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Qusay Z.M. Bahlool*, Alf Skovgaard, Per W. Kania, Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

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ABSTRACT

Excretory/secretory (ES) products are molecules produced by parasitic nematodes, including larval *Anisakis simplex*, a parasite occurring in numerous marine fish hosts. The effects of these substances on host physiology have not been fully described. The present work elucidates the influence of ES substances on the fish immune system by measuring immune gene expression in spleen and liver of rainbow trout (*Oncorhynchus mykiss*) injected intraperitoneally with ES products isolated from *A. simplex* third stage larvae. The overall gene expression profile of exposed fish showed a generalized down-regulation of the immune genes tested, suggesting a role of ES proteins in immunomodulation. We also tested the enzymatic activity of the ES proteins and found that lipase, esterase/lipase, valine and cysteine arylamidases, naphthol-AS-BI-phosphohydrolase and α-galactosidase activities were present in the ES solution. This type of hydrolytic enzyme activity may play a role in nematode penetration of host tissue. In addition, based on the notion that *A. simplex* ES products may have an immune-depressive effect (by minimizing immune gene expression) it could also be suggested that worm enzymes directly target host immune molecules which would add to a decreased host immune response and increased worm survival.

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1. Introduction

Third stage larvae of the parasitic nematode *Anisakis simplex* is commonly found in marine fishes including salmonids acting as paratenic (transport) hosts. Marine mammals serve as final hosts and obtain infection by ingesting larva bearing fish [1–3]. Internal pathological reactions in infected fish depend on the number of worms, their tissue location and the host species [4]. Both mechanical and chemical factors may contribute to the pathogenic effect of the parasites but we have no exact knowledge on the interactions between these molecules and fish physiology. It is known that excretory/secretory (ES) proteins produced by *A. simplex* comprise several components of major clinical importance [5]. Kennedy et al. [6] found two proteinases in *A. simplex* secretions and a later study on secreted neutral proteases from *A. simplex* revealed both a metallo-aminopeptidase and a trypsin-like serine protease [7]. ES proteins have also been shown to have anti-coagulatory properties with regard to human blood [8]. The allergenic nature of ES products has urged investigations on effects in mammals showing that Ani s1 is one of the major secretory allergens. It has been described in detail [9,10] and Arrieta et al. [11] suggested that this 21 kD protein has a troponin-like structure. Another allergen is Ani s5, which was found to elicit an antibody response (IgE) in humans [12]. The product induces inflammation and intradermal injection of ES proteins into guinea pigs elicits a profound accumulation of eosinophils 1 h post-injection [13]. However, our knowledge on the effects of *A. simplex* ES proteins on fish hosts immune response is still poor due to the fact that merely few nematode-fish models are established in comparison to mammalian laboratory animals [14]. In this study, we have investigated the early effect of ES molecules on the immune system of fish by measuring the expression of different immune-related genes in rainbow trout following intraperitoneal injection with these substances produced *in vitro* by *A. simplex*.

2. Materials and methods

All chemicals and reagents were purchased from Sigma—Aldrich, Denmark, unless otherwise stated.

* Corresponding author. Stigbøjlen 7, DK-1870 Frederiksberg C, Denmark. Tel.: +45 3533 1437.
  E-mail addresses: qusay@life.ku.dk, qusay.bahlool@gmail.com (Q.Z.M. Bahlool).

2.1. Parasites

Third stage larvae were recovered from the body cavity of freshly caught herring (*Clupea harengus*) purchased from a local fishmonger in Copenhagen. Sub-samples of these recovered *Anisakis* larvae used in our experiment were identified as *A. simplex* using molecular tools as described by Bahlool et al. [4].

2.2. ES products

ES proteins were extracted as described by Baeza et al. [15] with minor modifications. To ensure the removal of nematode surface proteins and any remaining fish proteins, nematodes were washed several times in a phosphate buffered saline (PBS) solution. The PBS solution contained 200 μg/ml of ampicillin and 400 μg/ml of kanamycin to prevent bacterial contamination. In order to describe any temperature dependent production of ES-antigens nematodes were incubated at various temperatures (4, 15, 22 and 37 °C) in sterile Nunclon™ cell culture (12-well) plates (Nunc A/S, Denmark) containing PBS (2.5 ml/well). The four temperature levels chosen were based on the fact that rainbow trout exists at 0–27 °C and that the nematode also occurs in warm water species. The influence of parasite density on ES protein production was also investigated by incubating nematodes at the above temperatures at four different nematode densities (1, 3, 5 and 7) per well. After 5 days of incubation, nematodes were removed and eight random samples of worm substrate were inoculated on blood agar plates at 37 °C for 48 h to ensure the absence of any bacterial growth. The liquid was filtered using 0.22 μm filters (Sartorius Minisart®, VWR, Denmark) and frozen at −40 °C until further analysis. Protein content of the filtrate was measured by NanoDrop 2000© (Saveen & Werner ApS, Denmark) using the Bradford-Protein preconfigured module. ES-containing filtrates were then diluted in PBS to reach 100 μg/ml.

2.3. Analysis of enzyme activity

The activity profile with regard to 19 enzymes was determined using APIZYM (Biomerieux Aps, Denmark) according to the manufacturer's instructions by adding 65 μL of ES filtrate to each well and subsequently incubating for 8 h at 37 °C. Following incubation, reagents (ZYM A and ZYM B) were added to each well, and the reaction was allowed to proceed for 5 min. The resulting colour development was evaluated using the manufacturer's colour chart. Enzyme activity was tested only for ES proteins recovered from nematodes incubated at 37 °C.

2.4. Fish

Pathogen-free rainbow trout (*Oncorhynchus mykiss*) were hatched (eggs originating from Fousing trout farm, Jutland, Denmark) and reared for four months at the Bornholm Salmon Hatchery (Nexø, Denmark). They were then brought to the University of Copenhagen fish keeping facility at Frederiksberg and acclimatized three weeks prior to experimentation. A total of 80 fish weighing 4.7 ± 0.2 g were used in our experiment. Fish were kept in 85 l glass aquaria with municipal freshwater (Frederiksberg County). Each aquarium contained internal biofilters (Eheim, Germany) and a source of aeration securing high oxygen saturation (100%) while the water was changed daily. Water temperature was kept between 14 and 15 °C during the whole period. Light/dark cycle was set to 12/12 h. Water quality parameters were tested (Merckoquant®, Merck, Germany) and kept under safe limits: Ammonia (NH_4^+/NH_3) less than 1 ppm, nitrate (NO_3^-) less than 5 ppm and nitrite (NO_2^-) less than 0.3 ppm.

2.5. Experimental design

Fish were divided into four aquaria (two control groups and two experimental groups). Fish were anaesthetized using immersion into 40 mg/l tricaine methane-sulfonate (MS 222). Each fish to be exposed to ES was intraperitoneally injected with 0.5 ml of the ES protein solution, whereas the same volume of PBS was injected into each control fish. Anaesthetized fish were then transferred to a small observation aquarium until they recovered and subsequently placed in the 85L fish tanks. Sampling was performed at 0, 1, 24 and 48 h after experimental injection and 2×5 fish (for both treated and control groups) were investigated at each time point. Spleen and liver (0.5 cm^3) were sampled from each fish and preserved in RNAlater®. The samples were placed at 4 °C for 24 h and then stored at −20 °C until further processing.

2.6. RNA isolation

RNA was extracted from 40 mg of liver and 20 mg of spleen using the GenElute™ Mammalian Total RNA Kit according to the manufacturer's instructions. Homogenization of tissue samples was performed on ice by sonication in the provided lysis buffer (Sonicator Ultrasonic Liquid Processor Model XL 2020, Bie & Berntsen, Denmark). Genomic DNA was removed by DNAse treatment using Sigma AMPD1. Concentration of RNA was measured on a NanoDrop 2000 at 260 nm, and the purity was assessed by the ratio of A260/A280. The integrity of the RNA and efficiency of DNase treatment was assessed by 2% agarose gel electrophoresis visualized by ethidium bromide staining.

2.7. Synthesis of cDNA

cDNA was synthesized using Taq Man® Reverse Transcription reaction with random hexamers according to the manufacturer's instruction (Applied Biosystems, Denmark). In all cases, 800 ng RNA served as template. Reactions of 10 μl were used for most samples, but 20 μl reactions were used when samples contained an RNA concentration below 80 ng/ml. Negative controls containing cDNA template and all the RT-reagents except the reverse transcriptase (RT minus) was used. The cDNA was diluted in DNase/RNase-free water into a total volume of 200 μl.

2.8. Real-time quantitative PCR (qPCR)

A total of 2.5 μl of the diluted cDNA was used as template in a 12.5 μl qPCR reaction using Brilliant II® qPCR master mix (AH-Diagnostic A/S, Denmark) in a Stratagene Mx3005P™ real time PCR system (AH Diagnostics, Denmark). Cycle conditions for all reactions were one cycle at 95 °C for 15 min, and 45 cycles consisting of a denaturation step at 95 °C for 30 s followed by a combined annealing and elongation step at 60 °C for 30 s with endpoint measurement. Negative controls included RT minus samples and no cDNA samples served as negative controls in each assay. Primers and probes are listed in Table 1. Elongation factor (EF 1-α) was used as reference gene based on previous demonstrations of its stable and superior expression in rainbow trout [16,17]. Immune genes tested are shown in Table 1. The efficiencies of all qPCR assays were within $100\% \pm 5\%$. All the qPCR assays were tested with SYBR green and melting curve analysis in order to ensure that only one product was present and that primer/dimer formation were negligible.

2.9. Data analysis

Statistical comparison of the concentration of ES protein contents from different incubation wells was performed using

Table 1
Primer and probe sequences used for gene expression analysis by quantitative real time PCR. Amp* denotes the length of the amplicons in basepairs.

Genes	Forward primer	Reverse primer	Probe	Amp* (bp)	Accession no.
ELF-1α	accctcctcttggtcgtttc	tgatgacaccaacagcaaca	gctgtgcgtgacatgaggca	63	AF498320
IL-1β	acattgccaacctcatcatcg	ttgagcaggtccttgtccttg	catggagaggttaaagggtggc	91	AJ223954
IL-4/13a	atccttctcctctctgttgc	gagtgtgtgtgtattgtcctg	cgcaccggcagcatagaagt	139	NM_001246341
IL-6	actcccctctgtcacacacc	ggcagacaggtcctccacta	ccactgtgctgatagggctgg	91	DQ866150
IL-8	agaatgtcagccagccttgt	tctcagactcatcccctcagt	ttgtgctcctggccctcctga	69	AJ279069
IL-10	cgactttaaatctcccatcgac	gcattggacgatctctttcttc	catcggaaacatcttccacgagct	70	AB118099
IL-17C1	ctggcggtacaggata	gagttatatccataatcttcgtattcggc	cgtgatgtccgtgccctttgacgatg	138	FM955453
IL-17C2	ctggcggtacagcatcgata	cagagttatatgcatgatgttgggc	cgtggtgtccaggccctttaatgatg	134	FM955454
IL-22	atgaccaccaccacagcatt	attcctttcccctcctccat	ctttccgcaagaagttgtccgag	64	AM748537
SAA	gggagatgattcagggttcca	ttacgtcccagtggttagc	tcgaggacacgaggactcagca	79	AM422446
Precereb-ellin	tggtgttgctttgctgttgt	gccactttgtttgctctc	atggttgagactcagacggagagtg	116	AF192969
Hepcidin	gaggaggttggaagcattga	tgacgcttgaacctgaaatg	agtccagttggggaacatcaacag	95	AF281354
TGF-β	tctgaatgagtggctgcaag	ggtttcccacaatcacaagg	ctggagaggagcagggattccaat	75	X99303
TNF-α	ggggacaaactgtggactga	gaagttcttgccctgctctg	gaccaatcgactgaccgacgtgga	75	AJ277604
C3	atttggcctgtccaaaacaca	agcttcagatcaaggaagaagttc	tggaatctgtgtgtctgaacccc	85	AF271080
MHCII	tgccatgctgatgtgcag	gtccctcagccaggtcact	cgcctatgacttctaccccaaacaaat	67	AF115533
CD4	cattagcctgggtggtcaat	cccttttctttgacagggaga	cagaagagagagctggatgtctccg	89	AY973028
CD8α	acaccaatgaccacaaccatagag	gggtccaccttttcccacttt	accagctctacaactgccaagtcgtgc	74	AF178054
IgM	cttggcttgttgacgatgag	ggctagtggtgttgaattgg	tggagagaacgagcagttcagca	72	S63348
IgT	agcaccagggtgaaacca	gcggtgggttcagagtca	agcaagacgacctccaaaacagaac	73	AY870265
Foxp3α	tcctgccccagtactcatccc	gctcctctggctctttagtgg	cttggcagcagatggagtgccacg	80	FM883710
Foxp3β	ctacaggcacagcctgcctgtcactagg	gctcctctggctctttagtgg	ccagaaccgagtggagtgtcacg	75	FM883711

Kruskal−Wallis one way analysis of variance of ranks among groups. Duplicate groups in all experiments were tested by Student's *t*-test and combined as they were not significantly different (probability level $p > 0.30$). A pair-wise multiple comparison procedure (Holm-Sidak method) was used to assess statistically significant difference in between groups. The qPCR expression data were analysed according to the $2^{-\Delta\Delta Ct}$ method [18]. Change of threshold cycle number (ΔCt) was calculated as the difference between Ct values of the target gene and the reference gene for each individual sample. The ΔΔCt values of the ES-injected fish were calculated as the differences between the ΔCt values of the treated groups and the mean ΔCt value of the control groups at each time-point. The expression data were presented as the fold increase/decrease of the ES-injected fish ($n = 10$) compared to control PBS-injected fish ($n = 10$) at each sample point. In order to ensure that the experimental setup was subjected to no changes during the time course of the experiment, the control groups at the different sampling time points were tested by *t*-test against the 0 time point group. The data are presented as the mean value of the fold change of the 10 fish at each sampling point. A probability level of 0.05 was chosen. The minimum acceptable fold change was set at 2-fold in order to limit misinterpretations due to biological variation. Regulation of a gene was only considered significant when both these parameters were satisfied.

3. Results

3.1. Excretion/secretion (ES) protein measurement

The concentration of ES proteins per well produced by *A. simplex* increased with the number of nematodes incubated, except at 4 °C where the concentration was stable regardless of the number of nematodes incubated (Fig. 1). Thus, the highest ES concentrations were found in wells containing 7 nematodes at all higher incubation temperatures, while the lowest concentrations were found in wells containing only one nematode. Nematodes (5 or 7 per well) incubated at 37 °C usually released higher amounts of ES proteins when compared with the release from a corresponding number of nematodes incubated at lower temperatures.

3.2. Enzyme activity

High enzyme activity of the ES protein solution was recorded with regard to esterase/lipase, naphthol-AS-BI-phosphohydrolase and α-galactosidase. Both lipase and valine arylamidase showed medium activity. Low activity was shown in cystine arylamidase, β-glucuronidase and *N*-acetyl-β-glucosaminidase. No activity was recorded for the other enzymes as well as the control well (Table 2).

3.3. Gene expression

The expressions of 22 genes were investigated in spleen and liver of both ES-injected fish and control fish. Table 3 shows the relative difference of gene regulation (in folds up- or down-regulation) for the infected trout compared to control trout injected with only PBS.

3.3.1. IL-1β and TNF-α
We measured significant down-regulations of IL-1β and TNF-α genes (4.4 and 5.6 folds, respectively) in the spleens of ES-injected rainbow trout compared to PBS injected controls at 48 h post
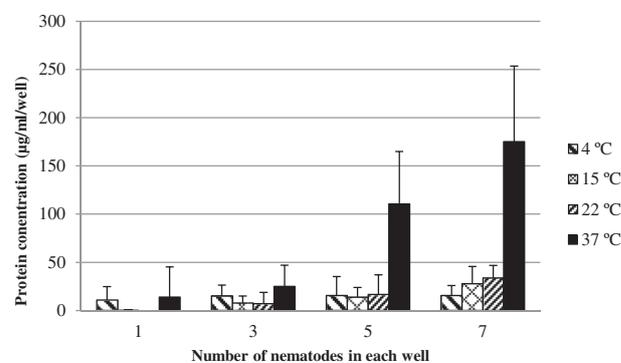

Fig. 1. Mean concentration of ES proteins produced by *Anisakis simplex* incubated in 2.5 ml PBS and measured after 5 days of incubation at 4 different temperatures (4, 15, 22 and 37 °C). *X* axis shows the number of nematodes in each well, while *Y* axis shows ES proteins concentration in μg/ml. Each point represents the average of 5 independent replicates.

Table 2
Enzyme activity of excretory/secretory (ES) proteins extracted from *Anisakis simplex*. Phosphate buffer saline (PBS) used as negative control. Enzyme reactions are ranked on a scale from 0 (no reaction) to 5 (strong reaction).

Enzymes	Reaction	
	ES proteins	PBS
Alkaline phosphatase	0	0
Esterase (C4)	0	0
Esterase lipase (C8)	5	0
Lipase (C14)	3	0
Leucine arylamidase	0	0
Valine arylamidase	3	0
Cysteine arylamidase	2	0
Trypsin	0	0
α-Chymotrypsin	0	0
Acid phosphatase	0	0
Naphthol-AS-BI-phosphohydrolase	5	0
α-Galactosidase	4	0
β-Galactosidase	0	0
β-Glucuronidase	2	0
α-Glucosidase	0	0
β-Glucosidase	0	0
N-acetyl-β-glucosaminidase	2	0
α-Mannosidase	0	0
α-Fucosidase	0	0

injection. The liver showed a significant up-regulation with regard to these two genes at the same time point (2.5 and 5.7 folds respectively) (Table 3).

3.3.2. IL-10 and TGF-β

We observed a general down-regulation of the IL-10 gene at all-time points in the spleens of ES-injected fish but this depression was only significant at 48 h post-injection (3.9 folds). Expression of TGF-β was not affected significantly.

3.3.3. IL-8 and IL-22

The IL-8 gene expression was depressed in spleens 48 h after injection of ES-products. No expression of the IL-22 gene was detected (neither in ES injected nor in control rainbow trout).

3.3.4. Complement factor (C3)

No significant difference in the expression of the C3 gene between ES-exposed and control rainbow trout was detected in any organ.

3.3.5. Acute phase proteins: SAA, hepcidin and precerebellin

No significant changes were detected with regard to genes encoding acute phase proteins. Hepcidin expression could not be detected in most samples (both ES-injected and control fish).

3.3.6. Immunoglobulins (IgM and IgT)

Both IgM and IgT transcripts were measured in spleen and liver. The expression of IgM was significantly down-regulated in the liver of ES-injected rainbow trout at 24 h post injection (5.9 folds). Expression of IgT was found significantly up-regulated in the liver at 1 h and 48 h post injection compared to control group (3.5 and 15.4 folds, respectively). However, it should be noted that IgT expression in the liver was extremely low which will make even slight changes significant.

3.3.7. Cell markers (CD4 and CD8)

The CD4 gene expression in the investigated organs showed a general down-regulation in both organs at almost all time points. Significant down-regulation was found in liver samples taken at 24 h post injection (8.3 folds). The CD8 gene expression did not reveal any significant change in spleen and liver at any time point.

3.3.8. Regulatory transcription factor (IL-17)

No expression of IL-17 in any sample was detected.

3.3.9. Foxp3 (α and β)

Expression of both Foxp3 (α and β) genes was not affected except in liver at 48 h post-injection where a down-regulation occurred.

3.3.10. MHCII

Expression of the MHCII gene was significantly decreased in spleen of ES-exposed fish compared to control fish at 48 h post

Table 3
Expression of immune relevant genes in rainbow trout larvae (avg. wt. 4.7 g) after injection with ES proteins of *Anisakis simplex* at three different time points (1, 24 and 48 h). Data are presented as mean expression as fold change with standard error (fold change ± SE). Differences are considered significant at $p < 0.05$[*]. $p < 0.01$[**]. $p < 0.001$[***]. NA: denotes the inability to detect the expression of the genes at the time points referred to in the table.

	Spleen			Liver			Average Ct value of control	
	1 h	24 h	48 h	1 h	24 h	48 h	Spleen	Liver
# of controls	9	6	9	9	7	7		
# of infected	9	9	5	8	8	7		
C3	−1.9 ± 2.9	1.4 ± 9.7	1.4 ± 2.1	1 ± 11.9	−1.5 ± 1.5	2.8 ± 29.8	34.3 ± 2.5	21 ± 2.9
IL1-β	1.5 ± 4.7	1.4 ± 2.7	−4.4 ± 0.5*	−1.5 ± 0.8	−2.5 ± 0.3	2.5 ± 7.8	33.3 ± 2.3	36.7 ± 1
IL-4	−1.8 ± 1.2	1.5 ± 4.1	1.6 ± 1.8	−1.5 ± 1.0	1.4 ± 1.9	1.6 ± 2.8	33 ± 1.8	34.3 ± 2
IL-6	−1.5 ± 6.7	1.2 ± 6.4	−1.3 ± 1.7	NA	NA	NA	35.8 ± 2.5	NA
IL-8	−2.0 ± 1.2	1.4 ± 2.2	−2.8 ± 0.2*	−1.9 ± 0.6	−1.5 ± 0.4	−1.5 ± 4.2	29.2 ± 1.2	31.7 ± 2.4
IL-10	−2.2 ± 0.6	−2.1 ± 1.8	−3.9 ± 0.5*	1.2 ± 0.1	2.1 ± 25	NA	33.9 ± 1.2	36 ± 0.9
IL-22	NA	−1.5 ± 1.2	NA	NA	NA	NA	38 ± 1.8	NA
CD4	−1.2 ± 3.7	−1.1 ± 2.4	−3.0 ± 0.6	−2.2 ± 1.9	−8.3 ± 0.1*	3.4 ± 6.0	31.1 ± 3.6	37 ± 2.7
CD8	1.6 ± 5.5	−1.5 ± 1.7	−1.1 ± 1.2	2 ± 4.1	−1.7 ± 1.7	2.4 ± 9.5	29.2 ± 2.3	36.2 ± 1.2
TNF-α	−1.8 ± 4.8	1.3 ± 1.3	−3.1 ± 0.5*	−1.8 ± 1.8	−2.4 ± 12.8	5.7 ± 26.2*	35.1 ± 2.5	38 ± 1.3
TGF-β	−1.4 ± 5.1	1.4 ± 2.5	−2.4 ± 0.6*	−1.6 ± 0.7*	−1.1 ± 1.9	2.0 ± 5.4	26.6 ± 1.4	32.6 ± 1.6
MHCII	−2.1 ± 0.5**	1.2 ± 1.7	−3.3 ± 0.7*	1.0 ± 2	−2.8 ± 0.2***	1.6 ± 4.4	23.3 ± 2.9	27.9 ± 1.9
SAA	−2.1 ± 2.9	1.7 ± 1.5	−1.5 ± 0.9	−1 ± 1.4	−1.1 ± 1	−1.1 ± 1.8	25.5 ± 2	28 ± 4.2
Precerebilin	−2.2 ± 2	1.3 ± 4	−1.2 ± 1.7	−1.2 ± 2	1.1 ± 1.9	1.6 ± 6.4	30.9 ± 2.5	21.7 ± 2.8
Foxp3 α	−4.7 ± 0.5	−1.3 ± 3.8	−1.2 ± 4.4	−2.3 ± 0.8	−2.6 ± 0.5	−1.2 ± 2.5	32 ± 1.6	31 ± 1.3
Foxp3 β	−4.1 ± 1.5	−1.3 ± 3.2	−1.2 ± 4.8	−2.8 ± 0.6	−2.0 ± 14.5	1.2 ± 5.9	31.6 ± 1.6	31 ± 1.2
IgM	−1.9 ± 0.5**	−1.0 ± 2.8	−1.1 ± 0.8	−1.4 ± 0.7	−5.9 ± 0.3**	2.3 ± 1.8**	19.4 ± 1.7	28.2 ± 2.2
IgT	−2.8 ± 4.2	−1.3 ± 3.4	−23.9 ± 2.5	3.5 ± 4.7*	−2.7 ± 2.5	15.4 ± 49.4*	30.6 ± 3.1	36.6 ± 2.2

injection (3.3 folds). The expression was also significantly decreased in liver samples at 24 h after injection (2.8 folds).

4. Discussion

Our experiment has elucidated the effect of *A. simplex* ES proteins injected intraperitoneally on the immune gene expression in rainbow trout. The overall gene expression profile of the injected hosts showed in many cases a down-regulation of the immune genes tested, suggesting that ES products form this nematode larva can minimize immune reactions of rainbow trout. Such a function of nematode products is in line with the recent demonstration that numerous genes in a related parasite, the large roundworm *Ascaris suum*, are involved in immune escape reactions [19]. Down-regulation of immune gene expression was demonstrated with regard to some cytokine genes (IL-1β and TNF-α) in the spleen of ES-injected rainbow trout. These pro-inflammatory cytokines stimulate inflammation and early innate immune reactions in rainbow trout [20] and a repression of these reactions appears beneficial for the invading nematode. Also IL-8, a CXC chemokine subfamily member considered to have a chemo-attractive effect on neutrophils in trout [21] was down-regulated which support the notion that *A. simplex* reduce host reactions. In the liver of the ES injected trout, the expression of MHCII present on antigen presenting cells and the T-cell marker CD4 were significantly down-regulated at 24 h post injection. The gene encoding immunoglobulin (IgM) was also significantly down-regulated at 24 h post ES-injection. Antibodies produced by the host during nematode infections have in fact been suggested to be essential factors in immunity of fish against nematodes [22,23] and an inhibition of this host reaction would increase ability of invading nematodes to survive.

It is well documented that both innate and adaptive responses are prominent actors during nematode infection in mammalian hosts [5,14]. Genes encoding molecules such as CD4, CD8 [24], MHC class I and II [24,25] and cytotoxic T cells [26] are present in rainbow trout and they may also in this host be involved in protection of fish against nematode invasion. Therefore, the demonstration of an immune-depressive effect of *Anisakis* ES proteins on these genes may explain survival of the nematode in the host tissues.

ES production in a well increased with the number of nematodes incubated but the production per worm was relatively stable at a given temperature. At the lowest temperature tested, 4 °C, the production per worm was that low that the additive effect was non-measurable. This frames the temperature-dependent production of ES-compounds.

The production and concentration of ES at 37 °C was the highest among all incubation temperatures regardless of the number of nematodes incubated. Further investigations should be performed to elucidate if other chemical/immunological factors (from hosts and/or worms) play a role in triggering the production of ES proteins.

The APIZYM kit has previously been used to demonstrate different enzymatic activity in the skin mucus [27] and leucocytes [28] of rainbow trout. Lipase, esterase/lipase, valine and cysteine arylamidases, which we found to be present in ES proteins, are hydrolytic enzymes that could play a role in nematode penetration of the host by disrupting the tissues in the infected hosts. Arylamidases are enzymes that have the ability to hydrolyse amino acid-naphthylamide substrates and are suggested to function at some stage in protein catabolism [29]. Another two enzymes that were relatively highly reactive were naphthol-AS-BI-phosphohydrolase and α-galactosidase. The latter is an enzyme that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. The enzymes found active using the APIZYM kit in the ES

protein solution may not only facilitate *A. simplex* penetration in the host tissue, but it cannot be excluded that these enzymes also may target certain immune molecules (e.g. immunoglobulins and complement factors) in the host and thereby suppress the host reaction against nematode infections.

The study showed that ES-compounds may be important factors during host-invasion and penetration and further suggested an immune-depressive role of ES-products which would be beneficial for an invading parasite.

Acknowledgements

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