Cod cathelicidin: Isolation of the mature peptide, cleavage site characterisation and developmental expression

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ABSTRACT

Cathelicidin antimicrobial peptides are multifunctional peptides that are important in the innate immune system of mammals. Cathelicidins have been identified in several fish species. In this study we have isolated cathelicidin from Atlantic cod (Gadus morhua) and identified the cleavage site from the cathelin propart. This is the first isolation of a cathelicidin from teleost fish. The mature cathelicidin was found to be a 67-residues peptide, highly cationic with a pI of 13. Reversed phase chromatographic fractions containing the purified peptide had pronounced antimicrobial activity and the activity of the mature peptide was confirmed using a synthetic peptide. We examined the expression of cathelicidin during cod larvae early development using real-time PCR and detected expression that varied in the course of the first 68 days post hatching (dph). Two groups of larvae having a different food regime were compared. Cathelicidin expression was found to differ between the two groups and this could be linked to their food input. The presence and rapid adjustment of cathelicidin expression in the larvae indicate that the immune system of cod is active from early on in development and responds to external stimuli by the production of antimicrobial peptides.

1. Introduction

The innate immune system is the first line of defence against pathogens. Antimicrobial peptides (AMPs) are an important part of this defence system and act as natural antibiotics. Due to the cationic and often amphipathic character of these peptides, they attack the negatively charged microbial cellular membrane (Zasloff, 2002). AMPs have been intensively studied in mammals and have been found, in addition to their direct antimicrobial action, to also be involved in many other processes in infection and inflammation, such as promotion of wound healing and initiation of adaptive immune responses (Lai and Gallo, 2009). Cathelicidins are a class of antimicrobial peptides that have so far only been identified in vertebrates. They are produced as prepropeptides with a conserved cathelin-like N-terminal domain (cathelin propart) and a diverse C-terminal antimicrobial domain (Fig. 1), reviewed in (Tomasinsig and Zanetti, 2005). In mammals, the cathelin part is encoded mainly by exons 1–3 and the sequence identity of this region is approximately 40% between species. The mature antimicrobial peptide is encoded by exon 4 and it is hypervariable with respect to size and sequence (Tomasinsig and Zanetti, 2005). This variability in the antimicrobial region is thought to have evolved after gene duplication through sequence remodelling by point mutations rather than exon shuffling (Zhu, 2008; Zhu and Gao, 2009).

Cathelicidins have been identified in several fish species and their mRNA expression was found to increase after bacterial infection (Chang et al., 2005, 2006; Maier et al., 2008a,b), indicating a role of cathelicidins in fish innate immunity. The fish cathelicidin genes identified so far have the typical four-exon structure, but interestingly a CDNA for cathelicidin in the Salvelinus genus lacks exon 3 (Maier et al., 2008a; Scocchi et al., 2009), deleting a part of the cathelin region of the proform. In mammals the antimicrobial peptide-coding sequence and the 3′-untranslated region are located in exon 4 with the processing site of the peptide close to the N-terminus of exon 4 (Tomasinsig and Zanetti, 2005). This thought to be true also for fish cathelicidins (Fig. 1). The amino acid identity of fish cathelicidins found so far is approximately 30%, both

Abbreviations: HPLC, high performance liquid chromatography; RP, reversed phase; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; kDa, kilodalton; CFU, colony forming units; dph, days post hatch.

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Cathelicidins are produced as prepropeptides with a signal sequence, cathelin propart and a mature antimicrobial peptide. The activating processing enzyme releases the antimicrobial peptide from the conserved cathelin region. The amino acid sequence of the predicted cod cathelicidin in the indicated region is shown and one aim of this study was to determine the actual cleavage site of the peptide. The shaded black amino acid residues (LT) represent the exon 3/4 boundary, which has been confirmed by sequencing (Maier et al., 2008a). The cleavage site of the processing enzyme was expected near the N-terminal boundary of exon 4. The cod cathelicidin antibody used in this study is raised against the underlined amino acid sequence.

for the cathelin and the mature peptide region. The relatively high sequence identity at the antimicrobial peptide region is unusual and is likely to reflect a conserved functional significance (Maier et al., 2008a), provided by positive selection as has been found in the mature peptides of primate cathelicidin (Zelezetsky et al., 2006). The cathelicidin peptides in fish can be divided on their putative secondary structure into peptides forming a disulphide bond and peptides forming an extended structure (Maier et al., 2008a). Examples of the latter are the newly identified Atlantic cod cathelicidins, with predicted mature peptides rich in arginine, glycine and serine (RGS). Little is known about the function of cathelicidins in fish, but in vitro studies using a synthetic fragment of a predicted rainbow trout cathelicidin have shown it to have antimicrobial activity (Chang et al., 2006). However, no mature teleost cathelicidins have been isolated to homogeneity or studied in detail.

The aims of this study were to purify cod cathelicidin, identify the proteolytic in vivo cleavage site of the proform (Fig. 1), and examine the mRNA expression during the development of cod larvae. Cod larvae have a very high mortality rate during the first 4–8 weeks after hatching, with a typical hatching survival of 10–20%. In aquaculture this is partly due to rearing problems, such as fish density and food (Steinarsson, 2004), but infections also play an important part. Natural antibodies (IgM) are present in fish serum independent of antigenic stimulation and are thought to have an important role in the innate immune system of Atlantic cod (Magnadottir et al., 2009). Studies on newly hatched cod larvae did not detect any immunoglobulin producing cells until at least 56 dph (Schröder et al., 1998) and no IgM was detected in the first 2 months of the larvae (Magnadottir et al., 2004). Similarly, the pentraxin CRP (C-reactive protein), a lectin important in the acute phase response, could not be detected at this stage (Magnadottir et al., 2004). These findings suggest that during the first 2 months after hatching the larvae depend mainly on other innate immune factors for their defence against pathogens.

In the present study we have isolated the mature cod cathelicidin from head kidneys, structurally characterised it and shown it to exhibit antimicrobial activity. The antimicrobial activity, as well as its increased transcription during infection, suggests a role of cathelicidin in fish immunity. We show that cathelicidin transcription is detected in cod eggs and larvae, which indicates that antimicrobial peptides provide a protection from infection in the early stages of development, before the appearance of other immune factors.

2. Materials and methods

2.1. Collection of cod material

For the infection study ten juvenile cod (60–120 g) were injected intraperitoneally (i.p.) with 10⁶ CFU of Aeromonas salmonicida ssp. achronogena (Asa) and as control 10 additional cod were injected with PBS as described earlier (Maier et al., 2008a). Twenty-four hours after injection the fish were dissected, samples were taken from various organs and immediately snap frozen in liquid nitrogen. Atlantic cod eggs were either taken from female cod in December (called immature eggs) after dissection of the fish or in April from running females ready to spawn (called mature eggs) and snap frozen immediately. For the protein extraction cod head kidneys were collected from adult, cultured cod and tissues were immediately snap frozen in liquid nitrogen.

In order to compare the effect of different feeding regimes on cod larvae, rearing trials were conducted at the Marine Research Institute’s Experimental Station, Stadur, Grindavik, Iceland, in the spring of 2009. Two groups of larvae (groups 1 and 2) in commercial hatchery tanks were reared on different start-feeding protocols. They were monitored from the egg stage, through the larval stage and well into the pre-juvenile stage. Rearing conditions were identical for both groups. Eggs were incubated at 7 °C and sterilized with Pyceze (Novartis Animal Vaccines Ltd.) at ~8 and ~2 days pre-hatch using 100 parts per million (ppm) for 30 min. Upon completed hatching, two hatchery tanks (3200L) were stocked with 170,000 larvae each. Antibiotics (25 ppm Lincospectin, Pfizer Ltd.) were routinely administered on 1, 4 and 7 dph. Light was provided at 3 dph (150 lux at surface) and increased incrementally to 700 lux from 10 dph onwards. The photoperiod was initially fixed at a 18:6 ratio (Light:Dark hours), but adjusted to 24:0 from 22 dph onwards. Rearing temperatures were held at 8–10°C until 7 dph and gradually increased to 12–13°C from 34 dph onwards. The water flow was 3.5 L/min at 3 dph and increased incrementally to 13 L/min from 45 dph onwards. Concentrated Nannochloropsis algae (Instant algae, Reed Mariculture) were added for shading twice daily (60–80 mL per day).

The group 1 larvae were fed enriched (Algamac 3000, Aquafuana Bio-Marine) rotifers (Brachionus plicatilis) from 3 to 30 dph (26–75 million per day), Artemia (Artemia salina) nauplii and metanauplii from 16 to 50 dph (0.5–60 million per day) and dry feed (Gemma Micro Diamond, Skretting) from 20 dph onwards. The group 2 larvae received the same rotifer protocol but were not fed Artemia. Dry feeding commenced at 14 dph in this group (Fig. 6C).

Larvae were collected directly into RNAlater (Ambion) at the days indicated in Fig. 6. The number of larvae was different at various sampling timepoints, with 50 larvae for the first dph, approximately 10–20 larvae until day 40 and thereafter 2–4 pre-juveniles for each timepoint.

2.2. RNA isolation, cDNA synthesis and quantitative real-time PCR

Atlantic cod larvae were homogenized with a Pellet Pestle Cordless Motor (Kimble-Kontes) and RNA was extracted using TRI Reagent (Ambion). For the infection and egg studies approximately 50 mg of frozen tissue was ground with a pestle in a mortar and total RNA was extracted using TRI Reagent. RNA was pre-
pared using manufacturer's instructions and dissolved in 30 μL RNase-free water. To minimise contamination with DNA, 10 μg of RNA were treated with DNase (New England Biolabs), followed by ethanol precipitation and the resulting RNA pellet was dissolved in 20 μL RNase-free water. Quantity and quality of the resulting RNA was assessed using NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). The integrity of the RNA from approximately half of the samples was evaluated by agarose gel electrophoresis.

For the following real-time analysis cDNA was prepared from 300 ng of RNA using the First Strand cDNA synthesis kit (Fermentas), according to manufacturer’s protocol. The absence of genomic DNA was confirmed by preparing several samples without reverse transcriptase in the reaction. The cDNA was diluted 10-fold (infection study) and 100-fold (larvae study) in water for further use in quantitative real-time PCR. The RNA (1000 ng) extracted from cod eggs was reverse transcribed into cDNA and amplified by PCR with cathelicidin and β-actin specific primers as described previously (Maier et al., 2008a).

Real-time PCR was performed in 96 well-PCR plates on an ABI 7500 real-time PCR System (Applied Biosystems) using Power SYBR green PCR Master Mix as recommended by the manufacturer (Applied Biosystems) with the exception of using 10 μL final reaction volume. Reactions were run in duplicates. Real-time primers for cathelicidin were designed in PerlPrimer (Marshall, 2004) and the sequence for the forward primer used was: 5′GCGTGAAACTGTATACCGAGGC3′ and the reverse primer 5′AATCTTGTGGCAATGTC3′. The relative expression of cathelicidin was measured against one or two reference genes encoding ubiquitin and ribosomal protein S 9 (RPS 9) as these genes had been shown to have stable expression levels in both adult cod (Olsvik et al., 2008) and larvae (Saele et al., 2009). The sequences of the primers were obtained from these studies. Efficiencies for all primers and tissues were calculated and shown to lie within the 90–110% required in order to employ the 2−ΔΔCt method (Livak and Schmittgen, 2001) for the calculation of the fold differences in expression. For the infection study the expression detected in skin from healthy fish was set as value 1 and all other tissues were compared to this value. One representative experiment is shown, corresponding to one healthy and one infected individual. In the larvae analysis the reference gene encoding ubiquitin was used and the expression detected on the hatching day (0 dph) was set as value 1. Other measurements of mRNA expression were compared to this value. Statistical differences between tissues and treatments were assessed from three cod for each condition, with a one-tailed t-test unequal variance. Values below 0.05 were considered significant.

2.3. Peptide isolation

Head kidney tissue from healthy farmed cod was collected in liquid nitrogen and kept at −80 °C until used. Frozen tissue (approximately 6 g) was ground with a pestle and mortar and proteins were extracted from the sample by overnight shaking in 60% acetonitrile/1% TFA at 4 °C. Supernatants were subsequently collected by centrifuging for 40 min at 10,000 × g and lyophilised. Oasis HLB cartridges (Waters) were used for desalting and enrichment of peptides and proteins in the supernatants. The cartridges were activated with 100% acetonitrile and then equilibrated with reverse three washes of 0.1% TFA. The samples were dissolved in 0.1% TFA and applied to the cartridges. The cartridges were rinsed with 0.1% TFA and then with 10% acetonitrile in 0.1% TFA. Proteins were eluted from the cartridges with 80% acetonitrile in 0.1% TFA and the samples were lyophilised before further processing. Subsequently a 5 mL HiTrap CM FF Sepharose column (GE Healthcare) was employed for cation exchange chromatography. The samples were dissolved in starting buffer (0.2 M acetic acid) and elution took place in a segmented gradient of 1.5 M ammonium acetate and a flow rate of 0.5 mL/min. Eluted fractions were lyophilised, reconstituted in water and an aliquot of each fraction was screened by Western blot analysis for the presence of cod cathelicidin. Lyophilised fractions positive for cathelicidin were pooled, dissolved in starting buffer (2% acetonitrile/0.1% TFA) and subjected to reversed phase chromatography. This was carried out on a Discovery C18 HPLC column (Sigma–Aldrich) in a linear gradient of 80% acetonitrile in 0.1% TFA and a flow rate of 1 mL/min. Aliquots of eluted and lyophilised fractions were screened by Western blotting.

Chromatographic runs were monitored at 280 and 214 nm. The chromatograms and silver stained protein gels were used to assess purity of the eluted fractions.

2.4. Amino acid sequence analysis and mass spectrometry

A part of a chromatographic reversed phase fraction containing only the cathelicidin peptide, as determined by SDS PAGE, silver staining and Western blot analysis, was analysed for a mass value with MALDI-MS utilising an Applied Biosystems Voyager DE-PRO instrument.

For N-terminal sequence analysis the remainder of the above chromatographic fraction, was applied onto Applied Biosystems procise cLC sequencer (PE Applied Biosystems). The sequencing was confirmed using a second independent cathelicidin peptide isolation.

2.5. Immunoassays

An affinity purified antibody against a synthetic fragment of the cod cathelicidin putative peptide (sequence: SRSGRGSKGGRGG, underlined in Fig. 1) was generated in rabbit by GenScript. Lyophilised chromatographic fractions were reconstituted in water, mixed with NuPAGE LDS sample buffer (Invitrogen) containing 10% mercaptoethanol, heated at 70 °C for 10 min and loaded onto NuPAGE Novex Bis-Tris gels (Invitrogen) Electrophoresis was performed at 90 V for 10 min and at 120 V for 50 min. The ColorPlus prestained protein marker (New England Biolabs) was used for all SDS-PAGE runs. Proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore) for 120 min at 40 V. The blotted membrane was then blocked in 1% fat-free milk in PBS with 0.05% Tween 20 for 1 h at room temperature prior to incubating with the primary antibody (0.1 μg/mL) overnight at 4 °C in 0.1% fat-free milk. The next day after three 10-min washes in PBS containing 0.05% Tween 20 the membrane was incubated with an anti-rabbit secondary antibody conjugated with horse radish peroxidise (Sigma–Aldrich) (diluted 1:10,000) in 0.1% fat-free milk for 2 h at room temperature. The protein bands were visualised with ECL Plus Western Blotting Detection Reagent (GE Healthcare) on a Typhoon 9400 scanner (GE Healthcare).

For detection of cathelicidin in crude tissue, organs were extracted in 60% acetonitrile/1% TFA at 4 °C overnight, desalted and concentrated using Oasis HLB cartridges (Waters Corp.) as described above. The resulting pellet after freeze-drying was reconstituted in water and the protein concentration was measured on the NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies). Equal protein concentrations (10–30 μg) were loaded onto NuPAGE Novex Bis-Tris Gels (Invitrogen). Blotting and visualisation was performed as described above.

2.6. Peptide synthesis and antimicrobial assays

The characterised 67-residue cod cathelicidin peptide (Table 1) was produced synthetically (GenScript) with over 95% purity. The synthetic cathelicidin peptide and reversed phase chromatogra-
phy fractions containing cod cathelicidin peptide were assayed for antimicrobial activity against Bacillus megaterium strain Bm11. Single colonies of Bm11 were picked and used to inoculate 20 mL Luria Bertani (LB) medium. Bacteria were grown at 37 °C until the optical density reached 0.6 (at 595 nm). Thin 1% agarose plates (1 mm) of LB media containing 3 × 10^8 bacteria/ml were poured and wells 3 mm in diameter were punched in the agarose layer. Three microlitres of the tissue extracts (10–20 µg) or the synthetic peptide dissolved in water at indicated concentrations were loaded into each well. The agarose plates were incubated at 37 °C and the following day inhibition zone diameters were measured.

The minimal inhibitory concentration (MIC) of the synthetic cathelicidin peptide was determined using colony counting assays as described elsewhere (van Dijk et al., 2007). Briefly, 10 µL of a log phase bacterial suspension of Bm11 or Escherichia coli strain D21 adjusted to approximately 10^6 bacteria/ml were mixed with 10 µL of a 2-fold serial dilution of the synthetic peptide ranging from 20 µM to 0.3125 µM. The incubation took place in diluted LB medium (1000-fold diluted in water) at 37 °C for 2 h and thereafter serial 10× dilutions of the suspensions were made and spread out on LB agar plates. Bacterial growth was assessed after an overnight incubation at 37 °C and the lowest concentration of synthetic cathelicidin peptide, which inhibited bacterial growth, was deemed the MIC.

3. Results

3.1. Expression of Atlantic cod cathelicidin mRNA

We have previously shown that bacterial infections of fish cause an increased expression of cathelicidins (Maier et al., 2008a). The fish pathogenic bacterium Aeromonas salmonicida ssp. achronomgenes (Asa) causes atypical furunculosis, a systemic disease in many fish species (Gudmundsdottir and Bjornsdottir, 2007). In the present study cathelicidin expression in healthy and Asa infected cod was compared using real time PCR. Increased cathelicidin expression was observed in all seven tissues examined, after injection of the cod with bacteria, compared to fish injected with PBS alone (Fig. 2). Due to the high individual variation a representative experiment of one individual for each condition is shown. For the statistical analysis three healthy fish and three infected fish were compared. A one-tailed t-test with unequal variance showed a statistically significant increase in cathelicidin expression in all tissues (p < 0.05) analysed except for gills, when comparing infected to healthy fish. Constitutive expression was found to be most pronounced in kidney and spleen. The high basal expression of cathelicidin in kidney or spleen was found to be statistically significant (p < 0.01 for kidney and p < 0.05 for spleen), when compared to all other tissues. Due to the relative high expression of cathelicidin in uninfected kidney and due to the availability and size of the organ, this tissue was used in the subsequent isolation of the mature cathelicidin peptide.

3.2. Isolation of the mature peptide of cod cathelicidin

Proteins were extracted from healthy cod kidneys and initially separated by high performance liquid chromatography (HPLC) using a cation exchange column (Fig. 3A). Fractions were screened for the presence of cathelicidin by Western blot analysis, with an antibody raised against a synthetic N-terminal fragment of the cathelicidin peptide (Fig. 1, underlined amino acids). The resulting positive fractions were pooled, separated by reversed phase (RP) HPLC (Fig. 3B) and cathelicidin was again detected by Western blot analysis. The presence of cathelicidin in the fractions was confirmed by peptide-mass fingerprinting using endoproteinase Lys-C: a positive fraction was separated by SDS-PAGE and silver stained. The band around 7 kDa, predicted to correspond to the mature cathelicidin peptide from the respective Western blot, was excised from the gel and digested with Lys-C. Three of the expected four fragments were identified by mass spectrometry (data not shown). The fourth band corresponding to the N-terminus of the predicted peptide could not be detected. To identify the exact N-terminal sequence of the isolated cod cathelicidin peptide and thereby the processing site, fractions containing cathelicidin were further analysed. Cathelicidin was eluted from the reversed phase column in fractions 19 and 20 (Fig. 3B). Although fraction 20 had larger amounts of cathelicidin protein, silver staining revealed that fraction 19 contained only one protein, while fraction 20 contained at least five additional proteins (data not shown). The pure protein in fraction 19 was analysed by MALDI-MS and gave a mass value of 6239 Da. Sequence analysis of the first 10 amino acid residues of the peptide in this fraction and from another independent preparation revealed the following sequence: SRSGRGSGXXG. This sequence showed that the N-terminus of the mature peptide started after the fifth amino acid in exon 4 (see Fig. 1). Confirming a processing site between Arg 126 and Ser 127. A cleavage at this site results in a mature peptide with a predicted mass value of 6235 Da and this is in agreement with the measured mass value of 6239 Da. Key characteristics of the cod cathelicidin are summarised in Table 1.

Table 1

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<td>6235</td>
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Fig. 2. Cathelicidin expression is increased during infection. Cod were injected with PBS (Healthy) or Aeromonas salmonicida ssp. achronomgenes (Infected) and incubated for 24 h. Tissues were sampled as indicated. Relative cathelicidin expression was analysed using quantitative real time PCR and the fold induction was compared to expression of healthy skin. Ubiquitin and RPS9 were used as internal reference and a representative result from one individual fish for each treatment is shown.
3.3. Antimicrobial activity of cod cathelicidin and its tissue expression

For the activity studies, cathelicidin was purified from cod head kidneys using cation exchange and reversed phase chromatography as described above. Fractions containing cathelicidin were identified (Fig. 4A) and used to examine the antibacterial activity against B. megaterium (Bm11). In this purification run the peptide eluted in fraction 23 and 24 from the reversed phase column and pronounced antimicrobial activity was observed in the fractions positive for cathelicidin. The subsequent fractions, but not the previous fractions were also shown to have antibacterial activity, due to additional antibacterial component(s) in the preparation. Fractions 25 and onwards also had a much higher abundance of proteins than previous fractions (corresponding to fraction 21 and onwards in Fig. 3B, upper inset). The antimicrobial activity of the mature cod cathelicidin peptide was confirmed with a synthetic peptide and a dose–response curve against the gram-positive bacterium Bm11 is shown in Fig. 4C. The minimal inhibitory concentration (MIC) of the peptide against Bm11 was determined to be 5 μM, while the MIC against the gram-negative bacterium E. coli D21 was 2.5 μM.

Cathelicidin peptide expression in different tissues from A. salmonicida infected cod was examined by Western blot analysis and a band of 7 kDa, corresponding to the mature peptide, was detected (Fig. 5). The expression was most pronounced in kidney and low expression was also observed in spleen.

3.4. Expression profile of cathelicidin mRNA during cod larvae development

In order to study cathelicidin expression during Atlantic cod early development, cod eggs were examined for transcripts and cathelicidin mRNA was detected in both immature and mature, unfertilised eggs (Fig. 6A). Subsequently cathelicidin expression
Fig. 5. Tissue distribution of the cod cathelicidin peptide. Western blot analysis detected the peptide in kidney and spleen of infected fish (indicated by arrows). K = kidney, L = liver, Sp = spleen, Sk = skin, PC = pyloric caeca, I = intestine, H = heart, G = gills. The antigen (14 amino acid residues long) was used as positive control for the antibody (+). Sizes of the protein marker (in kDa) are indicated to the left. Additional bands seen are interpreted as non-specific binding of the antibody.

was followed by quantitative real-time PCR for 68 dph in the larvae. Cathelicidin mRNA was found to be present at low levels in newly hatched larvae and its expression varied over the 68 days examined (Fig. 6B). A pilot study on larvae had indicated that the increase in cathelicidin expression was partly linked to the food of the larvae (data not shown). Therefore a study was set up to approach a direct effect of food on cathelicidin expression during development. One group of larvae was fed by a normal regime including mainly live rotifers (small zooplankton), live brine shrimp (Artemia sp.) and finally dry feed (group 1), while the other group (group 2) did not get any Artemia. The length growth rate of the larvae following the normal regime (group 1) was found to be approximately 3.5% per day, with a mean length of the pre-juveniles at 49 dph of 26.3 mm. The larvae fed without Artemia (group 2) also started off with a length growth rate of 3.5% but following premature dry feed weaning the rate decreased to only 2.5% per day and the mean length of the pre-juveniles at 49 dph was only 15.6 mm. Similarly the survival of the larvae in group 1 was 14% (50 dph), while the survival for group 2 was 6% (55 dph). Due to the lower survival in group 2 than in group 1 (10,000 versus 23,000 pre-juveniles, respectively) the amount of dry feed was adjusted accordingly (Fig. 6C). Comparison of the cathelicidin expression between the two groups showed a different expression profile. Both groups had an about 15–20 fold increase in cathelicidin expression around day 11 post hatch compared to 0 dph (Fig. 6B and C). Larvae raised on the normal food regime had an about 40-fold increase in cathelicidin expression at day 50, while a similar increase was seen in group 2 at day 37 post hatch. This increase in cathelicidin expression coincided with dry feed weaning in both groups.

4. Discussion

The primary aim of this study was to isolate the mature active peptide of cod cathelicidin from cod tissue. By three chromatographic separations we were able to isolate and characterise the mature cathelicidin peptide thereby identifying the processing site of the precursor protein. The cod cathelicidin peptide was found to consist of 67 amino acid residues, two residues shorter than initially predicted (Maier et al., 2008a). The peptide was detected in kidney and spleen of infected cod, both hematopoietic tissues. However so far we have not been able to detect the peptide in peripheral blood; serum or cells by Western blot analysis. Cathelicidin mRNA on the other hand could be detected in blood (data not shown) as well as in most tissues especially after infection. We have previously observed differences in mRNA and peptide levels for cathelicidin...
and proteinase 3 (Murakami et al., 2004; Panyutich et al., 1997; Sousa Abreu et al., 2009). The size of the cod cathelicidin peptide estimated by Western blot was larger than the confirmed molecular mass and this is thought to be due to the fact that cationic peptides may run more slowly in SDS-PAGE. Similar results have been reported by other groups for cationic peptides (Corrales et al., 2006), but so far not in cod or salmonids. The predicted proprotein convertase site found in all fish cathelicidins has the sequence R–X–R–R and we do not demonstrate here that the cod cathelicidin peptide is processed after this site. Similarly, the mature peptides of the ancient and modified cathelicidins in hagfish are processed after an Arg tetrad (Uzzell et al., 2003). These discoveries suggest that a proprotein convertase might be the processing enzyme, releasing the mature cathelicidin peptide from the proform in fish. Further cleavage of the cod cathelicidin mature peptide into smaller fragments could also occur and this would explain the low levels of the 6.2 kDa peptide detected. The antibody used in this study was raised against the N-terminal 14 amino acid residues of the mature peptide (Fig. 1). Therefore only peptides containing this region could be detected. Interestingly, further processing of the mature cathelicidin peptides has been detected in human skin (Murakami et al., 2004).

The peptide characterised in this study is very cationic and exhibits pronounced antibacterial activity. Antibacterial activity observed in fractions in which cathelicidin was not detected, originated from additional antimicrobial components, such as histones (Bergsson et al., 2005; Patat et al., 2004). The synthetic peptide confirmed the antimicrobial activity of the mature cod cathelicidin peptide against both gram-positive and gram-negative bacteria. In conclusion the increased production of cod cathelicidin during infection and the antibacterial activity of the mature peptide, suggests that cathelicidin is a contributing defence molecule in the innate immune system of Atlantic cod.

A second aim of this study was to follow the cathelicidin expression during the development of cod larvae. Cathelicidin mRNA was found to be present from very early on in development. Indeed the eggs might be already protected, as we have detected cathelicidin transcripts in immature, developing eggs as well as eggs ready to be fertilised. The presence of cathelicidin mRNA at this early stage is likely due to the transfer of maternal RNA. Transfer of maternal proteins (Magnadottir et al., 2004) and/or mRNA (Seppola et al., 2009) has been observed in several studies on cod eggs and larvae. Cathelicidin expression increased after hatching and first feeding with rotifers. The zooplankton given as live feed is grown in elevated temperatures and very susceptible to bacterial contamination (Olafsen, 2001). We suggest that the bacteria in the live feed might cause the increase in cathelicidin transcription observed. Interestingly, in larvae examined in this study, but not in our initial pilot study, an antimicrobial compound (Sanocare ACE, IVE) was used to wash the Artemia before feeding the cod larvae. This change in rearing practice coincided with a (4- to 6-fold) lower level of cathelicidin transcription around 30 dph than seen before. Comparison of two different rearing methods showed remarkable differences in cathelicidin expression during 30–60 dph. Larvae fed without Artemia also exhibited an about 40-fold increase in cathelicidin expression, but this increase was much earlier in the development i.e. at around 37 dph. The enhancement of cathelicidin expression coincided with the end of live feeding and the increase in dry feeding. Cod larvae prefer to be fed on live prey if supplied and therefore the termination of live feeding is a significant step for the developing pre-juveniles (Agnar Steinarsson, unpublished observation). Group 1 pre-juveniles were moved from one tank to another at day 49 post hatch, therefore an excess of Artemia were given at this timepoint in order to settle the larvae (Fig. 6B, 50 dph). However, due to the size of the larvae at this stage, this is thought to have little effect on the overall food consumption of the pre-juveniles. Group 2 pre-juveniles were moved at 55 dph and did not show an enhancement in cathelicidin expression at this timepoint. Therefore stress during movement is not thought to have an effect on cathelicidin expression in the larvae. The large increase in cathelicidin transcription observed in both groups could either be due to the stress during food exchange or alternatively due to components in the dry feed.

In conclusion, our results suggest that the innate immune system of cod larvae is active and responds to external stimuli. Results from a Norwegian group that analysed the transcription of several immune genes during ontogeny (Seppola et al., 2009) showed an increase in transcription of the antimicrobial peptides hepcidin and cathelicidin, comparable to our study for cathelicidin during the normal feeding regime. Additional immune factors such as pentraxin and lysozyme on the other hand showed very low levels during the first weeks after hatching and exhibited a gradual increase in transcription during development (Seppola et al., 2009).

Our study emphasises the fact that the larvae are sensitive to changes in food composition. Probiotic bacteria have been used in aquaculture in recent years to control diseases and shown to have many beneficial effects on fish health, including increased growth and reduced mortality after infection (Olafsen, 2001; Perez et al., 2010). How these probiotic bacteria work is not fully understood, but might include enhancement of the innate immune system through pattern recognition receptors, such as the Toll-like receptors and downstream signalling components (Magnadottir, 2010). We have previously shown that bacterial products can cause an increase in cathelicidin transcription in fish cells, independent of the bacterial viability or virulence (Maier et al., 2008b). Our present results may explain the beneficial effects seen by treatment of the larvae with probiotics, since the increase in production of the antimicrobial peptide cathelicidin would strengthen the innate immune system of the larvae and increase their survival during disease.

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