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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

Effect of the *Eubothrium rugosum* (Cestoda) Extract on Intestinal Proteolytic Activity in Various Fish Species

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Abstract—The extract of the fish tapeworm *Eubothrium rugosum* was found to inhibit activity of intestinal proteases in its host, the burbot. A decrease in proteolytic activity of the intestinal mucosa, when using the *E. rugosum* extract as an inhibitor, is comparable with that of a serine protease inhibitor PMSF. This implies that the tapeworm-produced inhibitor inactivates major intestinal serine proteases, trypsin and chymotrypsin. The tapeworm extract was also demonstrated to inhibit proteolytic activity in the intestinal mucosa of the two other fish species, zope and bream. Using the Gini index, it was found that the distribution of percent inhibition levels shows some nonuniformity across the given set of species. Namely, the tapeworm extract exerts a more selective inhibitory effect on intestinal proteases in different fish species as compared to PMSF. This indicates a partial though quite explicit species-specific selectivity of the tapeworm-produced inhibitor toward the host's proteolytic activity which may contribute to the formation of specific host–parasite interrelationships.

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INTRODUCTION

One of the fundamental problems of parasitology concerns the host–parasite interrelationship. To establish close and specific relationships with a host organism, parasite has to be well adapted to its novel environment. Besides, helminthes inhabiting the intestine of vertebrates have to be resistant to host's proteolytic enzymes. One of the major protective mechanisms against these enzymes is the secretion of inhibitors able to effectively inactivate host's proteases [1]. It has long been believed that parasites can use protease inhibitors to survive in a host. There have been identified and characterized quite a lot of protein-based inhibitors [2] that refer to a family of serine protease inhibitors [3]. Due to the epidemiologic significance of varied nematode species, a wide range of these

Fish species analyzed in this study

Fish species (*n**)	Reservoir	Capture time	Body length, cm	Feeding type
burbot *Lota lota* (5)	Rybinsk Reservoir	February 2016	44–48	predator–optional benthophage
zander *Sander lucioperca* (4)	same	August 2016	48–54	predator
zope *Ballerus ballerus* (4)	same	same	29.4–30.1	planktophage
bream *Abramis brama* (3)	same	same	35.5–41.6	benthophage
pike *Esox Lucius* (3)	same	May 2016	48–50	predator
trout *Oncorhynchus mykiss* (4)	fish farm Ladoga Lake	September 2015	28.8–30.0	same
perch *Perca fluviatilis* (4)	same	same	14.5–14.7	same

**n*—the number of fish individuals of the given species studied herein.

parasites has been examined to reveal inhibitors of all protease families (aspartic, serine, cysteine and metalloprotease). Cestodes are far less studied in this regard. By now, they have been established to have only inhibitors of serine proteases, trypsin and chymotrypsin [4]. A series of studies were devoted to the capacity of the cestode *Hymenolepis diminuta* that parasitizes in the rat intestine to inhibit host's proteases [1, 5, 6]. However, the inhibitory capacity can far not always be detected in cestodes and its specific carriers be isolated from these parasites [7, 8].

The aim of this study was to explore the capacity of a widespread cestode *Eubothrium rugosum,* parasitizing in the burbot intestine, to inhibit activity of the host's intestinal alkaline proteases and to find out if the tapeworm-produced protease inhibitor is burbot-specific.

MATERIALS AND METHODS

Animals. The objects of study were cestodes *E. rugosum* from the intestine of the burbot *Lota lota*. The tapeworms were studied in 5 burbots captured during the winter season in the Rybinsk Reservoir. The number of parasites per fish varied from 1 to 10 and their weight from 0.31 to 1.88 g. All manipulations (dissection, tapeworm extraction, varied preparative procedures) were carried out in an ice-cold bath.

Tapeworms were taken out from the dissected intestine and washed three times in 10 mL of Ringer's solution (pH 7.5) for poikilothermic animals to remove host's enzymes.

Preparation of tapeworm and fish intestinal mucosa homogenates. Tapeworms were divided into 5 similar-weight groups and homogenized, while the homogenates were then diluted by Ringer's solution at a weight-to-volume ratio of 1:9.

To assay host's intestinal proteolytic activity and to study the tapeworm inhibitory activity, burbot intestinal mucosa homogenates were prepared. To test the hypothesis about a putative specificity of tapeworm inhibitory activity toward host's proteases, intestinal mucosa homogenates from different fish species were prepared (see Table). To do this, after intestine dissection and chymus removal, the mucosa was scraped out, homogenized and diluted 1:49 by Ringer's solution. All homogenates were prepared using Sartorius AG glass homogenizers (Germany).

Tapeworm and mucosal homogenates were centrifuged at 6500 g and 4°C for 5 min, and the resultant supernatants were used to assay proteolytic and inhibitory activities.

Inhibitory activity assay. Supernatants (extracts) of tapeworm homogenates were used as a source of tapeworm inhibitory activity. To assay inhibitory activity, 50 μL of the tapeworm extract were added to the experimental medium (reaction mixture), containing 500 μL of intestinal mucosa homogenate, and incubated for 15 min at 20–22°C. In a series of tentative experiments, it was established that an increase in the tapeworm extract volume entails no further inhibition, hence the above-mentioned 1:9 ratio was used as optimal for the extract preparation. Simultaneously, the same volume of buffer was added to an appropriate con-

trol sample. After incubation, proteolytic activity was assayed in the samples as described below.

To compare with inhibitory activity levels in *E. rugosum* extracts, the inhibitory effect of 50 μL PMSF (phenylmethylsulfonyl fluoride), a serine protease inhibitor, was determined at a concentration of 100 mM in dimethyl sulfoxide (DMSO) relative to proteolytic activity of the intestinal mucosa from different fish species.

Proteolytic activity assay. Total protease activity in fish intestinal mucosa homogenates (trypsin EC 3.4.21.4; chymotrypsin EC 3.4.21.1; dipeptidases EC 3.4.13.18) was assayed using 0.3% azo-casein in Tris buffer (pH 7.5) as a substrate [9]. The substrate and the enzymatically active preparation were co-incubated for 60 min at 20–22°C. The reaction was stopped by the addition of 1 mL of 0.3 N trichloroacetic acid (TCA), and the resultant precipitate was removed by centrifugation at 6500 g for 5 min. The staining intensity (proportional to enzyme activity) was measured in a supernatant at 440 nm using the Lambda 25 spectrophotometer (PerkinElmer).

Protease activity (UA) was expressed as a difference (ΔAbs) between spectrophotometric readings for substrate-containing and blank (substrate-free) samples (the latter contained an appropriate volume of the buffer instead of the enzyme and inhibitor) calculated per unit mass of the intestinal mucosa per 1 min ($\Delta Abs \times g^{-1} \times min^{-1}$). All biochemical measurements were thrice repeated.

Quantitative evaluation of species-specific selectivity of inhibitors. Selectivity of the inhibitory effect of the tapeworm extract and PMSF on host's and other species' proteases was evaluated using the Gini coefficient [10]. This index is calculated for a multitude of percent inhibition values obtained at one and the same inhibitor concentration. The values were ranked in the order of an increasing inhibitory effect, summated and normalized. Then, the Gini coefficient was calculated by analyzing the correlation between the cumulative fraction of total inhibition and that of the number of the species studied. The index can take values from zero (no selectivity) to one (absolute selectivity). Calculations were carried out in two variants: (1) for a whole set of the seven species studied, and (2) for a narrower range of the five predatory species, i.e. excluding the bream and the zope.

Statistics. The results are presented as means ± SEM. Data treatment was carried out using Microsoft Excel 2010 and STATISTICA 8.0 (StatSoft, Inc., Tulsa, OK). The inhibitory effect was assessed using one-way ANOVA and the Tukey multiple comparison test at $p < 0.05$. Statistical significance of differences in the percent inhibition values between the tapeworm extract and a synthetic inhibitor PMSF was evaluated using the Mann–Whitney U-test for each individual fish species.

RESULTS

It was established that the addition of 50 μL of the *E. rugosum* extract to the reaction mixture decreased proteolytic activity of the burbot intestinal mucosa almost 2 times from 0.587 ± 0.068 to 0.323 ± 0.023 $\Delta Abs \times g^{-1} \times min^{-1}$. PMSF decreased protease activity of the burbot intestinal mucosa to 0.214 ± 0.038 $\Delta Abs \times g^{-1} \times min^{-1}$, and this was not significantly different from the values obtained for the tapeworm extract used as an inhibitor source. However, when calculating the percent inhibition induced by the tapeworm extract vs. PMSF, these values exhibited statistically significant differences: the tapeworm extract inhibited intestinal protease activity by 45.4%, whereas PMSF by 64.3% (Mann–Whitney U-test, $p < 0.05$).

Testing the hypothesis about the specificity of *E. rugosum* inhibitory activity toward host's proteases demonstrated that the tapeworm extract inhibited protease activity not in all fish species (Fig. 1). Specifically, the addition of the tapeworm extract significantly decreased intestinal mucosa protease activity in three out of seven fish species studied herein: burbot (host), zope and bream ($p < 0.05$). At the same time, PMSF significantly decreased intestinal mucosa protease activity in all species except the zander. It should be noted that for the burbot, zope and bream intestinal mucosa no significant differences in the rate of proteolytic activity decline were found when using the tapeworm extract and PMSF, whereas in the other species PMSF decreased protease activity to a much greater extent than the tapeworm extract (Fig. 1).

Calculation of the percent inhibition of protease activity in the intestinal mucosa of different

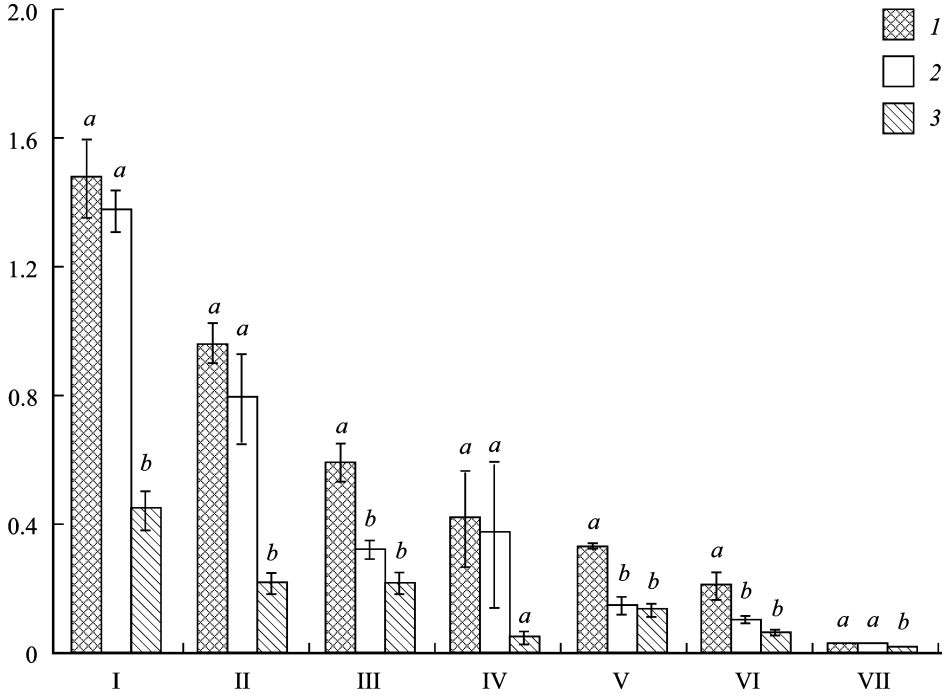

Fig. 1. Proteolytic activity of the intestinal mucosa in various fish species (*1*) as affected by the tapeworm *E. rugosum* extract (*2*) and PMSF (*3*). *Ordinate*: proteolytic activity, $\Delta Abs \times g^{-1} \times min^{-1}$; *abscissa*: I—trout, II—perch, III—burbot, IV—zander, V—zope, VI—bream, VII—pike. Mean values denoted by different lettering (*a*, *b*) differ significantly ($p < 0.05$) for each fish species.

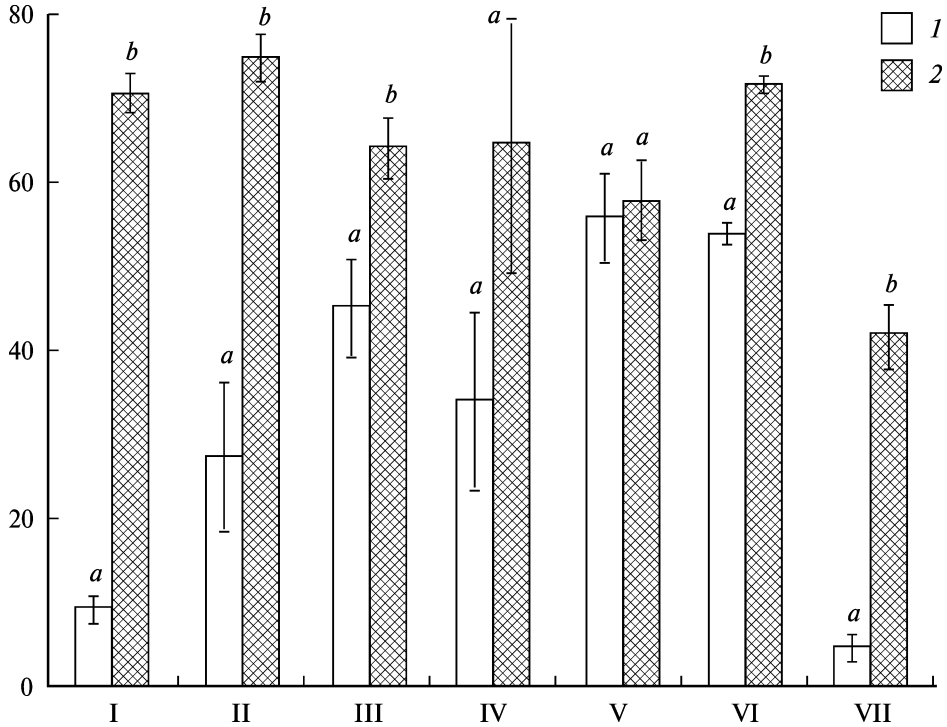

Fig. 2. Percent inhibition (%) of proteolytic activity of the intestinal mucosa in various fish species induced by the tapeworm *E. rugosum* extract (*1*) and PMSF (*2*). *Ordinate*: %; other designations as in Fig. 1.

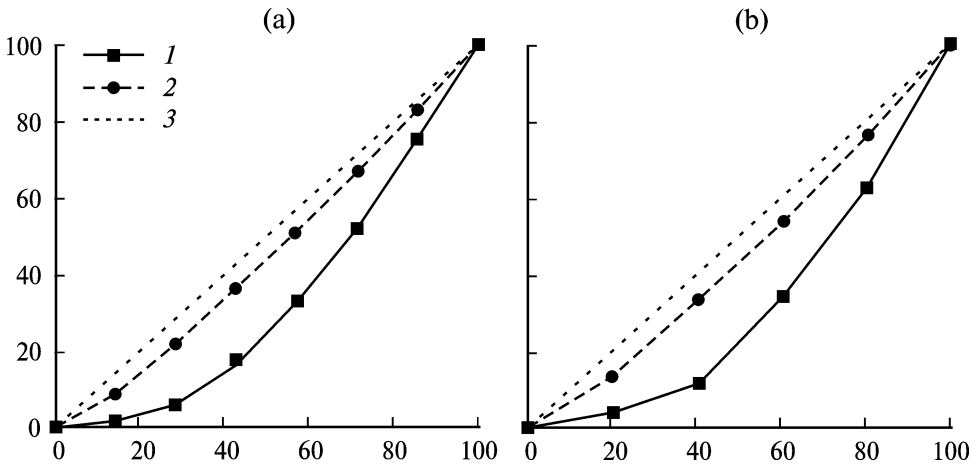

Fig. 3. Selectivity analysis of the inhibitory effects induced by the *Eubothrium rugosum* extract (*1*) and PMSF (*2*) using a whole data set of seven fish species (a) and a subset of five predatory species (b). *Ordinate*: cumulative fraction of total inhibition (%); *abscissa*: cumulative fraction of the number of species studied (%).

fish species revealed that only in the zope and zander this activity is equally inhibited both by the *E. rugosum* extract and PMSF. In all other fish species studied, PMSF inhibited proteolytic activity of their intestinal mucosa significantly stronger than the *E. rugosum* extract ($p < 0.05$) (Fig. 2). Thus, the PMSF-induced percent inhibition of proteolytic activity in different species fluctuates from 41.8 (pike) to 75.2% (perch), while the *E. rugosum* extract inhibits the same activity within the range from 4.6 (pike) to 56.2% (zope). The lowest *E. rugosum* extract-induced percent inhibition was obtained in the trout and pike, while the highest in the bream and zope (Fig. 2).

One-way ANOVA showed that a fish species as a factor had a significant effect on the percent inhibition value as a dependent variable both in the case of the tapeworm extract ($F = 103.90$, $p < 0.0001$) and PMSF, albeit to a much lesser extent ($F = 9.55$, $p < 0.001$). A series of multiple pairwise comparisons demonstrated that the efficacy of the tapeworm-produced inhibitor's effect on host's intestinal proteolytic enzymes exhibited no significant differences from the appropriate values of the analogous enzymes in the bream, zope and zander ($p < 0.05$). At the same time, mucosal proteases in the burbot proved to be more sensitive to the *E. rugosum* extract compared to intestinal proteases in the other predators (trout, pike and perch) ($p < 0.05$).

As demonstrated by the Gini coefficient, species-specific selectivity of the inhibitory effect of the tapeworm extract is more explicit than in the case of PMSF solution (Fig. 3). For example, under the effect of the tapeworm-produced inhibitor, cumulative curves are much farther from the graph's diagonal line, indicative of a more nonuniform data distribution for different fish species than under the effect of PMSF. Accordingly, the Gini coefficient reaches 32.4% for the tapeworm extract but does not exceed 8.6% for PMSF (when using a whole data set for the seven fish species). Similar results were obtained in the analysis of analogous data separately for predatory fish species (35.2 and 9.3%, respectively).

It should also be noted that under the effect of the tapeworm extract the mean value of the percent inhibition for the host species (45.4%) was notably higher as compared to the mean level for all the other fish species (31.0%). When using PMSF, these levels were virtually identical (64.3 and 64.4%, respectively). By the Mann–Whitney U-test, differences between the samples compared were statistically significant ($p < 0.05$) in the first case (under the effect of the tapeworm extract) but insignificant in the second one (under the effect of PMSF). These data indicate a certain degree of specificity of the tapeworm-produced inhibitor toward host's proteases and also its more pronounced selectivity compared to the PMSF effect.

DISCUSSION

The use of PMSF, a serine protease inhibitor,

enabled establishing the prevalence of activities of these proteases (represented by trypsin, chymotrypsin and elastase [11]) in all fish species studied herein. In all likelihood, differences in activity levels of the serine proteases in these species are due to different habitats, capture time and feeding types [12]. The available literature data also indicate a higher variability of the fraction of serine proteases in total proteolytic activity in different fish species. The prevalence of serine protease activities in total proteolytic activity was reported for fish with dissimilar feeding types: herbivorous, omnivorous and predatory [13]. Specifically, in the predatory Asian arowana *Scleropages formosus* a 77.1% PMSF-induced inhibition of serine proteases was established [11]. In the pyloric caeca of some salmonids, 44.09–70.27% of serine proteases are inhibited [14]. In the intestine of the three carp species (*Catla catla*, *Labeo rohita* and *Hypophthalmichthys molitrix*), PMSF inhibits 59.1–79.6% [15], while in the common snook *Centropomus undecimalis* up to 60% [16] and in the thicklip grey mullet *Chelon labrosus* up to 87% of protease activity [17]. By far lower protease activity inhibition induced by this agent was reported for the predatory gilt-head bream *Sparus aurata* and deepwater redfish *Sebastes mentella* (43.7 and 46.3%, respectively), and quite insignificant (11.5%) for the turbot *Scophthalmus maximus* [18]. A modest PMSF-induced inhibition (26%) was established in the intestine of the omnivorous freshwater brycon *Brycon orbignyanus* [19]. Different levels of serine protease inhibition in fish sharing one the same feeding type may be due to species-specific and physiological features of these fish [12] as well as putative species-specific structural peculiarities of serine proteases and dissimilar methods used by different authors for detecting activity of these enzymes.

A decrease in proteolytic activity of the burbot intestinal mucosa induced by the *E. rugosa* extract indicates the presence of a protease inhibitor in this extract. Protease inhibitors are well known to be secreted to the environment in negligible amounts [20]. Since the volume of the reaction mixture, when determining proteolytic activity, is 1.5 mL and for determining the inhibition level this volume is added with 50 μL of the 10-fold diluted tapeworm extract (i.e. the final dilution relative to the initial tapeworm weight is 300 times), we are free to suggest that *E. rugosum* produces small amounts of a highly efficient protease inhibitor. Moreover, since a decrease in intestinal mucosa proteolytic activity induced by the *E. rugosum* extract and PMSF is more or less the same, we can assume that the inhibitor produced by the tapeworm inactivates the major intestinal serine proteases, trypsin and chymotrypsin. Despite the percent inhibition of protease activity induced by the *E. rugosum* extract is far lower than that induced by PMSF, in the intestine the tapeworm is resistant to proteolytic enzymes. Evidently, the produced amount of the inhibitor is quite enough to ensure the parasite with such resistibility. As some authors believe, the interaction of host's proteases with inhibitors produced by cestodes occurs directly on the adsorptive surface of the latter, leading to an irreversible inactivation of the enzyme [5, 6].

Our data on the inhibitory effect of the cestodal extract on host's protease activity are consistent with the literature data. For example, inhibition of proteolytic activity by extracts of the *Ligula intestinalis* plerocercoids from the bream body cavity, as well by extracts of adult tapeworms from the duck intestine was established previously [21]. Subsequently, it was found that extracts prepared from larval and adult *Bothriocephalus acheilognathi* inhibited *in vitro* trypsin and chymotrypsin activities in the carp intestine [22]. At the same time, it is well known that most of the characterized protein inhibitors in helminthes refer to serine protease inhibitors [3].

The original working hypothesis suggested that, since *E. rugosum* parasitizes only in the burbot intestine, the inhibitor that this tapeworm produces may be specific to host's proteases. This suggestion was also based on the views of some authors that the parasite-to-host specificity depends on parasite's protease inhibitors [23]. However, the outcomes of our studies turned out to be not that unequivocal. On the one hand, the tapeworm-produced extract significantly decreased activity of mucosal proteases not only in the burbot but also in the two other fish species (zope and bream). This shows that specificity of the given inhibitor toward host's and other species' proteases is far from being absolute. At the same time, there is evidence of a partial selectivity of this inhibitor. By the one-way ANOVA, the in-

hibitory capacity of the tapeworm extract was significantly species-dependent, indicating a selective effect of this inhibitor. Statistically, the percent inhibition values for burbot intestinal proteases were not lower than for the analogous enzymes in the other fish but at the same time significantly higher than for the most of such enzymes in the predatory species. The Gini coefficient also argues in favor of a partial selectivity of the *E. rugosum*-produced inhibitor, indicating a nonuniform distribution of percent inhibition levels across a set of the species studied herein. The Gini coefficient values were not high (32.4–35.2% depending on a calculation method). At the same time, they were quite comparable to those obtained in testing some other inhibitors. For example, Gini coefficients calculated for 40 kinase inhibitors, each of which affected 85 different kinases, varied within a wide range from 9.3% for a nonselective inhibitor to 90.5% for a highly selective and 56.8% for a moderately selective ones [10]. It is noteworthy that the tapeworm extract was distinguished by its explicit action selectivity compared to PMSF. In the case of a whole data set (including the seven fish species studied), the Gini coefficient characterizes an overall nonuniformity of percent inhibition (i.e. action selectivity) rather than a specificity as such, since formally the host fish is not the topmost species on the cumulative curve. At the same time, in the sample of predatory fish species the burbot demonstrates top values of inhibition, and in this scenario the Gini coefficient equally reflects both a selectivity of the inhibitor and its specificity toward proteases of a certain host. An additional argument in favor of the tapeworm extract's action specificity is the fact that the mean value of percent inhibition for burbot mucosal proteases is notably above the appropriate mean level calculated for analogous enzymes in all the other fish species.

There is another evidence for the action specificity of tapeworm-produced inhibitors toward varied proteases. For example, it was hypothesized that the interaction between a rat intestinal parasite *H. diminuta* and trypsin is specific because other proteolytic enzymes (subtilysine, pepsin and papain) are not inactivated by this cestode [6]. Selective specificity of protease inhibition was demonstrated for sexually mature cestodes *Taenia pisiformis* from the canine intestine [24]. In extracts of these worms, there was found inhibitory activity toward bovine, canine and rabbit trypsin and chymotrypsin, although the same inhibitor did not affect hydrolytic activity of subtilisin, elastase, collagenase, pepsin, renin and papain. It was also shown that serpin of the intracellular type produced by the cestode *Echinococcus multilocularis* inhibits trypsin and trypsin-like plasmin as well as porcine pancreatic and human neutrophil elastase, probably promoting inactivation of proteolytic enzymes in the host's intestine [25].

Thus, we have established that the *E. rugosum* extract inhibits protease activity in the host's (burbot) intestine. A decrease in proteolytic activity of the intestinal mucosa when using the *E. rugosum* extract as an inhibitor is comparable with that when using PMSF, a serine protease inhibitor, whereas the percent inhibition in the former case is somewhat smaller than in the latter. Inhibition of proteolytic activity of the intestinal mucosa in different fish species by the tapeworm extract indicates a moderate but quite explicit specificity of the tapeworm-produced inhibitor toward the host's proteolytic activity.

The significance of such specificity for host–parasite interrealtionships is yet to be elucidated in further experiments on several species of Cestoda using isolated and purified inhibitors and on a wider range of fish species, both harboring the parasites of interest and being used in comparative studies.

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COMPLIANCE WITH ETHICAL STANDARDS

All applicable international, national and institutional principles of handling and using experimental animals for scientific purposes were observed. This study did not involve human subjects as research objects.

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