stored in Tyrod liquid at the temperature of +4°C. To prepare the 0.1% solution of bovine trypsin (*ca* 1000 BAEE, Polskie Odczynniki Chemiczne, Gliwice) and porcine trypsin (274 j/g, Wytwórnia Surowic i Szczepionek of Warsaw) as well as papain (1:350, Loba-Chemie, Vienna), 0.066 M Sörensen buffer with pH 7.6 was used. In preparing the 0.1% solution of pepsin (14 mA/mg Serva, New York), 0.1M buffer of citric acid – sodium citrate with pH 3.0 was used.

Twenty larvae were inserted into test tubes containing 5 ml of the enzyme solution and then the tubes were placed in a heater at the temperature of 37°C. The solution of enzyme containing no larvae was used as control. The tests concerning changes in the activity were done for each of the enzymes in three repetitions. The enzyme activity in all solutions was measured after 1 hour from placing larvae in them. The following measurements were made after 10, 15, 20, and 25 h. At the same time the identity of the enzymes in the controls was measured.

The activity of the enzymes was measured by Anson method (Kłyszejko-Stefanowicz 1982). Two percentage hemoglobin denatured using urea with the pH 7.5 was used as the medium for measurement of trypsin and papain activity while in case of pepsin 2% hemoglobin in 0.06 N solution of hydrochloric acid was used.

RESULTS

The results of tests on the level of activity of the enzymes in the media with the *A. simplex* larvae incubated in them and for the controls are presented in the Table 1.

In all cases, after the first hour of larvae incubation in the experimental media, there was no difference in the measured enzyme activity between the solutions with the larvae and the controls.

After 10 h, in the solutions containing bovine trypsin and the larvae, a higher activity of protease as compared to the controls and the value recorded after 1 h was measured. In the specimens examined after 15 and 20 h, a lower level of trypsin activity was recorded in the solutions containing the

Table 1. Influence of *A. simplex* stage III larvae upon the activity of proteases

Hours of incubation	Activity of proteases [mUA]. Arithmetic mean (± SD) for three repetitions							
	Bovine trypsin		Porcine trypsin		Porcine pepsin		Papain	
	+ larvae	control	+ larvae	control	+ larvae	control	+ larvae	control
1	13.69±0.66	13.0±0.53	40.76±0.73	40.25±0.14	34.66±0.94	34.52±0.56	16.22±0.86	16.00±0.36
10	20.17±0.44	12.93±0.88	26.73±0.68	31.67±0.76	25.30±0.81	32.11±0.76	33.88±0.89	9.20±0.85
15	15.06±0.95	11.50±0.57	25.29±0.89	22.87±0.99	21.90±0.89	28.86±0.92	18.80±0.9	8.23±0.31
20	13.45±0.91	9.43±0.58	23.53±0.50	20.43±0.92	18.40±0.80	25.51±0.75	15.20±0.98	7.50±0.61
25	10.50±0.62	9.05±0.48	22.15±0.34	19.35±0.57	15.30±0.72	20.34±0.68	10.50±0.73	6.00±0.27

larvae and in the controls. In the solutions with the larvae the activity of trypsin decreased faster than in the controls examined after the same time. After 25 h of incubation, the activity of trypsin in the solutions containing larvae was slightly higher than in the controls.

In case of solutions containing porcine trypsin, after 10 h of incubation, a lower activity of that enzyme was observed both in the solutions with the larvae and in the controls. However, the recorded activity of the same enzyme was lower in the solutions containing the larvae than in the controls. During the following hours of incubation, a lower activity was observed in the controls than in the experimental samples. In the specimens examined after 25 h, a slightly higher activity of the enzyme was recorded in the media containing the larvae than in the controls.

The dynamics of the pepsin activity decrease in the media containing *A. simplex* larvae was higher as compared to the controls. After 10, 15, 20, and 25 h of incubation a lower activity of pepsin in samples containing the larvae recorded was lower than that in the controls.

The dynamics in changes of papain activity in the experimental samples and the controls was similar to the dynamics observed in cases of activity changes of bovine trypsin. After 10 h, the activity of papain in the solutions containing the larvae was higher by more than twice than in the samples examined after 1 h of incubation. In the controls a gradually decreasing activity of that enzyme was observed. After longer incubation (15, 20, 25 h), a progressively lower activity of papain was recorded both in the solutions containing the larvae and in the controls. The recorded activity of the enzyme in the samples containing the larvae was higher than that in the controls during the entire time of the experiment.

Concluding, it should be emphasized, that *A. simplex* larvae incubated in solutions of enzymes had the most pronounced influence on decreasing the activity of pepsin. The activity of that enzyme in the samples containing the larvae was significantly lower than in the controls during the entire period of incubation. No similar trends were observed in case of the solutions with other proteases. The activity of those enzymes in the solutions containing the larvae

was higher than in the controls. Only the activity of porcine trypsin after 10 h of incubation was slightly lower in the experimental sample than in the control, however, during the later hours the dynamics of the activity decrease of that enzyme in the controls was higher than in the experimental samples.

DISCUSSION

This study investigated, in the *in vitro* environment, the influence of *Anisakis simplex* larvae upon the activity of proteases through incubation of the larvae in solutions of those enzymes. The results of earlier studies on survivability of *A. simplex* larvae in solutions containing proteases (Dziekońska-Rynko et al. 1997a) indicated that the larvae are resistant to animal proteases such as trypsin and pepsin, while they lack resistance to vegetable protease such as papain. The lower activity of pepsin in the media containing the larvae as compared to the controls was probably caused by binding of that enzyme with an inhibitor contained in the larvae. According to the authors quoted above, *A. simplex* were most resistant to influence of pepsin. In that solution they lived the longest and showed the highest mobility. That suggests that the larvae have an inhibitor protecting them against that enzyme activity.

On the basis of the results obtained, it is not possible, as in the case of pepsin, to clearly define the influence of *A. simplex* larvae on the activity of bovine and porcine trypsin. After 10 h of incubation a higher activity of bovine trypsin was recorded in the solutions containing the larvae as compared to the controls. The higher activity in solutions containing the larvae might have resulted from low compatibility of the inhibitor contained in the larvae to that protease and by probability of measuring the activity of protease excreted by the *A. simplex* larvae to the incubation environment together with the activity of bovine trypsin. Matthews (1982, 1984) and Sakanari and McKerrow (1990) concluded that *A. simplex* larvae incubated *in vitro* produce proteases excreted to the environment. Those are proteases with a similar optimum activity as the bovine trypsin in the solutions. In this study, during consecutive hours of incubation measurements gradually recorded lower and lower activity of trypsin in both experimental and control samples. The dynamics of the activity decrease was higher in the experimental samples than in the controls.

Different trends were recorded in case of solutions containing porcine trypsin. After 10 h of incubation, a lower activity of that enzyme was recorded in the solutions containing *A. simplex* larvae than in the controls. Those results indicate a closer compatibility of the trypsin inhibitor produced by *A. simplex* larvae with the porcine trypsin than the bovine one. A gradually lower activity of that enzyme in both experimental samples and in the controls during the later hours (15, 20, 25) was recorded. It was probably caused by a decreased binding of the enzyme by the inhibitor as a result of its saturation.

In this experiment bovine and porcine trypsin was used. It was not trypsin originating from hosts specific for *A. simplex* larvae, i.e. marine mammals. It could be assumed that their internal inhibitor protects them against the proteases of the specific host and that is why in the specific host the parasite may complete its development. It was confirmed that the parasite does not establish in a non-specific host. The larvae may go through a single moult and as pre-adults are subject to degeneration after ca. 3 months or earlier. Maybe lack of effective protection against proteases of non-specific host making establishment impossible is the cause of incomplete development of that parasite in non-specific hosts. The different influence of *A. simplex* larvae upon the activity of bovine and porcine trypsin may also be explained by high specificity of the inhibitors of proteases limited to specific species. Hawley and Peanasky (1992) compared the properties of trypsin inhibitors obtained from *A. suum* and *A. lumbricoides*. The inhibitor from *A. suum* was binding human trypsin 10 000 weaker than the porcine one, while the inhibitor from *A. lumbricoides* bound porcine trypsin only 3 times weaker than the human one. The inhibitor contained in *A. simplex* larvae is probably closer to porcine trypsin than bovine trypsin and as a consequence in this study, after 10 h of incubation a lower activity of that enzyme was recorded in experimental samples than in the controls.

In the available literature no papers were found on the influence of *A. suum* larvae or larval stages of other parasites upon activity of animal proteases in the *in vitro* environment. Juhasz and Matskasi (1979), while incubating adult *A. suum* in trypsin and α-chymotrypsin solutions, observed a lower activity of those enzymes after 24 and 48 h in solutions containing the nematodes than in the controls. Adult *A. suum*, according to Juhasz (1979), in *in vitro* environment do not excrete proteases into the environment. Pappas and Read (1972 a, b) while incubating the adults of *Hymenolepis diminuta* in a solution of trypsin and α- and β-chymotrypsin, found that it caused irreversible inactivation of those enzymes. The phenomenon was explained by the authors quoted above by binding the enzymes by inhibitors contained in that parasite. On the other hand, they did not confirm production of its own proteases to the incubation environment by the parasite.

In this experiment, a very high increase of papain activity after 10 h was found in the solution containing the larvae of *A. simplex*, as compared to that activity before inserting the larvae and the activity recorded for the controls. An increase in the activity of that enzyme was explained by high mortality of the larvae during the initial hours of incubation, and supplying the additional medium for that protease. Another reason for that high increase in the activity of papain could result from including in the result the additional activity of proteases produced by *A. simplex* larvae during the excretion-secretion (ES) process, similar to what was observed in the case of trypsin activity. The activity of that enzyme was definitely higher than in the case of trypsin, which

could prove production of a higher volume of those products, maybe as a defensive reaction of the *A. simplex* larvae against papain. After 15 h of incubation, the difference between the results for media containing the larvae and the controls was much lower, but it was still very high.

The activity of proteases in solutions containing *A. simplex* larvae could also be influenced by other factors such as the products of their metabolism as well as various toxins secreted as excretion-secretion process. In ES products of numerous nematodes presence of enzymes such as hialuronidase (Hotez et al., 1994), acetylocholinesterase (Opperman and Chang 1992) and protease (Matthews 1982, 1984; Sakanari and McKerrow 1990) was found. The total activity of those enzymes and the enzymes added to the medium, could influence the changes in activity of those later ones during the later hours of incubation.

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