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Published in:
Fish and Shellfish Immunology

DOI:
10.1016/j.fsi.2013.06.007

Publication date:
2013

Document version
Peer reviewed version

Citation for published version (APA):
Effects of excretory/secretory products from *Anisakis simplex* (Nematoda) on immune gene expression in rainbow trout (*Oncorhynchus mykiss*)

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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.

**Keywords:**

*Anisakis simplex*  
ES proteins  
Fish immunology  
Rainbow trout  
ES enzymes

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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.
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 °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 filtrate 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 (Nexa, 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 (NH₄/NH₃) less than 1 ppm, nitrate (NO₃) less than 5 ppm and nitrite (NO₂) 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 cm³) 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 sonicating 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 nm, 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% ± 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
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^ΔΔCt method [18]. Change of threshold cycle number (ΔCt) was calculated as the difference between Ct values of the ES-injected fish (Ct) versus the control PBS-injected fish (Ct). 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^ΔΔCt method [18]. Change of threshold cycle number (ΔCt) was calculated as the difference between Ct values of the ES-injected fish (Ct) versus the control PBS-injected fish (Ct). A pair-wise multiple comparison procedure (Holm-Sidak method) was used to assess statistically significant difference in between groups.

### 3.2. Enzyme activity

High enzyme activity of the ES protein solution was recorded with regard to esterase/lipase, naphthol-AS-BI-phosphohydrolase and β-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 infection.
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 precrebellin

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 injection.

Table 2

<table>
<thead>
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<th>Reaction</th>
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Table 3

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Table 3: Expression of immune relevant genes in rainbow trout larvae (avg. wt. 4.7 g) after injection with 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.
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-attractant 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 immune-depressive role of ES-products which would be beneficial for an invading parasite.

Acknowledgements

This work was produced under the Danish Fish Immunology Research Centre and Network DAFINET (www.dafinet.dk) and supported by the Danish Research Council for Strategic Research.

References

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