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Research Brief

Akirins in sea lice: First steps towards a deeper understanding

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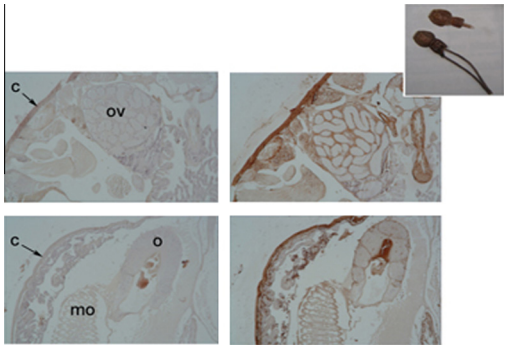
HIGHLIGHTS

- We obtained the my32-Cr cDNA ends by RACE-PCR.
- We cloned the uncharacterized *L. salmonis* akirin-2 (my32-Ls) based on EST sequence.
- These genes/proteins have the main characteristics of akirins from invertebrates.
- 3D models predicted a α-helical structure in the 'non-disordered' C-terminal region.
- Immunization with purified my32-Ls elicited a specific antibody response in mice and fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Sea lice (*Copepoda, Caligidae*) are the most widely distributed marine pathogens in the salmon industry. Vaccination could be an environmentally friendly alternative for sea lice control; however, research on the development of such vaccines is still at an early stage of development. Recent results have suggested that subolesin/akirin/my32 are good candidate antigens for the control of arthropod infestations, including sea lice, but background knowledge about these genes in crustaceans is limited. Herein, we characterize the my32 gene/protein from two important sea lice species, *Caligus rogercresseyi* and *Lepeophtheirus salmonis*, based on cDNA sequence isolation, phylogenetic relationships, three dimensional structure prediction and expression analysis. The results show that these genes/proteins have the main characteristics of akirins from invertebrates. In addition, immunization with purified recombinant my32 from *L. salmonis* elicited a specific antibody response in mice and fish. These results provide an improvement to our current knowledge about my32 proteins and their potential use as vaccine candidates against sea lice in fish.

1. Introduction

Infestations with sea lice (*Copepoda*, *Caligidae*) have grown to become the greatest challenge in salmon aquaculture. The salmon louse *Lepeophtheirus salmonis* is responsible for the main disease outbreaks on salmon farms in the northern hemisphere. In the southern hemisphere, *Caligus rogercresseyi* is the most important species affecting Chile's salmon industry (Johnson and Fast, 2004; Bravo, 2010).

A wide range of medicines has been used to control sea lice infestations (MacKinnon, 1997; Stone et al., 2000); however, as only a few chemicals are currently available, the potential for lice to develop resistance is high, and has been reported several times

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in both *L. salmonis* and *C. rogercresseyi* (Denholm et al., 2002; Bravo et al., 2008; Lees et al., 2008). Treatment-resistant lice, and the necessity to reduce costs and threats to the environment, highlight the importance of developing new methods for parasite control, a fact that is fully recognized by the industry, authorities and society. To date, there are no commercial vaccines available against sea lice.

Akirin (AKR) and subolesin (SUB) are evolutionarily conserved orthologous proteins that affect the expression of signal transduction and innate immune response genes in vertebrates and invertebrates (Macqueen and Johnston, 2009; Goto et al. 2008). SUB/AKR is a well characterized, protective antigen, highly conserved across vector species and thus potentially useful for the development of a broad-spectrum vaccine for the control of arthropod infestations, including hard ticks, mosquitoes, sand flies and the poultry red mite *Dermanyssus gallinae* (Almazán et al., 2005a,b; Canales et al., 2009; Galindo et al., 2009; Harrington et al. 2009).

Sea lice could also be targeted by such a vaccine if it could be proven that sea lice AKRs are conserved and induce protective immune responses in salmon. Recently, we characterized a novel gene (denoted as my32-Cr) from *C. rogercresseyi*. It has been shown that immunization with this protein elicited protection against sea lice in a vaccine trial in *Salmo salar* (Carpio et al., 2011). The objective of the present work is to continue the characterization of the sea lice my32 gene/protein. As reported in this paper, we obtained the my32-Cr full-length complementary DNA (cDNA) by the Rapid Amplification of cDNA Ends (RACE) technique. In addition, the coding region for *L. salmonis* my32 (my32-Ls) was cloned, based on the reported sequence of an expressed sequence tag (EST). Sequence analysis, three-dimensional (3D) structure prediction, phylogenetic analysis and gene/protein expression characterization support the notion that my32 is an AKR ortholog in sea lice. Finally, it was demonstrated that my32-Ls is able to induce specific antibodies after immunization in mice and fish. These results are important steps in the future development of a sea louse vaccine based on my32.

2. Materials and methods

2.1. Animals

C. rogercresseyi and *L. salmonis* were collected from cage-cultured Atlantic salmon (*Salmo salar*) in Chile and Norway, respectively. C57Bl/6 mice (12 weeks of age) were provided by CENPALAB, Cuba. Tilapia (*Oreochromis niloticus*, ~50 g) were provided by the Aquaculture Research Station at the Centre for Genetic Engineering and Biotechnology (CIGB), Cuba. Tilapia were kept alive in aerated freshwater under a 12 h light/12 h dark photoperiod. Water temperature was maintained at 26 ± 2 °C.

All experiments were conducted in accordance with Chilean, Norwegian and Cuban animal-welfare regulations.

2.2. RNA isolation

Adult female lice were collected with forceps from anaesthetized fish and stored in RNA-later (Ambion) at room temperature until RNA extraction. For RNA extraction, the RNA-later solution was discarded and the Tri-reagent (Promega) was added. Then, the tissue was homogenized by means of mortar and pestle. RNA isolation was performed according to the manufacturer's instructions. Total RNA concentration and quality were determined by UV absorbance at 260 nm, the A_{260}/A_{280} ratio and by denaturing gel electrophoresis. Contaminating genomic DNA was eliminated by DNase I digestion (Invitrogen).

2.3. Cloning of my32-Cr cDNA ends and my32-Ls cDNA

The characterization of 5′ and 3′ ends of my32-Cr was carried out by RACE. To obtain the 3′ end, the first-strand cDNA synthesis reaction contained the components of the Promega Reverse Transcription System (RT) kit (Promega, USA), with the exception of a modified oligo dT (OdTmod) (Table 1). For the first round of Polymerase Chain Reaction (PCR) amplification, the A gene-specific primer and the OdTmod were used. The reaction was performed using 50 pM of primers and the master Mix PCR (Promega, USA). PCR consisted of an initial denaturing step at 95 °C for 5 min, 35 amplification cycles (denaturing at 95 °C for 30 s, annealing temperature at 63 °C, and extension at 72 °C for 3 min), and a final extension step at 72 °C for 5 min. For a nested PCR, 2 μL of the first round PCR product and the pair of primers B-OdTmod were used. PCR conditions were identical to those in the first reaction. DNA fragments were purified, cloned and sequenced. The recombinant plasmids were isolated using the Minipreps DNA purification system (Promega). The nucleotide sequences were determined by the standard dye terminator chemistry using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

To obtain the 5′ end of the my32-Cr gene, a homopolymeric dC tail was added to the 5′ end of cDNA using Terminal Deoxynucleotidyl Tranferase (Invitrogen, USA), and the tailed cDNA then used directly for PCR amplification assays, using KOD HOT Start DNA Polymerase (Novagen). For the first round of PCR, a gene-specific reverse primer named C and an Oligo dG primer (OdG) were used. PCR consisted of an initial denaturing step at 95 °C for 2 min, 35 amplification cycles (denaturing at 95 °C for 30 s, annealing temperature at 63 °C, and extension at 68 °C for 2.5 min), and a final extension step at 68 °C for 10 min. The second round of PCR amplifications (semi-nested) were performed with the Oligo dG primer and the specific-gene reverse primer D. DNA fragments were cloned and sequenced as described above.

For cloning of my32-Ls cDNA, five micrograms of *L. salmonis* RNA were reverse-transcribed into cDNA using the RT System (Promega), according to the manufacturer's instructions. The primer combinations E-F, designed based on the reported EST sequence (GenBank ADD38399) (Table 1), were used to obtain the cDNA sequence of my32-Ls. The amplifications were performed using *Pfx* polymerase (Invitrogen) according the manufacturer's instructions and the following cycling conditions: initial denaturation at 95 °C for 2 min, 30 cycles of denaturing at 95 °C for 30 s, annealing temperature at 55 °C for 30 s and extension at 68 °C for 1 min. The purified PCR products were cloned into pMOS-Blue after phosphorylation. DNA fragments were cloned and sequenced.

Table 1
Primers used to amplify my32-Cr cDNA ends and my32-Ls cDNA.

Primers	Sequence 5′-3′[a]	bp	Direction
OdTmod	GGCCACGCGTCGACTAGTAC$(T)_{17}$	37	Reverse
A	GGCTTCACCAACACATTCACAAAG	24	Forward
B	CCACTTTCACGCTCAAGCAAATGACGC	27	Forward
OdG	ATAAGAATGCGGCCGCTAAA$(G)_{15}$	36	Forward
C	AGCAGTAGCAGCAGCCAATGTTCC	24	Reverse
D	TGCACAACGGGGGACTCTCTG	21	Reverse
E	**CCATGG**CTTGTGTTACTCTTAAACG	25	Forward
F	TTAGGACAAATAACTCGGAGTTT	23	Reverse
G	TCTTAAACGTCCCTGCGATT	20	Forward
H	ACTTGATCCGTACGTTCTTT	20	Reverse
I	GATGAAGCCCAATCCAAGAGAG	22	Forward
J	AGGCATAGAGGGAGAGGACAG	21	Reverse
K	**AAGCTT**ATAACTCGGAGTTTGGGAGTC	27	Reverse

[a] Restriction endonuclease sites are indicated in bold.