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Lepeophtheirus salmonis secretory/excretory products and their effects on Atlantic salmon immune gene regulation

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SUMMARY

We have previously shown that Lepeophtheirus salmonis *produces trypsin and prostaglandin E_2 (PGE_2) that are most likely responsible for the limited inflammatory response of Atlantic salmon to infection. After removal of the dopamine and PGE_2, the immunomodulatory activity of unfractionated and pools of the fractionated secretions was determined by examining the effects of the secretions on Atlantic salmon immune gene expression. Incubation of macrophage-enriched isolates of Atlantic salmon head kidney cells with the unfractionated secretion + PGE_2 revealed a significant inhibition of interleukin-1β (IL-1β) and major histocompatibility class I gene expression. Inhibition of lipopolysaccharide-induced IL-1β expression in the Atlantic salmon head kidney cell line (SHK-1) was observed when three pools of the secretory/excretory products were tested. Further purification of products within these pools revealed that fraction 1-2 could account fully for the inhibition of IL-1β expression in SHK-1 cells observed in pooled fraction 1. This study demonstrates that there are other immunomodulatory compounds produced by* L. salmonis*, in addition to PGE_2 and trypsin, that can inhibit the expression of Atlantic salmon immune-related genes* in vitro.

Keywords *gene expression, immunomodulation,* Salmo salar *sea lice*

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INTRODUCTION

The emergence of arthropod parasites as economically important organisms that hamper animal production in both terrestrial and aquatic systems, has stimulated studies into their interactions with their hosts. *Lepeophtheirus salmonis* is a marine ectoparasitic copepod that feeds on mucus, skin and blood of salmonids. This species has a direct life cycle consisting of two free-living planktonic nauplius stages, one free-swimming infectious copepodid stage, four attached chalimus stages, two pre-adult stages and one adult stage (1). Despite its economic importance, gaps of knowledge exist with respect to *L. salmonis* biology, physiology and host–parasite interactions. In particular, the means by which *L. salmonis* limits host immune responses in species such as Atlantic salmon (*Salmo salar*), which are highly susceptible to infection, have yet to be fully understood (2,3). Improvements in our understanding in these areas are critical to the development of more effective treatment and control measures for *L. salmonis*.

There is growing literature concerning secretions released by arthropod ectoparasites and their possible host immunomodulatory capabilities. Proteases, phosphatases and prostaglandins are major salivary constituents of numerous arthropod parasites (4); however, macrophage migration-inhibitory factors, apyrases, peroxidases, and many other as yet unidentified products probably exist in the secretions of these parasites (4,5). Trypsin-like enzymes and prostaglandin E_2 (PGE_2) have been identified in the secretions of arthropod parasites, including *L. salmonis* (2,6–18). It is thought that the trypsin-like proteases play important roles in the establishment and maintenance of *L. salmonis* on hosts. Based on the study of a variety of other parasitic diseases, these roles may include aiding in the invasion of host tissues and evasion of host immune response (6,8). In the host–parasite relationship, prostaglandins, such as PGE_2, are thought to regulate vasodilation, anti-coagulation and T-lymphocyte regulation (19,20). Recently, we reported the discovery of PGE_2 in the secretions of *L. salmonis* (7). The presence of

PGE_2 is viewed as a mechanism by which *L. salmonis* evades host immune responses, possibly through its anti-inflammatory effects (7).

Because products other than trypsin-like enzymes and PGE_2 have been identified in the secretions of other arthropod parasites, the present experiments were designed to identify whether *L. salmonis* secretes other immunomodulatory products. We employed biochemical, proteomic and molecular techniques to partially separate these secretions and characterize their biological significance. After removal of dopamine and PGE_2, the immunomodulatory activity was determined for unfractionated and pools of fractionated secretions, using macrophage-enriched head kidney cell isolates and a head kidney cell line (SHK-1) derived from Atlantic salmon.

MATERIALS AND METHODS

Sources of *Lepeophtheirus salmonis*

Pre-adult and adult *L. salmonis* were collected from farmed Atlantic salmon held at various seawater net cage sites in the Bay of Fundy, Canada, and from wild Pacific salmon caught off the coast of Vancouver Island, Canada. After collection, the copepods were washed with sterile seawater (SSW) and maintained off hosts in SSW for 24 h prior to incubation with dopamine.

Collection of *Lepeophtheirus salmonis* secretory/excretory products

Secretory/excretory products (SEPs) were obtained from approximately 1200 pre-adult and adult *L. salmonis* collected in New Brunswick and British Columbia, using methods previously described (7). Briefly, live *L. salmonis* were washed in SSW (and then incubated at 10–15°C in SSW with 0·1 mM dopamine (DA) (Sigma, Missassauga, ON, Canada) for 45 min. Approximately 80–100 *L. salmonis* were incubated (per tube) at two lice/mL. *Lepeophtheirus salmonis* were then removed and the samples spun through 3000 Da molecular weight cut-off columns to concentrate constituents and remove SSW and DA. These solutions are referred to as SEPs.

The same manipulations were carried out using DA + SSW in the absence of *L. salmonis*. These samples were used as controls in cell culture experiments if DA removal was incomplete. After centrifugation, all of the concentrated samples were re-suspended in dd H_2O and were stored at −80°C.

Size-exclusion chromatography and protein determination

Prior to analysis, SEPs were lyophilized and reconstituted with 1·0 M ammonium acetate (pH 6·0). An Agilent 1100 high performance liquid chromatography (HPLC) (Agilent, Missassauga, ON, Canada) equipped with a diode array detector (monitoring at 230 and 256 nm) and a Taso Haas (G3000PWX2, 6 μm d_p (7·8 mm × 300 mm)) column were used to separate proteins/peptides. Fractions were collected using a Waters Fraction collector (Waters, Missassauga, ON, Canada) according to the time-intervals shown in Table 1. Six separate HPLC runs were performed and fractions were pooled for each time-interval. These samples were freeze-dried prior to protein determination. The column was kept at room temperature and eluted isocratically with 98 : 2 ammonium acetate: acetonitrile for 30 min at 0·2 mL/min. Standard solutions of bovine serum albumin (20 μg, 2·0 μg, and 0·2 μg), SSW + DA (1×10^{-4} M), and porcine trypsin (40 μg) were run as controls for peak comparison with SEPs.

Protein concentrations of *L. salmonis* secretory fractions were determined using a dye-binding method with bovine γ-globulin as a standard (21). All assays were run on a spectramax 384 Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). These fractionated samples were used in both proteomic analysis and cell-based functional assays.

Table 1 Fraction interval times and protein concentrations of size-exclusion chromatography of *Lepeophtheirus salmonis* secretory/excretory products

Fraction number (SHK-1-Trial 2)	Fraction collection start (min)	Protein concentration (ng/μL)	Fraction collection finish (min)	Pooled fraction grouping (SHK-1-Trial 1)
Fraction 1-1	6	50	9·5	1
Fraction 1-2	9·51	149	12	1
Fraction 2-3	12·01	69	15·5	2
Fraction 2-4	15·51	13	17·5	2
Fraction 2-5	17·51	17	19·5	2
Fraction 2-6	19·51	11	21·5	2
Fraction 3-7	21·51	31	24·0	3
Fraction 3-8	24·01	18	26·0	3
Fraction 3-9	26·01	7	28·0	3
Fraction 3-10	28·01	0	29·0	3

Proteomic analysis

Prior to mass spectrometry (MS)-MS analysis, 10% of each fraction (by volume) was analysed using SDS-PAGE as previously described (22,23). Briefly, samples were diluted 1 : 1 with SDS-PAGE sample buffer containing DL-dithiothreitol (5%) and heated prior to loading. Proteins were electrophoresed in 12% acrylamide gels at 100 V and silver-stained as described previously (22,23).

Protein digestion

Proteins within the SEPs were resistant to digestion using trypsin alone, and therefore digestion was performed using cyanogen bromide cleavage followed by trypsin. Cyanogen bromide (Sigma) digestion was conducted following Crimmins *et al*. (24). Briefly, 88% formic acid (80 µL) was added to distilled deionized water (15 µL of ddH_2O) and CNBr in acetonitrile (5 µL of 0·53 g/mL) and incubated with the sample (0·05–10 µg protein) in an opaque container at room temperature for 19 h. The protein was vortexed to assure complete solubilization using an opaque container to minimize side reactions with other amino acid side chains. Following incubation, 10 volumes of ddH_2O were added to each reaction and the reactions were lyophilized for 19 h to remove the CNBr. Samples were resolubilized in 0·1 M ammonium bicarbonate (50 µL) containing trypsin (at 1 : 50 final enzyme: substrate concentration). The tryptic digestion was carried out for 19 h at 37°C and the reaction stopped by the addition of trifloroacetic acid (2%). The reaction mixture was speed-vacuumed to obtain a final volume of 10 µL for mass spectrometry analysis.

Mass spectrometry

Both fractionated and unfractionated SEPs were analysed and compared against SSW and SSW + DA controls using LC-MS/MS analysis to obtain partial sequence data. All samples were analysed by liquid chromatography (LC)-MS/MS using an LC Packings high performance liquid chromatography (HPLC) system equipped with a 5 cm × 300 µm PepMap C_{18} column (Dionex Ltd, Oakville, ON, Canada). The separation was carried out using a linear gradient from 10% to 50% B over 20 min (A: 5% acetonitrile, 0·5% formic acid; B: 90% acetonitrile, 0·5% formic acid) at 5 µL/min. The HPLC was interfaced to an MDS SCIEX QStar Pulsar *i* mass spectrometer (Applied Biosystems, Foster City, CA, USA) via a nanoflow source. Data were acquired in the information-dependent acquisition mode, i.e. the *m*/*z*-values of the tryptic peptides were measured using a time of flight (TOF)-MS scan and this scan was used to generate a peak list of peptides for tandem MS analysis. The tandem MS spectra were submitted to the database search program MASCOT (Matrix Science Ltd, Boston, MA, USA) in order to identify the proteins and the NCBI nucleotide database was searched.

Macrophage isolation and manipulation

Macrophages were isolated from 10 Atlantic salmon anterior kidneys as previously described (25). Briefly, anterior kidneys were removed aseptically and placed immediately into 5 mL L-15 media supplemented with 2% foetal bovine serum (FBS), 100 units/mL penicillin/streptomycin (P/S) and 10 units/mL heparin. Tissues were stored on ice until further processing. Individual head kidneys from each fish were dissociated by repeated passage through a 3-mL syringe and fragments allowed to settle for 10 min prior to removal of suspended cells. Cell suspensions were pelleted (500 ***g*** for 10 min at 4°C) and washed twice prior to layering on Percoll gradients (34/51%). Cells were centrifuged at 400 ***g*** for 20 min at 4°C and the macrophage-enriched fraction was collected at the 34/51% interface. Cells were re-suspended in 10 mL L-15/2% FBS, pelleted by centrifugation at 500 ***g*** for 10 min at 4°C, washed with 10 mL of L-15/2% FBS and then resuspended in L-15/5% FBS with 100 units/mL P/S. Viable cells were counted using the trypan blue exclusion method and cell density was adjusted to 1×10^7 cells/ml in L-15/0·1% FBS. Cells were plated at 100 µL per well on 96-well plates and incubated at 18°C for 2 h. After 2 h, media and non-adherent cells were removed and an equal volume of L-15/5% FBS was added. A minimum of 10 wells per condition, per fish, were plated. Cells were maintained for 1·5 days at 18°C prior to manipulation, then media was removed and 100 µL of fresh L-15/5% FBS with or without (control) 5 µg/mL *Escherichia coli* lipopolysaccharide (LPS, Sigma), 5 µg/mL LPS + 1×10^{-8} M PGE_2, 5 µg/mL LPS + 660 ng/mL SEPs, 5 µg/mL LPS + 1% DA + SSW, and 5 µg/mL LPS + 1×10^{-8} M PGE_2 + 660 ng/mL SEPs was added. Stimulation of cells was carried out for 4 h at 18°C before media was removed, and the cells were stored in RNAlater (Ambion, Austin, TX, USA) at −80°C until RNA extraction. All cell culture materials were supplied by Invitrogen (Burlington, ON, Canada) unless otherwise stated.

SHK cell culture

SHK-1 cells were cultured following methods previously described (26). Briefly, SHK-1 cells were cultured at 18°C in 75 cm (2) tissue-culture-treated flasks (Fisher Scientific, Ottawa, ON, Canada), in L-15 medium (with 300 mg/L L-glutamine) supplemented with 500 µL gentamycin sulphate (50 mg/mL distilled in water), 365 µL 2-mercaptoethanol (55 mM in D-PBS) and 5% FBS. All media components were purchased from Invitrogen. Confluent flasks were passaged

weekly by dividing cells and medium evenly between flasks from a stocked larger volume flask and adding an equal volume of new medium to each flask. Cells used in this study were passaged between 64 and 68 times.

SHK-1 cells were seeded at approximately 4×10^6 cells/flask in L-15 medium supplemented as described above. Cell stimulation followed the procedure given in Fast *et al*. (26). Briefly, following a 48-h period to allow any manipulation-induced gene expression to return to constitutive levels, medium was removed and 20 mL fresh medium was added. Lipopolysaccharide was added to all flasks, except the controls, to obtain a final concentration of 5 μg/mL.

In the first experiment, SEP fractions obtained from size-exclusion HPLC were pooled into three groups (Table 1), each containing equal time ranges (10 min) and volumes from the chromatography effluent. This resulted in 13 μg/mL (pooled fraction 1), 8·0 μg/mL (pooled fraction 2) and < 1.0 μg/mL (pooled fraction 3) being added to each flask. These incubations were carried out for 4 h at 18°C, after which the medium was removed, and the cells were transferred into RNAlater and stored at −80°C until RNA extraction.

In the second experiment, blanks, and SEP fractions 1-1 and 1-2 (Table 1), resulting from concentrating four size-exclusion HPLC runs, were added at 1·0 and 1·4 μg/mL, respectively. The unfractionated SEPs (660 ng/mL) were incubated as a positive control. Both SHK-1 experiments were repeated twice with triplicate flasks for each condition.

Isolation of RNA and cDNA synthesis

Total RNA was isolated from SHK-1 cells and macrophage-enriched cell cultures using the Nucleospin RNA II kit (Clontech, Mountain View, CA, USA). For macrophage-enriched cell cultures, multiple wells containing cells from individual fish under a single incubation condition were pooled. RNA samples were subjected to PCR to verify the lack of DNA contamination. For reverse transcription, 1·0 μg of total RNA from each sample was dissolved in molecular biological grade water.

Real-time PCR

Sequences for real-time PCR primers were designed using PRIMER 3 software and Dr Michael Zuker's mfold server (http://www.bioinfo.rpi.edu/applications/mfold) (27). Primers were generated from available Atlantic salmon (*Salmo salar*) sequences (β-actin: AF012125; MH class I: AF508864; MH class II: X70166; and IL-1β: AY617117) and EST databases (cyclo-oxygenase-2; COX-2) (Table 2) (26). The $TGF\beta_{1/5}$-like gene primers were designed from comparisons of the highly conserved regions of that gene in rainbow trout (*Oncorhynchus mykiss*) and plaice (*Pleuronectes platessa*) (28–30). The gene product developed for Atlantic salmon $TGF\beta_{1/5}$-like gene product exhibited 107/108 (99%) identity to rainbow trout. All primer sets were tested on head kidney cells isolated from Atlantic salmon to confirm single amplification products. PCR products of β actin, MH class I, MH class II, COX-2, $TGF\beta_{1/5}$ and IL-1β were cloned into the TA-cloning vector (above) and sequenced to confirm sequence of amplified products. Plasmid vectors were isolated and used as standards for real-time studies (26).

Real-time quantitative PCR was performed using an iCycler iQ™ real-time detection system and SYBR green kits (Invitrogen). The SYBR green Mastermix kit was used according to the manufacturer's instructions with the following exceptions. Mastermix was added (25 μL) to template cDNA (2·5 μL), water (17·5 μL) and specific primers (125 nM forward and reverse final concentration) giving a total volume of 50 μL prior to dividing into separate wells for duplication of readings. Primer concentrations were optimized at 125 nM after testing a range of concentrations from 90 to 900 nM. To ensure no genomic DNA contamination was added to the quantified cDNA, non-RT controls for each RNA isolation

Table 2 Sequences of oligonucleotide primers used in real-time PCR

Genes	Primers	Sequences (5′–3′)
β-actin	β actin – forward	^{230}CAACTGGGACGACATGGAGA^{249}
	β actin – reverse	^{318}AGTGAGCAGGACTGGGTGCT^{299}
Cyclo-oxygenase-2	COX-2 – forward	^{238}CAGTGCTCCCAGATGCCAAG^{257}
	COX-2 – reverse	^{337}GCGAAGAAGGCGAACATGAG^{318}
MH class I	MH I – forward	^{974}TGCTCGTCGTTGCTGTTGTT^{993}
	MH I – reverse	^{1067}TCAGAGTCAGTGTCGGAAGTGC^{1048}
MH class II	MH II – forward	^{722}AAGGCTTGAAGACACGTTGC^{741}
	MH II – reverse	^{828}CAGTCCAGCAGTAACGTCCA^{809}
IL-1β	IL-1β – forward	^{197}ATGCGTCACATTGCCAAC^{214}
	IL-1β – reverse	^{287}GGTCCTTGTCCTTGAACTCG^{268}
$TGF\beta_{1/5}$	$TGF\beta_{1/5}$ – forward	ATCGGAGAGTTGCTGTGTGC
	$TGF\beta_{1/5}$ – reverse	GGGCCGATGCAGTAGTTAGC

were run under the same PCR conditions and observed by 2·5% agarose gel electrophoresis.

The PCR profile was as follows: a 4-min denaturation step at 95°C, followed by 40 cycles of denaturation (15 s at 95°C), annealing (30 s at 58°C) and extension (30 s at 72°C), finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products, such as primer dimers, indiscriminately detected by SYBR green (i.e. SYBR green binds to all double-stranded DNA), were evaluated by amplifying 10-fold dilutions of the clones (1 to 10^{-6} ng) and duplicate samples, as well as by performing a blank without cDNA with each run. The relationship between the threshold cycle (Ct) and the log [RNA] was linear ($-3{\cdot}30 < \text{slope} < -3{\cdot}14$) for all reactions. Real-time experiments were conducted at least twice for each gene in each sample.

Single-product amplification was further verified by melt-curve analysis. Melting curves were obtained following 40 cycles of amplification on the LightCycler (Bio-Rad) by integrating the signal every 0·1 s during a linear temperature transition from 95°C to 75°C. Fluorescence data were converted by iCycler software (Bio-Rad), in which background fluorescence and the effect of temperature on fluorescence was removed.

Statistical analysis

All gene expression is presented as expression changes relative to β-actin (ERB). Under the conditions used in this study, expression of β-actin has previously been shown to remain relatively constant in these cell-types and reliable as a housekeeping gene (25,26). Statistical analyses were performed using SigmaStat for windows version 3·0 (SPSS). All non-normal data were transformed (i.e. $\log_{10}$) prior to analysis. All values shown are means ± SEM. The statistical significance of differences was assessed using one-way ANOVA ($P < 0{\cdot}05$).

RESULTS

Lepeophtheirus salmonis SEPs were run under size-exclusion HPLC. A large negative peak that was eluted at *c*. 13 min and a small peak at 14 min, both seen in the SSW + DA control, were observed in *L. salmonis* SEPs, suggesting some carryover of salts and DA, respectively (Figure 1a,c). Despite concentration of numerous *L. salmonis* + SSW + DA incubations to obtain reasonable SEP protein concentrations, the DA concentration was still much lower in SEPs than in the DA + SSW control (Figure 1a,c). The SEPs exhibited peaks with similar retention time to porcine trypsin (*c*. 10·8 and 22 min elution times) (Figure 1d).

Proteomic analysis

The protein concentrations of the SEP fractions were relatively low in all samples (Table 1). However, silver-stained SDS-PAGE gels revealed protein bands in fractions 1-1, 1-2, 2-3 and 3-7 (Figure 2). Fraction 1-2 was enriched in two proteins at *c*. 40 kDa, whereas fraction 1-1 exhibited numerous proteins, all of equally low concentrations (Figure 2). Protein bands in fractions 2-3 and 3-7 were extremely faint and difficult to detect. Using MS-MS analysis, three peptide sequences (^{215}FIDWIAEHQ^{223}, ^{71}IAVSDITYHEK^{81} and ^{115}DQEVVVSGWGTISSSGPPSPVLK^{141}) showed significant identity to *L. salmonis* trypsins types 1-4. In agreement with size-exclusion chromatography on porcine trypsin, *L. salmonis* tryptic peptides were observed

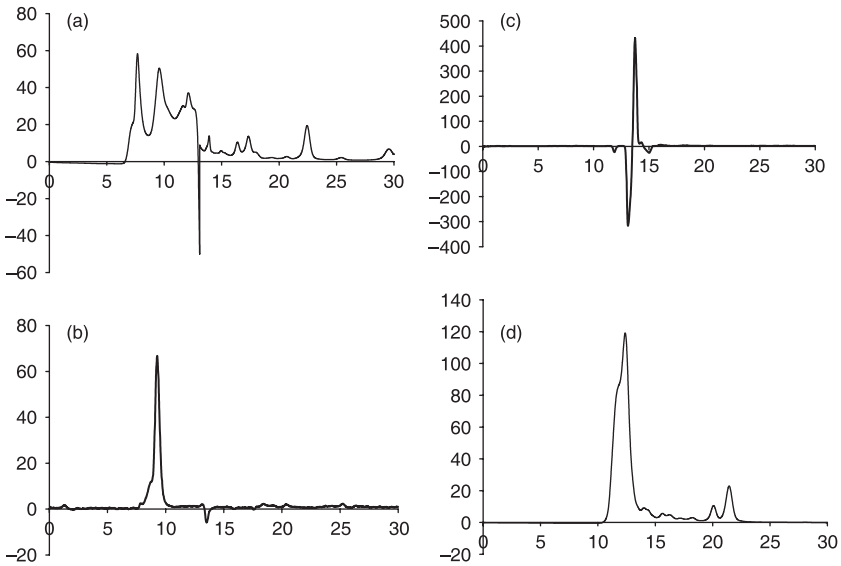

Figure 1 Size-exclusion HPLC chromatogram (254 nm) of *Lepeophtheirus salmonis* secretory/excretory products (SEPs) (—) (a), bovine serum albumin (b), sterile seawater and dopamine ($1{\cdot}0 \times 10^{-4}$ M) (c) and porcine trypsin (d). The column was kept at room temperature and eluted isocratically with 98 parts ammonium acetate (1 M): 2 parts acetonitrile for 30 min at a flow rate of 0·2 mL/min. Injections of 20 µL were made of samples and standards dissolved in 1·0 M ammonium acetate.

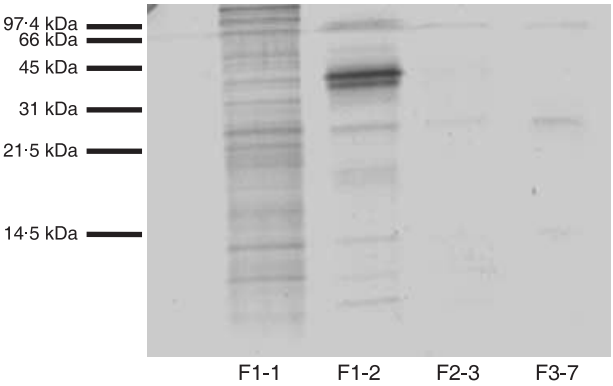

Figure 2 Protein profiles of *Lepeophtheirus salmonis* secretory fractions using SDS-PAGE (12% acrylamide). Molecular masses are along the left hand side of the gel (kDa). F1-1 indicates fraction 1-1, F1-2 indicates fraction 1-2, F2-3 indicates fraction 2-3, and F3-7 indicates fraction 3-7.

in SEP fractions 1-1, 1-2 and 2-6. The protein identifications were manually verified by comparing the experimentally obtained MS-MS spectrum with the predicted peptide sequence.

Effects of SEPs on immune gene expression in adherent head kidney leucocytes

The effects of SEPs, with and without PGE_2, on immune gene expression were studied in LPS-stimulated macrophage-enriched head kidney isolates (HKMs) by real-time PCR (Figure 3a,b,c). To control for the carryover of DA in SEP preparations, cells were also stimulated by LPS + SSW + DA.

The expression of COX-2 was significantly up-regulated by the addition of LPS, as well as LPS + SSW + DA (Figure 3a). There was a slight reduction in COX-2 expression upon incubation with LPS + PGE_2, LPS + SEP, and LPS + PGE_2 + SEP. However, when compared to the LPS and LPS + SSW + DA incubations, this reduction was not significant (Figure 3a). Similar to COX-2, IL-1β expression was induced in HKMs following LPS and LPS + SSW + DA incubation (Figure 3b). However, unlike COX-2, addition of LPS + PGE_2 or LPS + PGE_2 + SEPs significantly reduced the magnitude of the LPS-induced stimulation (Figure 3b). Decreased expression of IL-1β was observed in cells stimulated with LPS + SEP; however, this decrease was not significant when compared to LPS + SSW + DA-stimulated cells.

Lipopolysaccharide stimulation also significantly increased MH class I gene expression when compared to the unstimulated control (Figure 3c). Addition of SSW + DA or PGE_2, to LPS, resulted in no significant change in expression when compared to HKMs stimulated only with LPS. Incubation of HKMs with both LPS + SEPs resulted in significantly

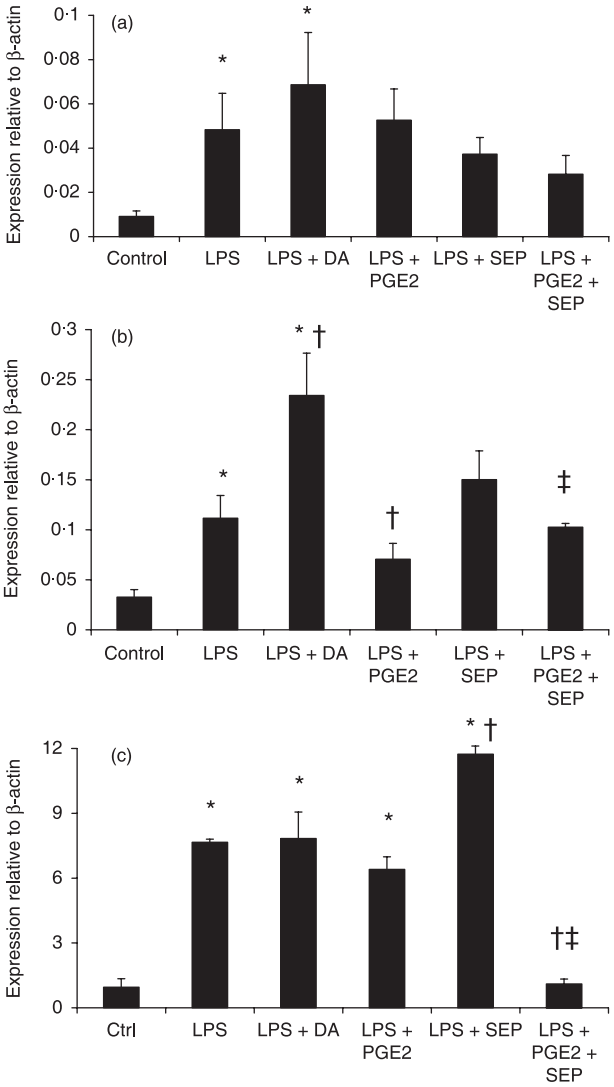

Figure 3 Mean (± SEM) real-time PCR expression of cyclo-oxygenase-2 gene (a), interleukin-1β gene (b) and major histocompatibility class I gene (c), relative to β-actin, in Atlantic salmon head kidney-isolated macrophages incubated with and without (control) lipopolysaccharide (LPS), sterile seawater (SSW) + dopamine (DA), prostaglandin E_2 (PGE_2), and *Lepeophtheirus salmonis* secretory/excretory products (SEPs). *Indicates significant differences from control; †indicates significant differences from LPS; ‡indicates significant differences from LPS + SSW + DA (n = 10).

increased expression of the MH class I gene when compared to the unstimulated control, LPS and LPS + SSW + DA-stimulated cells. However, incubation with LPS + PGE_2 + SEP resulted in a significant reduction in MH class I gene expression to a level similar to that seen in unstimulated controls (Figure 3c).

The expression of MH class II and TGFβ-like genes was significantly increased, following incubation with LPS

(0·13 ± 0·01 and 0·14 ± 0·04 expression relative to β-actin, respectively), in comparison to unstimulated controls (0·05 ± 0·02 and 0·02 ± 0·01 ERB, respectively). Incubation with LPS + DA resulted in significantly lower MH class II gene expression when compared to LPS alone (0·08 ± 0·02 ERB). The addition of LPS + PGE_2, LPS + SEPs or the combination of LPS + PGE_2 + SEPs had no further effect on the expression of either of these genes (data not shown).

Effects of SEPs on immune gene expression in SHK-1 cells

To avoid some of the inherent variability that was experienced during use of primary isolates of head kidney macrophages, it was decided to use the Atlantic salmon head kidney cell line (SHK-1) to observe the effects of SEP fractions on the expression of IL-1β. Because of the difficulties in obtaining sufficient SEP fractions, the study was limited to a single gene. As seen in HKMs, LPS induced a significant increase in the expression of IL-1β following 4 h incubation (Figure 4). Following incubation with LPS and pooled SEP fraction 1 (PF1), there was a significant inhibition of IL-1β expression when compared to LPS-stimulated cells, yet the expression was still significantly higher than that found in unstimulated controls (Figure 4). Pooled SEP fractions 2 and 3 not only abrogated LPS-stimulated IL-1β expression, but reduced expression levels significantly below that of the unstimulated controls.

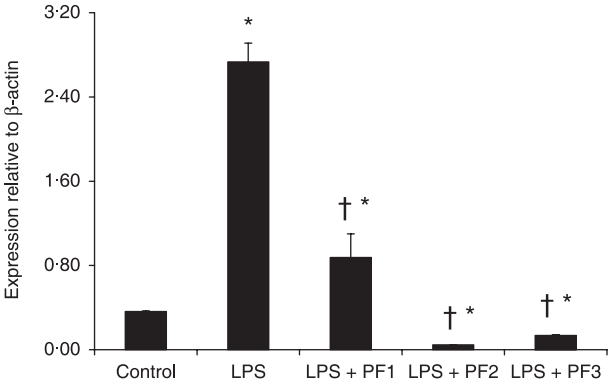

Figure 4 Mean (± SEM) real-time PCR expression of interleukin-1β gene, relative to β-actin, in SHK-1 cells incubated for 4h with and without (control) lipopolysaccharide (LPS), pooled *Lepeophtheirus salmonis* secretory/excretory product (SEP) fraction 1 (PF1), pooled *Lepeophtheirus salmonis* SEP fraction 2 (PF2), and pooled *Lepeophtheirus salmonis* SEP fraction 3 (PF3). *Indicates significant differences from control; †indicates significant differences from LPS. Each condition was replicated in triplicate flasks and these incubation experiments were carried out twice; $n = 6$.

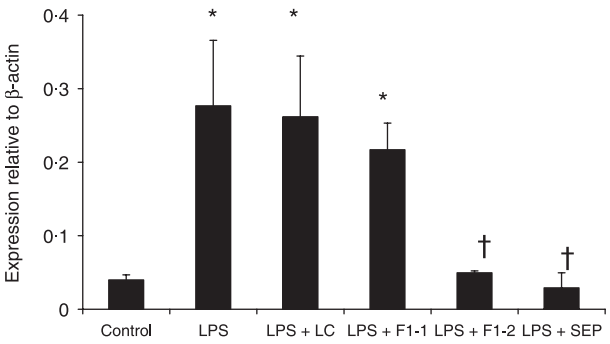

Figure 5 Mean (± SEM) real-time PCR expression of interleukin-1β gene, relative to β-actin, in SHK-1 cells incubated with and without (control) lipopolysaccharide (LPS), LPS and lyophilized liquid chromatography solvent (LC), *Lepeophtheirus salmonis* secretory/excretory product (SEP) fraction 1-1 (F1-1), *Lepeophtheirus salmonis* SEP fraction 1-2 (F1-2), and *Lepeophtheirus salmonis* unfractionated SEP. *Indicates significant differences from control; †indicates significant differences from LPS. Each condition was replicated in triplicate flasks and these incubation experiments were carried out twice; $n = 6$.

To discern whether both fractions 1-1 and 1-2 contributed to the inhibition previously observed from pooled fraction 1, they were individually incubated with LPS-stimulated SHK-1 cells (Figure 5). Although LPS-stimulation induced IL-1β expression at a much lower level than in the pooled trial, the relative change (sevenfold), with respect to the unstimulated controls was the same (Figure 5). Because the possibility remained that residual solvent from the separation procedure adversely affected the SHK-1 cells, a diluted lyophilized liquid chromatography solvent (LC solvent) control was incubated with LPS-stimulated SHK-1 cells. This LC solvent control showed no significant effect on IL-1β expression when compared to LPS-stimulated cells (Figure 5). Incubation of SHK-1 cells with LPS + fraction 1-1 resulted in no significant difference from LPS-stimulated cells (Figure 5). Incubation with LPS + fraction 1-2, however, significantly reduced LPS-stimulation of IL-1β gene expression to a level similar to that seen in unstimulated cells or those incubated with LPS + SEPs (Figure 5).

DISCUSSION

In the interests of obtaining a better understanding of the relationship between *L. salmonis* and Atlantic salmon, secretory products from *L. salmonis* were isolated and their biological activity studied. We recognized that the collection method used to obtain secretory products may result in some contamination of secretory products by excretory products. However, the use of this method was necessary because of the field conditions under which the samples were obtained.

In arthropod parasites that take blood meals, the timing of collection of salivary products can greatly affect secretion contents (31,32). In this study the time of collection was unlikely to have affected the results. This is because *L. salmonis* spend most of their time feeding on host mucus, tissue, and blood, and all of the collections of SEPs were pools from numerous individuals at different stages in their development and moult cycles (33).

The current study provides the first direct measurement of trypsin within SEPs, as evidenced by the presence of multiple trypsin fragments in MS analysis of fractions 1-1, 1-2 and 2-6. At least five trypsin genes have been characterized for *L. salmonis*; unfortunately the data obtained herein do not allow assignment of SEP trypsin fragments to individual genes (34,35). It is unknown whether these forms of trypsin have distinct roles in *L. salmonis*; however, they all appear to be up-regulated from non-feeding copepodid to adult developmental stages (35). Trypsin-like serine proteases are dominant digestive proteases found in several insect species and they have been linked to cleavage of the sheep IgG heavy chain (36,37). Previously, *L. salmonis* trypsin-like proteases were identified in the mucus of infected fish (6,8). *Lepeophtheirus salmonis* produces several different trypsins via several cell types lining the midgut (34,35).

In this study we used levels of PGE_2 and SEPs that are physiologically relevant, based on past studies. The PGE_2 concentration chosen for this work was based on a combination of concentration ranges observed in mammalian inflammatory situations, as well as ranges shown to be inhibitory to fish inflammatory gene expression (26,38,39). The range of SEP concentrations chosen for the cell-based assays in this study were based on Bergman *et al.*, where 20–70% suppression of ConA-stimulated T-cell proliferation was observed, using 0·5 μg of *Dermacentor andersoni* salivary gland extract and up to 90% inhibition using 4 μg of extract (40). Throughout this study, none of the conditions tested showed an effect on cell viability, morphology or adherence properties. This is consistent with reports on mammalian macrophages incubated with PGE_2 at similar concentrations (41). At these concentrations we were unable to detect a significant change in COX-2, MH class II and TGFβ-like gene expression in HKMs incubated with LPS + PGE_2, LPS + SEPs, or LPS + PGE_2 + SEPs.

With respect to MH class I, incubation with LPS + SEP resulted in a significant increase in gene expression when compared to the LPS and LPS + SSW + DA controls. However incubation with LPS + PGE_2 resulted in a significant decrease in expression. The addition of PGE_2 + SEPs acted synergistically, reducing MH class I LPS-induced expression to constitutive levels. Previous work on SHK-1 cells has also shown an inhibitory effect of PGE_2 on MH class I gene expression at this concentration (26).

Incubation with 0·66 μg of SEPs did not show significant inhibition of IL-1β in LPS-stimulated HKMs, but nearly 100% inhibition of LPS-stimulated expression of IL-1β in SHK-1 cells. In addition, higher concentrations of pooled and single fractions of SEPs significantly reduced the expression of IL-1β in LPS-stimulated SHK cells, often to levels below that seen in unstimulated cells. Salivary gland extract from *D. andersoni* has also been shown to reduce LPS-stimulated production of IL-1β and TNFα by splenic macrophages and peripheral-blood-derived macrophages (32,42).

Differences in our results between the primary isolated HKMs and the cell line (SHK-1) may result from inherent differences in the cell types that are present or from slight differences in culture conditions. The SHK-1 cell line is macrophage-like, and similar to a melanomacrophage precursor, but over lengthy passaging of this cell line may result in differences compared to freshly isolated HKMs (26,43). In this study the FBS lot number used in the HKM trial was different from that used in the SHK trial. Different lots of FBS may contain different ratios of bovine proteins, growth factors and glucocorticoids, all which might have differing effects on cytokine expression in fish cells.

In this study we treated samples of SSW + DA with the same ultrafiltration steps as the SEPs. Although DA is a small molecule, not all of it passed through the filters, as evidenced by the LC data. Although the amount of DA remaining was lower than that observed in the SSW + DA control, its final concentration was unknown. Because of the potential of residual DA in SEPs having an effect on the cells, the SEP + LPS incubations were compared with LPS + SSW + DA incubations. With the exception of MH class II, SSW + DA appeared to have either no effect or to act synergistically with LPS to increase the expression of HKM genes. Head kidney macrophage LPS-induced expression of the MH class II gene, however, appeared to be inhibited by DA. Thus, SEP inhibition of HKM LPS-induced expression of MH class II could not be discerned from the effects of DA.

In mammalian systems, DA at concentrations in the range of 10^{-5} to 10^{-7} M show mainly inhibitory effects on T-cell cytokine expression, but mixed results on proliferation (44–46). In macrophages, DA effects, however, are largely stimulatory at 10^{-6} to 10^{-7} M (44). Ferreira and Silva (47) also observed no effect of DA incubation on Con-A-induced proliferation of mice splenocytes (*c.* 1×10^{-5} M). The maximum possible concentration of DA in the cell incubations would have been 1×10^{-6} M if no DA was lost during concentration of samples through LMW cut-off filtration. As stated above, at this concentration no inhibitory effect on macrophage gene expression would be expected. There are mixed reports on whether PGE_2 is the main contributing factor to the immunosuppressive capabilities of other arthropod ectoparasite secretions (48,49). Although PGE_2

concentrations were not determined in SEP fractions, it was expected that they would be extremely low to non-existent for several reasons. *Lepeophtheirus salmonis* produce very small amounts of this prostaglandin under the incubation conditions used here, and the use of 3000 nominal molecular weight (NMW) filters to concentrate SEPs would remove most if not all of the PGE_2 (26).

In this study, its was shown that, although PGE_2 can act in an immunosuppressive way on its own and synergistically with SEPs, SEP fractions not containing PGE_2 also caused 100% inhibition of LPS-induced stimulation of the inflammatory gene IL-1β. It appears then, that *L. salmonis* secretions have immunomodulatory capabilities other than that attributed to PGE_2, a situation that is again similar to that found for the Rocky Mountain wood tick, *D. andersoni* (40,50).

This brings us to the interesting point of what biological role these compounds serve in the interactions between the parasite and its host? Based on past studies, the lack of an inflammatory response appears to be correlated with host susceptibility to *L. salmonis* infection (2,3). Chalimus stages of the parasite are fixed to one location on the host for several days and yet no tissue response is mounted in susceptible species (3,51). Furthermore, histology of host tissue at the site of attachment and feeding of adults shows inflammation to occur only in the periphery of the lesion and not in tissues beneath the cephalothorax (52). This is strong evidence that host immunomodulation is occurring at the site of feeding, and the results of the present work support this hypothesis. Interestingly, skin mucus from the relatively resistant host species, coho salmon (*Oncorhynchus kisutch*), is reported to stimulate SEP release from *L. salmonis* at a significantly reduced level in comparison to more susceptible hosts (i.e. Atlantic salmon), as evidenced by the lack of trypsin activity (6). This lack of SEP release or its changed composition may simply allow the process of inflammation in coho salmon to proceed as expected, which is not the case for susceptible hosts (i.e. Atlantic salmon).

From the parasite's vantage point, the release of PGE_2, through its vasodilatory action, should increase blood flow to the feeding site providing a food source for the parasite. However, a negative effect of this would be the increased exposure to antigen-presenting cells, antibodies, harmful reactive oxygen species and clotting agents. The simultaneous release of anti-inflammatory compounds may alleviate these potentially harmful effects. Prostaglandin E_2, although it may increase blood flow, can reduce leucocyte recruitment and antigen-presenting cell capabilities, through its inhibition of MH class I and II gene expression. One or more of the compounds found in fraction 1-2 (i.e. 40 kDa protein, trypsin, etc.), may also exhibit anti-leucocyte recruitment capabilities through their inhibition of IL-1β and its downstream effectors (IL-8, etc.).

Both pro-inflammatory and anti-inflammatory compounds have been isolated from ectoparasitic arthropod secretions (5,53). Here, we have observed an inhibitory effect on one centrally involved inflammatory gene (IL-1β) by *L. salmonis* SEPs; however, when further fractionated they appear to have different effects, as evidenced by the differences between pooled fractions and within fractions 1-1 and 1-2. Lawrie and Nuttall have demonstrated how the composition of parasite secretions changes over time, and may be a result of the parasites evolving a complex milieu of components within their secretions that provide favourable conditions for feeding and survival (31). These evolutionary traits appear to extend beyond simply terrestrial ectoparasitic arthropods, to ectoparasitic copepods, as described here, and possibly to other ecto- and endoparasitic organisms. These immunomodulatory secretory/excretory components provide *L. salmonis* with the means to evade and modulate the host's immune response. Their continual release may be responsible for the difference between successful and unsuccessful infections, as seen in relatively susceptible and resistant host species.

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