

Caspase-1 and IL-1 β Processing in a Teleost Fish

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Abstract

Interleukine-1 β (IL-1 β) is the most studied pro-inflammatory cytokine, playing a central role in the generation of systemic and local responses to infection, injury, and immunological challenges. In mammals, IL-1 β is synthesized as an inactive 31 kDa precursor that is cleaved by caspase-1 generating a 17.5 kDa secreted active mature form. The caspase-1 cleavage site strictly conserved in all mammalian IL-1 β sequences is absent in IL-1 β sequences reported for non-mammalian vertebrates. Recently, fish caspase-1 orthologues have been identified in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) but very little is known regarding their processing and activity. In this work it is shown that sea bass caspase-1 auto-processing is similar to that of the human enzyme, resulting in active p24/p10 and p20/p10 heterodimers. Moreover, the presence of alternatively spliced variants of caspase-1 in sea bass is reported. The existence of caspase-1 isoforms in fish and in mammals suggests that they have been evolutionarily maintained and therefore are likely to play a regulatory role in the inflammatory response, as shown for other caspases. Finally, it is shown that sea bass and avian IL-1 β are specifically cleaved by caspase-1 at different but phylogenetically conserved aspartates, distinct from the cleavage site of mammalian IL-1 β .

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Introduction

IL-1 β is the most studied pro-inflammatory cytokine, much due to its role in mediating auto-inflammatory diseases (see e.g., [1], [2], [3], [4]). It is mainly produced by activated macrophages, monocytes, and dendritic cells and affects almost every cell type, playing a central role in the generation of systemic and local responses to infection, injury, and immunological challenges, although it can also have detrimental effects (see e.g., [5], [6], [1], [2], [3], [4]). IL-1 β exerts its activity by binding to IL-1 type I receptor (IL-1RI), which then recruits IL-1 receptor accessory protein (IL-1RAP) forming a complex that triggers a series of phosphorylation events leading to the activation of I κ B kinase (IKK) and MAPK pathways (see e.g., [7], [4], [8]). These signaling pathways activate in turn the transcription factors NF- κ B and AP-1, resulting in the induction of genes encoding chemokines, cytokines, acute-phase proteins, cell adhesion molecules, and enzymes involved in the production of small pro-inflammatory substances [5]. In mammals, IL-1 β is synthesized as an inactive 31 kDa signal peptide-less precursor molecule (proIL-1 β) [9], [10]. In humans and mice, caspase-1, also known as ICE (interleukin-1 β converting enzyme) [11], [12], [13], specifically cleaves proIL-1 β after aspartate 116 and 117, respectively, yielding a C-terminal 17.5 kDa secreted active form [14], [15], [11], [12], [16], [17], [18]. Non-caspase-1-mediated cleavage mechanisms generating different active forms of IL-1 β have also been described (reviewed in [2], [19], [4], [5]).

IL-1 β has been identified in several fish species [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30]. However, although it was reported that processing of IL-1 β by a gilthead sea bream fibroblast cell line is abrogated by a specific caspase-1 inhibitor [31], neither direct evidence for the participation of caspase-1 in this processing event was provided nor the cleavage site in pro IL-1 β was defined. This last issue is particularly important because the caspase-1 cleavage site reported for mammalian proIL-1 β is absent in the proIL-1 β sequences of non-mammalian vertebrates. Putative trout [32], sea bass [33], and chicken [34], [35], [36] recombinant mature IL-1 β starting respectively at Ala⁹⁵, Ala⁸⁶, and Ala¹⁰⁶, equivalent sequence-wise to the N-terminal residue of mature mammalian IL-1 β , were shown to be biologically active. However, in none of these is the putative mature N-terminal preceded by an aspartate residue, making those sites rather improbable caspase-1 cleavage sequences. Furthermore, it was shown that in trout a 29 kDa IL-1 β precursor expressed in RTS-11 cells was processed to a 24 kDa peptide [37], whereas a 15 kDa band has been detected by Western blotting with an anti-IL-1 β antibody of phytohemagglutinin activated carp leucocyte culture supernatant [38], but the mechanism and exact cleavage site of processing were not clarified [39], [40], [41].

Caspase-1 is the prototype of a family of inflammatory caspases that contain a caspase recruitment domain (CARD) within their amino-terminal prodomains (see e.g., [5], [42]). Normally, it exists in the cytoplasm as a 45 kDa inactive precursor [43], being

activated by CARD oligomerization in a multiprotein complex known as the inflammasome (see e.g., [44], [45], [46], [47], [48], [49], [50], [51]). Once activated, caspase-1 processes the pro-inflammatory cytokines starting an inflammatory response. Caspase-1 was recently found to be also involved in pyroptosis, a rapid active cell death programme characterized by early plasma-membrane rupture and release of proinflammatory intracellular contents ([52]; reviewed in [53], [54]). In humans, caspase-1 is activated by cleavage after Asp¹⁰³, D¹¹⁹, D²⁹⁷ and D³¹⁶, releasing the 11.5 kDa prodomain and a 2 kDa linker peptide and originating the active heterodimer p20/p10 [17], [55], [18], [56], [57].

Fish inflammatory caspases have been primarily sequenced in zebrafish [58] and sea bass (GenBank accession no.: DQ198377), the later being a caspase-1 homologue that has recently also been reported for sea bream [59], but there is still no functional data from these enzymes. Here, the characterization of the sea bass caspase-1 homologue, its processing and activity are reported. It is shown that sea bass caspase-1 auto-processing follows the mechanism described for the mammalian enzyme, yielding p24/p10 and p20/10 active heterodimers. In addition, three other caspase-1 isoforms have been identified. Finally, analysis of sea bass IL-1 β processing by active sea bass caspase-1 revealed that the cleavage of the cytokine occurs at a phylogenetically conserved aspartate residue, distinct from the cleavage site in birds.

Materials and Methods

Ethics Statement

This study was carried out in accordance with European and Portuguese legislation for the use of animals for scientific purposes (Directive 86/609/EEC; Decreto-Lei 129/92; Portaria 1005/92). The work was approved by Direcção Geral de Veterinária, the Portuguese authority for animal protection.

Fish

Sea bass (*Dicentrarchus labrax*) were kept in a recirculating, ozone-treated salt-water (25–30‰) system at 20 \pm 1°C, and fed at a ratio of 2% body weight per day. For organ collection fish were euthanized with 2-phenoxyethanol (Panreac; >5 ml/10 L).

Cloning and sequencing of caspase-1

Messenger RNA was purified with the MicroPoly(A)PureTM kit (Ambion[®]) and transcribed to cDNA using the SuperscriptTM II First-Strand Synthesis System (Invitrogen). PCRs were done according to standard procedures. For amplifying full length sequences Pfu DNA polymerase was used in the reactions. DNA sequencing was performed using primers detailed in Table S1. Complementary DNAs used for 5' Rapid Amplification of cDNA Ends (5'RACE) experiments were purified using High Pure PCR Product Purification Kit reagents (Roche), and used with the 5'RACE System from Invitrogen (Version 2.0). The purified cDNA was dATP tailed using Recombinant Terminal Transferase (Roche).

Messenger RNA (~340 ng) extracted from the head kidney of a fish 1 h after stimulation with LPS was reverse transcribed using primer APv (Table S1). Degenerate primers were designed based on conserved regions of caspase-1 amino acid sequences from different vertebrates. The cDNA was first PCR amplified using primers CASP1FW1/CASP1RV1 followed by a semi-nested amplification using primers CASP1FW1/CASP1RV2 (Table S1). A third amplification with primers CASP1FW1/CASP1RV2 was carried out and a PCR product with the expected size (168 bp) was purified, cloned and sequenced. This sequence was used to design

the specific reverse primers DLCASP1RV1, DLCASP1RV2 and DLCASP1RV3 (Table S1), in order to obtain the 5' untranslated region (5'-UTR) using the 5'RACE strategy. The obtained PCR product was purified, cloned and sequenced and, because it did not include the start codon, the 5'RACE strategy was repeated using again the specific reverse primers DLCASP1RV1 and DLCASP1RV2, plus the reverse primer DLCASP1RV5, designed based on the sequence obtained from the previous 5'RACE (Table S1). The fragment obtained was cloned, sequenced and used to design the specific primer DLCASP1FW7 (Table S1) in the 5'-UTR. This primer was combined with primer AUAP (Table S1), in a PCR aiming to obtain the complete sequence of sea bass caspase-1. Four PCR products ranging from 1500 to 1680 bp were purified, cloned into the pGEM[®]-T Easy Vector (Promega) and sequenced.

Genomic DNA cloning and sequencing

Sea bass genomic DNA (gDNA) was isolated from erythrocytes of a single fish, as described by Stet and co-workers [60]. To obtain the complete caspase-1 gene, gDNA was amplified using the 5'-UTR primer DLCASP1FW_{EcoRI} and the 3'-UTR primer DLCASP1RV_{EcoRI} (Table S1), each containing a restriction site for EcoRI. The obtained PCR product was purified, digested with EcoRI (Fermentas) and cloned into the pET-32b vector (Novagen). Plasmid DNAs from three positive clones were purified and sequenced.

Southern blotting

Restriction enzymes EcoRI, SpeI, BamHI, NcoI, NdeI, ApaLI (all zero cutters in sea bass caspase-1 gene), HindIII (single cutter in intron 1 of sea bass caspase-1 gene), and XbaI (double cutter in intron 1 of sea bass caspase-1 gene) were used to digest sea bass gDNA from 4 fish, according to the manufacturer's instructions (Fermentas). Digestion products were subjected to 0.8% agarose gel electrophoresis and Southern blotting [61].

A fragment of the sea bass caspase-1 coding region was amplified with primers DLCASP1FW4 and DLCASP1RV6 (Table S1). The PCR product (367 bp) was purified as previously described and labelled using the Gene ImagesTM AlhPhos DirectTM Labelling and Detection System (Amersham Biosciences). For signal generation and detection the Chemiluminescent Signal Generation and Detection with CDP-*Star*TM protocol from the same kit was followed.

Sequence analysis of caspase-1

Full nucleotide and derived protein sequences from sea bass caspase-1 were compared with several caspase-1 sequences currently available at the GenBank database (www.ncbi.nlm.nih.gov). Multiple sequence alignments were made with CLUSTAL W [62]. Caspase-1 domains were based on PROSITE predictions (www.expasy.org/prosite). The Neighbour-joining phylogenetic tree was constructed using MEGA version 3.1 [63], using p-distance parameter and complete deletion of gaps. The phylogenetic tree was tested for reliability using 1000 bootstrap replications. The percentages of similarity and identity (Table S2) were calculated by pair-wise alignments by the program needle [64], with first and extending gap penalties of 10 and 0.5, respectively.

Expression analysis of caspase-1 isoforms

Complementary DNA was synthesized from total RNA extracted from spleens of 9 non-stimulated fish as described above. The cDNAs were amplified using the forward primer

DLCASPIFW11 (designed in an exon/intron boundary) for all isoforms and a reverse primer (Table S1) specific for different isoforms: isoform 1, DLCASPIRV18 (between exon 7 e 8); isoform 2, DLCASPIRV16 (intron 7); isoform 3/4m, DLCASPIRV19 (between exon 6 and 8); and isoform 4, DLCASPIRV17 (intron 5). The reverse primer DLCASPIRV19 amplifies isoforms 3 and 4 but, in combination with the primer DLCASPIFW11, different size products are obtained (989 bp for isoform 3 and 1076 bp for isoform 4). The PCR products were purified and sequenced.

Production of recombinant sea bass caspase-1 isoforms

The coding region of sea bass caspase-1 isoform 1 was amplified from the pGEM-T Easy plasmid DNA carrying the full length cDNA using specific primers DLCASPIFWNdeI and DLCASPIRVXhoI (Table S1). The PCR product was cloned into pGEM-T Easy, the plasmid DNA was digested with NdeI and XhoI (Fermentas), and the insert cloned into pET-28a (Novagen) in frame with N- and C-terminal His-tags. Recombinant sea bass caspase-1 was expressed in *E. coli* BL21 Rosetta (DE3) overnight at 37°C with 1 mM IPTG. The recombinant protein was extracted from bacterial cells as inclusion bodies and solubilised with 8 M Urea, 50 mM Tris-HCl, 0.2 M NaCl, 2 mM EDTA pH 8.0. The protein was refolded by dilution in 50 volumes of refolding buffer (50 mM Taps pH 8.5, 1.5 M Sorbitol, 1 mM TCEP, 24 mM NaCl and 1 mM KCl) overnight at 22°C. As a control, the refolding process was also performed in the presence of 100 μ M of a specific caspase-1 inhibitor (Ac-YVAD-CHO, Caspase-1 inhibitor I, Calbiochem). The refolded protein was bound to an IMAC column (HisTrap HP, GE Healthcare) in refolding buffer. The column was washed with the same buffer supplemented with 10 mM imidazole and the protein was then eluted in 4 steps with increasing concentrations of imidazole (50, 100, 250 and 500 mM). The purified protein was analyzed by SDS-PAGE and, after blotted onto polyvinylidene difluoride membrane (PVDF), fragments of 24, 20 and 10 kDa were subjected to N-terminal Edman sequencing (Proteome Factory AG, Germany), in order to determine the cleavage sites between the large and small subunits of sea bass caspase-1.

The cDNAs encoding caspase-1 isoforms 2, 3 and 4 were obtained with the forward primer DLCASPIFWNdeI together with DLISO2/3RVXhoI for isoform 2 and 3 and with DLISO4RVXhoI for isoform 4 (Table S1). The cloning strategy, expression, purification and refolding protocols were those described for isoform 1, except that isoform 2 was cloned in pET-30a (Novagen).

In vitro processing of caspase-1 isoforms

Two polyclonal antibodies directed against sea bass caspase-1 were produced (Davids Biotechnologie GmbH, Germany). An anti-p10 polyclonal antibody was raised against the peptide VHKEKDFISLLSST, and detected all caspase-1 forms containing the p10 domain. The antibody produced against the peptide QACRGNAGGAVLVSD corresponding to the carboxyl-terminal residues adjacent to the proteolytic cleavage site, detected processed forms that were cleaved at the p20 cleavage site (therefore, lacking the linker and the small subunit), but did not recognize unprocessed caspase-1.

Autoprocessing of the caspase-1 isoforms was analysed by SDS-PAGE and Western blotting. Samples of the isoforms collected after urea solubilisation or after an overnight refolding step in the presence or absence of 50–100 μ M caspase-1 inhibitor were subjected to SDS-PAGE. Proteins in the gels were stained with Coomassie brilliant blue or were transferred to nitrocellulose membranes and probed with the rabbit anti-p10 or anti-p20

antibodies (both at a 1/10000 dilution) for 1 h at room temperature. Goat anti-rabbit Ig conjugated with alkaline phosphatase (Sigma) was used as the secondary antibody and the detection performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).

The ability of active sea bass caspase-1 isoform 1 to process isoforms 2, 3 and 4 was tested by incubating 2 μ g of the purified isoforms with recombinant active caspase-1 (p24/p10) at a ratio of 2:1 (w/w) in a caspase-1 substrate buffer (0.1% v/w CHAPS, 0.1 M HEPES, 10 % v/w sucrose, 10 mM TCEP, pH 7.5) overnight at 22°C. As control, similar incubations were performed in the presence of 100 μ M caspase-1 inhibitor. The proteins in the samples were precipitated with TCA and analysed by SDS-PAGE and Western blotting using the anti-p10 and anti-p20 antibodies, as described above, or transferred to a PVDF membrane for N-terminal Edman sequencing.

Activity of recombinant sea bass caspase-1 isoforms

The activities of the refolded sea bass caspase-1 isoforms were tested by a fluorimetric assay using 0.1–2 μ g of enzyme and a specific substrate for caspase-1 (Z-YVAD-AFC, Caspase-1 substrate VI, Calbiochem). To control cleavage specificity, 100 μ M caspase-1 inhibitor was used. All assays were performed in duplicate. In each reaction, 71.5 μ l of reaction buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5) and 16 μ M of caspase-1 substrate were used. The mixtures were incubated at 25°C and the fluorescence recorded for up to 3 h with 20 min intervals on a Spectra Max Gemini XS fluorimeter (Molecular Devices) at an excitation wavelength of 405 nm and an emission wavelength of 492 nm. The results were expressed as relative fluorescence units (RFU).

In vitro production of sea bass, chicken and human IL-1 β by rabbit reticulocytes lysates

Sea bass. Plasmids harboring different IL-1 β forms have been produced as detailed below.

pET23proIL1 β [His]: the coding region of sea bass proIL-1 β was first amplified by PCR with primers DLIL1 β FWNde3 and DLIL1 β RVXho4 (Table S1) using a pGEX-4T-3 plasmid (GE Healthcare) carrying the proIL-1 β cDNA (previously amplified from sea bass head kidney) as template [23]. The PCR product was purified and cloned into the pGEM-T Easy vector. After digestion with NdeI and XhoI, the sea bass proIL-1 β was cloned into the expression vector pET-23a (Novagen) in frame with a C-terminal His-tag.

pET-23proIL1 β , for producing proIL-1 β : the histidine tag from pET-23proIL1 β [His] was removed by site-directed mutagenesis (QuickChange[®] Site-Directed Mutagenesis Kit, Stratagene) using primers listed in Table S1.

pET-23proIL1 β [D⁶⁰A], pET-23proIL1 β [D¹⁰⁰A], pET-23proIL1 β [D²⁵²A], and pET-23proIL1 β [D¹⁰⁰A/D²⁵²A], for producing mutated forms of proIL-1 β in D⁶⁰, D¹⁰⁰, D²⁵², or D¹⁰⁰/D²⁵²: each aspartate was mutated to alanine by site-directed mutagenesis using pET-23proIL1 β as template and primers listed in Table S1.

pET-23matureIL1 β , for producing mature sea bass IL-1 β (from S¹⁰¹ to Q²⁶¹ and non-tagged): produced using pET-23proIL1 β [-His] as template and primers listed in Table S1.

pET-23matureIL1 β [D²⁵²A], for producing mature sea bass IL-1 β mutated at D²⁵²: aspartate 252 was mutated to alanine by site-directed mutagenesis using pET-23matureIL1 β as template and primers listed in Table S1.

Chicken. The plasmids harboring the coding region of proIL-1 β from chicken (GGpGEX-6P-1proIL1 β), duck (APpET28-

proIL1 β), goose (AApET28proIL1 β) and turkey (MGpET28-proIL1 β) were kindly provided by Dr. Hsien-Sheng Yin and Dr. Long Huw Lee. The coding region of chicken proIL-1 β was first amplified by PCR with primers listed in Table S1 using the plasmid GGpGEX-6P-1proIL1 β as template. The PCR product was purified and cloned into the pGEM-T Easy vector. After digestion with NdeI and XhoI, the chicken proIL-1 β was cloned into the expression vector pET-23a (Novagen) rendering GGpET23proIL1 β .

GGpET23proIL1 β [D⁷⁷A], GGpET23proIL1 β [D⁸⁰A] and GGpET23proIL1 β [D⁸²A] for producing mutated forms of chicken proIL-1 β in D⁷⁷, D⁸⁰ or D⁸²: each aspartate was mutated to alanine by site-directed mutagenesis using GGpET23proIL1 β as template and primers listed in Table S1.

GGpET23mature1IL1 β , GG pET23mature2IL1 β , and GGpET23matureIL1 β for producing mature chicken IL-1 β forms starting at I¹¹⁹, I¹²² and S⁸¹: produced using GGpET23proIL1 β as template and primers listed in Table S1.

Human. HSpCMV-XL5proIL1 β [D¹¹⁶A], HSpCMV-XL5proIL1 β [D¹²⁸A] and HSpCMV-XL5proIL1 β [D¹¹⁶A/D¹²⁸A], for producing mutated forms of human proIL-1 β in D¹¹⁶, D¹²⁸ or D¹¹⁶/D¹²⁸: each aspartate was mutated to alanine by site-directed mutagenesis using HSpCMV-XL5proIL1 β (IL-1 β Human cDNA clone (NM_000576.2) in pCMV-XL5 plasmid from Origene (#SC122566)) as template and the primers listed in Table S1.

HSpET28mature1IL1 β and HSpET28mature2IL1 β , for producing mature human IL-1 β from A¹¹⁷ and from S¹²⁹: produced using HSpCMV-XL5proIL1 β as template and primers described in Table S1.

The above mentioned plasmids, harboring different IL-1 β forms, were used to synthesize the respective proteins using the TNT[®] T7 Quick Coupled Transcription/Translation kit (Promega) in the presence of [³⁵S] methionine (specific activity >1000 Ci/mmol; PerkinElmer Life Sciences) following the manufacturer's instructions.

Production of recombinant sea bass IL-1 β . His-tagged proIL-1 β (proIL-1 β [His]) was expressed in *E. coli* BL21 (DE3) CodonPlus at 37°C with 1 mM IPTG for 4 h, extracted from bacterial cells as inclusion bodies, solubilized as described for recombinant caspase-1 and purified under denaturing conditions using His-select[™] Nickel Affinity Gel (Sigma). The protein was then refolded by dialysis in three steps against 50 volumes of decreasing concentrations of urea (4 M for 12 h, 2 M for 12 h, 0 M overnight) in 10 mM sodium phosphate buffer pH 7.2 containing 184 mM NaCl at 4°C. The dialyzed protein was centrifuged at 12000 g and the supernatant analyzed by SDS- and Native-PAGE.

The cDNAs encoding mature IL-1 β (starting at S¹⁰¹) with or without a C-terminal His-tag were amplified from pET23-proIL1 β [His] with the forward primer DLIL1BFWNde4 and the reverse primers DLIL1BRVXho4 or DLIL1BRVXho1, respectively (Table S1), cloned in pET-23a and expressed in *E. coli* BL21 (DE3) CodonPlus using the approach described for proIL-1 β .

In vitro processing of proIL-1 β by caspase-1

The sea bass ³⁵S-labeled IL-1 β forms produced using rabbit reticulocyte lysates were incubated at 22°C for different periods with recombinant sea bass or human (Sigma) active caspase-1, with or without a caspase-1 substrate buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 20 mM TCEP, pH 7.5) and in the presence or absence of 100 μ M of caspase-1 inhibitor. The same procedure was done for the human and chicken ³⁵S-labeled IL-1 β forms, but incubating overnight at 37 °C and with or without 250 μ M of the

caspase-1 inhibitor. The duck, turkey and goose ³⁵S-labeled IL-1 β forms were also incubated with recombinant active sea bass caspase-1 as described for chicken IL-1 β . Samples were subjected to SDS-PAGE, and transferred to nitrocellulose membrane for autoradiography.

The ability of caspase-1 to process recombinant proIL-1 β was tested at 22°C by mixing recombinant active caspase-1 with recombinant proIL-1 β [His] at a ratio of 1:2 (v/v) in the caspase-1 substrate buffer, in the presence or absence of 20 μ M caspase-1 inhibitor. The samples were precipitated with TCA and subjected to SDS-PAGE. Proteins in the gels were stained with Coomassie brilliant blue for mass spectrometry (MS) analysis, or blotted onto a PVDF membrane for N-terminal Edman sequencing, or analyzed by Western blotting using rabbit anti-sea bass IL-1 β or anti-His (Santa Cruz) antibodies. For antibody production, dialyzed recombinant proIL-1 β [His] was further purified on a UNO[™] Q column (Bio-Rad) using 20 mM Tris pH 8.0 and 20 mM Tris pH 8.0 1.0 M NaCl as buffer system. The fractions containing proIL-1 β [His] were pooled and subjected to SDS-PAGE. The band corresponding to proIL-1 β [His] was excised from the gel and used to immunize rabbits (Davids Biotechnologie GmbH). This antibody recognizes proIL-1 β and processed IL-1 β fragments.

Sea bass IL-1 β processing in vivo

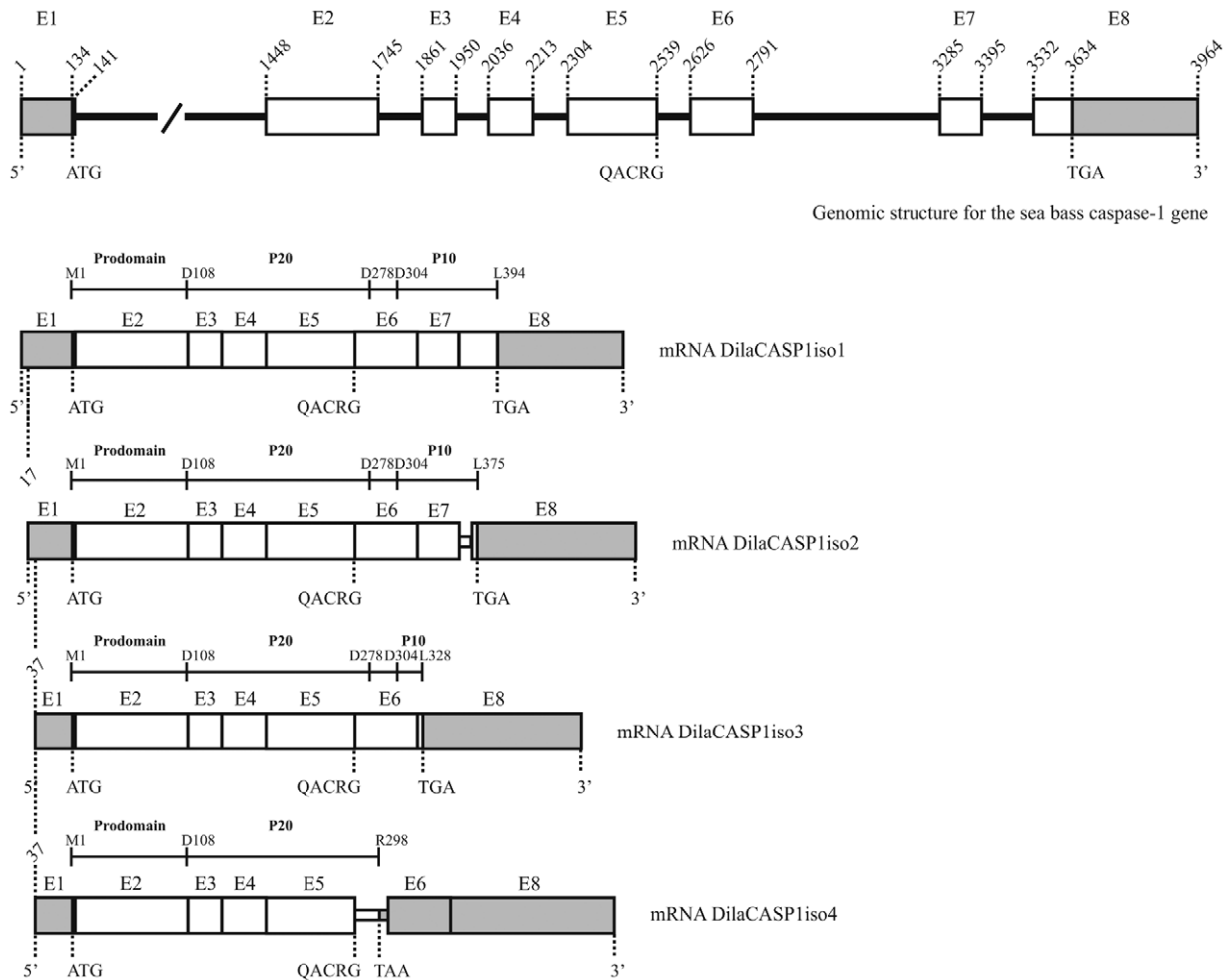
Over 20 fish were injected intraperitoneally with 200 μ l of *Photobacterium damsela piscicida* concentrated heat-inactivated extracellular products, prepared as described elsewhere [65]. After 3 h, cells from the peritoneal cavity were collected by washing with 10 ml of HBSS, centrifuged and resuspended in fresh HBSS [66], [67]. Cells were counted, diluted to a final concentration of 1×10^6 cells/ml and incubated for 3 h at 22°C in the presence or absence of 100 μ M caspase-1 inhibitor. One milliliter of cell suspension was centrifuged at 400 g, the cell pellet resuspended in gel loading buffer and the proteins in the supernatant precipitated with TCA. After SDS-PAGE, samples were analyzed by Western blotting, using the rabbit anti-IL1 β antibody, as described above.

Results

The sea bass caspase-1 transcript is alternatively spliced

The sea bass caspase-1 gene (GenBank accession number: DQ198377) was found to consist of 8 exons and 7 introns (Fig. 1), while the human caspase-1 gene has 10 exons and 9 introns (Ensembl transcript ID ENST00000260309) and presents much longer intronic regions (8266 bp vs. 2318 bp). Southern blot analysis suggested that, similar to the mouse and human caspase-1 genes [68], [69], [70], sea bass caspase-1 is a single copy gene (Fig. S1A), although four different transcripts encoding sea bass caspase-1 have been detected by RT-PCR using specific primers (Fig. 1, S1B and S1C). The corresponding deduced proteins are referred to as isoform 1–4 (DilaCASP1iso1-DilaCASP1iso4; GenBank accession numbers DQ198376, HQ398873, HQ398874 and HQ398875, respectively). DilaCASP1iso1 contains the typical caspase family p20 and p10 domain profiles and a caspase family active site signature (K²⁵⁷PKIIIQACRG), as well as a characteristic CARD-containing domain. DilaCASP1iso2 and DilaCASP1iso3 have a shorter p10 domain, whereas DilaCASP1iso4 contains a longer p20 domain and no p10 (Fig. S1B). All isoforms comprise the active center pentapeptide QACRG.

Pair-wise alignments confirmed the homology of DilaCASP1iso1 with caspase-1 sequences from other species (Table S1). The phylogenetic tree (Fig. S2) clustered the inflammatory and apoptogenic caspases into two different clades. Within the



Genomic structure for the sea bass caspase-1 gene

Figure 1. Schematic representation of the genomic organization and alternative splicing of sea bass caspase-1. The exons are represented by boxes (E1-E8) and introns by a solid line (introns larger than 800 bp are represented by an interrupted solid line). Coding and untranslated regions are indicated by white and shaded boxes, respectively. Nucleotide numbers are given above the boxes. Caspase-1 domains and cleavage sites are schematically represented on top of each transcript. ATG: starting codon; QACRG: caspase-1 pentapeptide active-site motif; TGA/TAA: stop codon.

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inflammatory caspase clade, the fish caspases cluster into a single distinct branch with two subgroups: one for sea bass and sea bream caspase-1, and another one for the two caspy sequences (caspase-a and -b) from zebrafish.

Sea bass caspase-1 is activated to p24/p10 and p20/p10 heterodimers in vitro

In order to characterize caspase-1 auto-processing and IL-1 β activation in fish, a recombinant form of sea bass caspase-1 (DilaCASP1iso1) was produced.

Solubilization and refolding of recombinant DilaCASP1iso1 from inclusion bodies originated three major polypeptides, two corresponding to putative large subunits (p24 and p20), and one corresponding to a putative small subunit (p10; Fig. 2A). N-terminal sequencing of the three polypeptides revealed that they were generated by proteolytic cleavage of sea bass caspase-1 after E⁸⁶, D¹⁰⁸ and D³⁰⁴ originating a p24, a p20, and a p10 polypeptide, respectively. Both p24/p10 and p20/p10 heterodimers (elutions #2 and #3, respectively, in Fig. 2A) displayed activity in a caspase-1 specific fluorimetric assay, which was abolished by the caspase-1 inhibitor Ac-YVAD-CHO (Fig. 2B).

The more abundant p24/p10 heterodimer was selected for subsequent experiments.

A kinetic analysis of the caspase-1 refolding process revealed a time-dependent decrease of the higher molecular weight forms and an increase of lower molecular weight forms (Fig. 3). The polypeptides detected in these experiments as well as the non-linear kinetics of processing, typical of an autocatalytic event, are remarkably similar to those described for the human protein [56], [57].

DilaCASP1iso2 and DilaCASP1iso3 have little auto-processing activity but are processed by DilaCASP1iso1

Considering that all spliced sea bass caspase-1 isoforms contain intact pro- and p20 domains (which include the active site), recombinant forms of sea bass caspase-1 isoforms 2, 3 and 4 were produced in order to test their auto-processing activity and/or processability by active DilaCASP1iso1 (Fig. 4). Although isoforms 2 and 3 showed auto-processing activity, which was totally absent in isoform 4 (Fig. 4), they do not generate active forms towards a specific substrate, as assessed by the fluorimetric assay. Moreover, isoforms 2 and 3, but not isoform 4, are readily processed by

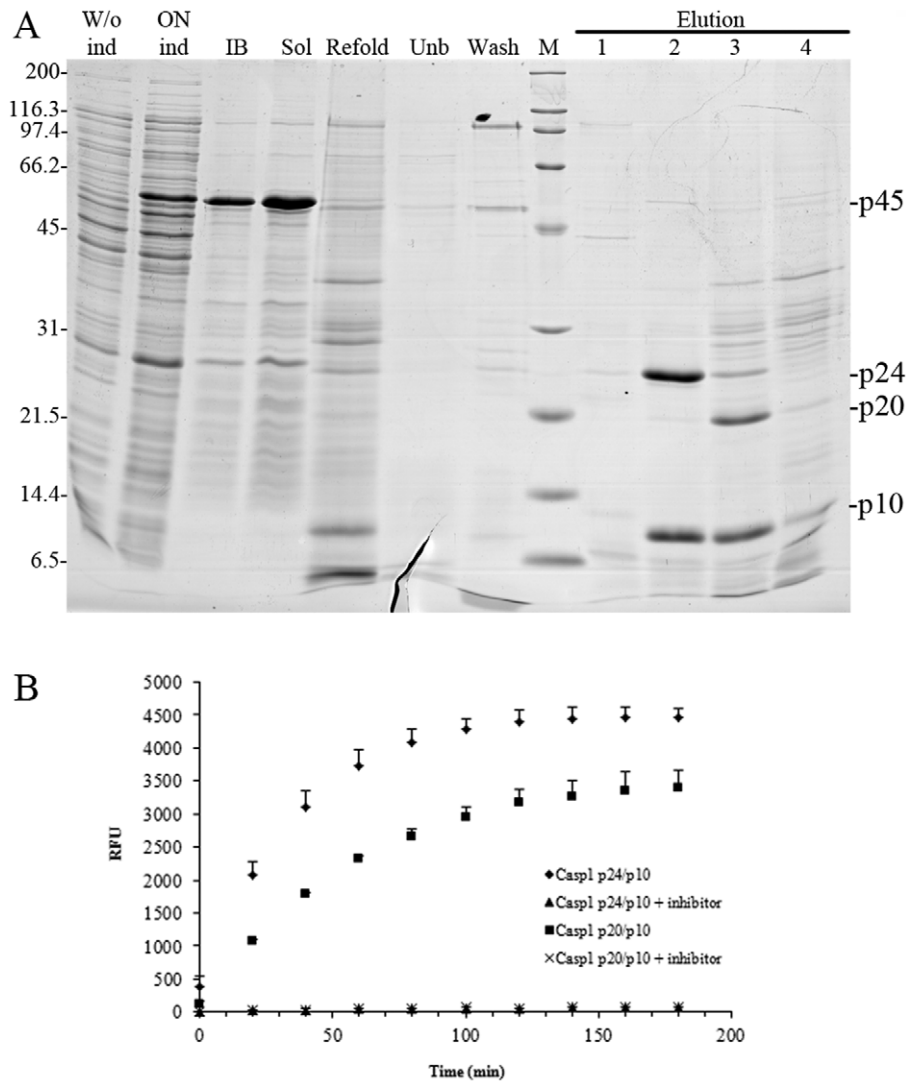


Figure 2. Recombinant sea bass caspase-1 displays enzymatic activity. (A) Production and purification of recombinant sea bass caspase-1. W/o ind: non induced; ON ind: overnight induction; IB: inclusion bodies' fraction; Sol: soluble fraction; Refold: overnight refolding; Unb, Wash and Elution: non-bound, washed and eluted (1–4) fractions, respectively, of Ni-NTA chromatography purified refolded caspase-1. The positions of the p45, p24, p20 and p10 fragments are indicated. Numbers on the left indicate the position of the molecular weight markers in kDa. (B) Enzymatic activity of recombinant p24/p10 and p20/p10 sea bass caspase-1 heterodimers. p24/p10 and p20/p10 were standardized for 1 μ g of p10 and were incubated with a specific fluorogenic substrate for caspase-1 in the presence or absence of the caspase-1 inhibitor Ac-YVAD-CHO. Hydrolysis of the substrate was followed by fluorimetry and results were expressed as relative fluorescent units (RFU). doi:10.1371/journal.pone.0050450.g002

DilaCASP1iso1 (Fig. 4) with a fragment corresponding to the shorter p10 of isoform 2 being detected (Fig. 4A and B). For isoform 3, the shorter p10 fragment could not be detected probably because it is too small to be detected in these gels (Fig. 4A and B). The identity of the fragments was confirmed by N-terminal sequencing of p36 (GSSHHHHHHM¹ADK; band 1 in Fig. 4A) and p10 (T³⁰⁵LHFVH; band 2 in Fig. 4A). Fragment 3 was a short fragment from the N-terminus (GSSHHHHHHM¹ADK; band 3 in Fig. 4A). These results suggest that both isoforms 2 and 3 are processed by isoform 1, releasing the prodomain and the p10 plus linker.

Evolutionary divergence of the caspase-1 cleavage site in proIL-1 β

One of the main cellular substrates of caspase-1 is proIL-1 β , although this has only been shown for mammals with no

information available for non-mammalian species [39], [40], [41]. To evaluate the involvement of sea bass caspase-1 in proIL-1 β processing, the enzyme was incubated either with *in vitro* translated sea bass IL-1 β forms or recombinant sea bass proIL-1 β .

As shown in figure 5, *in vitro* synthesized sea bass proIL-1 β was processed by sea bass active caspase-1 in a time-dependent manner giving rise to a band with an apparent molecular mass of 18 kDa that co-migrates with the putative mature sea bass IL-1 β MS¹⁰¹-Q²⁶¹. Mutation of D¹⁰⁰ abolished the generation of the 18 kDa fragment, with accumulation of higher molecular mass species (Fig. 5), whereas mutating D⁶⁰, a residue previously suggested as a possible caspase-1 cleavage site in fish [31], did not affect proIL-1 β processing (Fig. S3A). This suggests that cleavage of sea bass proIL-1 β by caspase-1 occurs C-terminal to D¹⁰⁰, an aspartate residue absolutely conserved in all IL-1 β sequences reported so far (Fig. S4).

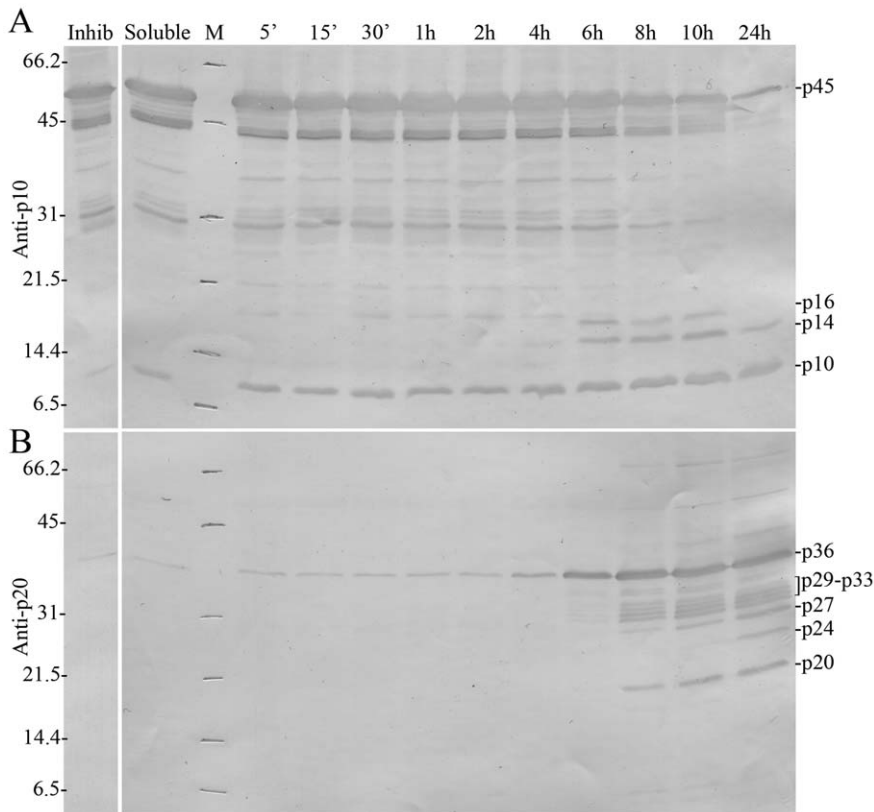


Figure 3. *In vitro* auto-processing of sea bass caspase-1. (A) Western blotting analysis of *in vitro* auto-processing of sea bass caspase-1 using a polyclonal antibody that detects all polypeptides containing the p10 domain. (B) Western blotting analysis of *in vitro* auto-processing of sea bass caspase-1 using a polyclonal antibody that detects only caspase-1 fragments that have been processed at the p20 cleavage site, i.e. that have released the linker plus the p10 domain. Soluble: solubilized DilaCASP1iso1. Inhib: sample incubated for 24 h in the presence of the specific caspase-1 inhibitor Ac-YVAD-CHO. The position of the different polypeptide fragments is indicated. Numbers on the left indicate the mass of the molecular weight (M) markers in kDa.

doi:10.1371/journal.pone.0050450.g003

An 18 kDa fragment was also generated from recombinant proIL-1 β (Fig. S3B), together with a shorter fragment (fragment 3') the intensity of which markedly increased with time. N-terminal sequencing of fragments 2' and 3' (Fig. S3B) revealed that they start at S¹⁰¹EKRSLVLP, confirming that they originated from a cleavage after D¹⁰⁰. Fragment 3' was also processed after D²⁵², as mutating D²⁵² abolished its formation (Fig. S3C). Using *in vitro* translated proIL-1 β it was confirmed that fragment 3' appears upon long incubation periods (Fig. S3A, C). Furthermore, only few fish species present a slightly longer C-terminal region including potential cleavable aspartates (Fig. S4).

As the caspase-1 cleavage site described for mammalian proIL-1 β is only present in mammals (Fig. S4), it is tempting to speculate that the phylogenetically conserved aspartate identified as the caspase-1 cleavage site in sea bass proIL-1 β is also the residue targeted by caspase-1 in the proIL-1 β of other non-mammalian vertebrates. To test this possibility, the processing of *in vitro* translated avian pro-IL1 β was investigated. Since avian caspase-1 is not available, sea bass or human caspase-1 was used. Both sea bass (Fig. 6A) and human (Fig. S5A) caspase-1 processed chicken proIL-1 β into a mature IL-1 β running in SDS-PAGE above the 21 kDa marker. A similar fragment was obtained when sea bass caspase-1 was incubated with duck, goose or turkey pro-IL1 β (Fig. S5B). The apparent molecular mass of this fragment suggests that avian proIL-1 β is cleaved at D⁷⁷ or D⁸⁰ or D⁸², which also align with other phylogenetically conserved aspartates (Fig. S4). Site-

directed mutagenesis suggested that D⁸⁰ is the preferential aspartate cleavage site, because it is the residue that upon mutation more drastically reduces the appearance of the putative mature form (Fig. 6A). Despite the concerns associated with the use of heterologous caspases, three observations suggest that they are cleaving the substrate specifically: (i) sea bass caspase-1 cleaved human proIL-1 β at the specific mammalian caspase-1 cleavage site, originating the same mature form obtained by incubation with the human enzyme (Fig. 6B); (ii) despite the presence of several aspartates in the sea bass pro-IL1 β , the human caspase-1 was unable to cleave sea bass pro-IL1 β (Fig. S5C); (iii) a similar result was obtained when chicken proIL-1 β was cleaved by caspase-1 from two phylogenetically distinct species.

***In vivo*-generated sea bass mature IL-1 β is an 18 kDa polypeptide.** In order to obtain evidence supporting the *in vitro* cleavage results described above, peritoneal leukocytes harvested from stimulated sea bass were incubated in the presence or absence of a caspase-1 inhibitor. Western blotting analysis using an anti-sea bass IL-1 β antibody, which recognizes proIL-1 β and processed IL-1 β fragments, showed a specific band with an apparent molecular mass of 18 kDa in culture supernatants and total extracts of cells incubated *ex vivo* without caspase-1 inhibitor (Fig. 7). Importantly, the 18 kDa protein co-migrates with a recombinant putative mature sea bass IL-1 β starting after aspartate D¹⁰⁰ (MS¹⁰¹-Q²⁶¹). As there are no aspartates in sea bass proIL-1 β sequence that could generate an 18 kDa polypep-

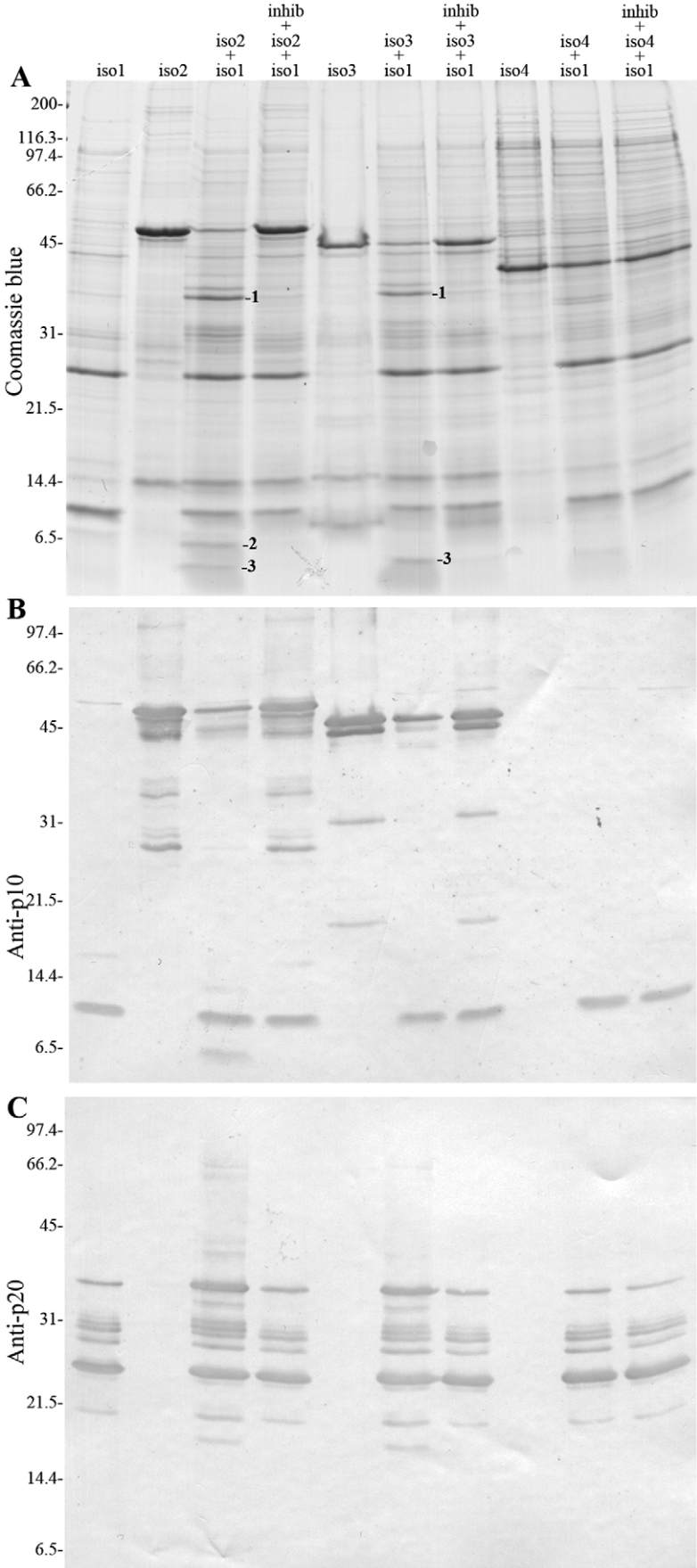


Figure 4. *In vitro* auto-processing and processing of sea bass caspase-1 isoforms 2, 3 and 4 by isoform 1. (A) Coomassie brilliant blue stained SDS-PAGE of sea bass DilaCASP1iso2 (iso2), DilaCASP1iso3 (iso3) or DilaCASP1iso4 (iso4) incubated alone or in combination with active (p24/p10) DilaCASP1iso1 (iso1), in the presence or absence of caspase-1 inhibitor Ac-YVAD-CHO (inhib). N-terminal sequencing revealed that the indicated fragments 1 and 2 correspond to p36 (GSSHSHHHHM¹ADK) and p10 (T³⁰⁵LHFVH), respectively. Fragment 3 corresponds to an N-terminal fragment of p45 (GSSHSHHHHM¹ADK). (B) Western blotting of the *in vitro* processing of caspase-1 isoforms as in A, using the anti-p10 polyclonal antibody that detects all polypeptides containing the p10 domain. (C) Western blotting of the *in vitro* processing of caspase-1 isoforms as in A, using the anti-p20 polyclonal antibody that detects only caspase-1 fragments that have been processed at the p20 cleavage site, i.e. that have released the linker plus the p10 domain. Numbers on the left indicate the mass of the molecular weight markers in kDa.
doi:10.1371/journal.pone.0050450.g004

tide upon cleavage, other than D¹⁰⁰, this suggests that *in vivo* sea bass proIL-1 β is processed at D¹⁰⁰, and does not suffer any additional cleavage at the C-terminal end (D²⁵²).

Discussion

Caspase-1 primary structure has since long been known in humans [17], [18], [70] and mice [68], [55], but was only recently determined for two fish: sea bass (GenBank: DQ198376 and DQ198377) and sea bream [59]. Comparative analyses of the gene and of the primary structure of sea bass caspase-1 indicate that a strikingly conserved structure has been kept throughout evolution, suggesting that its function, specificity and processing mechanisms are highly conserved from at least teleost fish to mammals.

In humans, in addition to the caspase-1 isoform α , four mRNA isoforms (β , γ , δ and ϵ) resulting from one or more alternative

splicing events were described [71]. In the present work, besides sea bass caspase-1 isoform 1 (DilaCASP1iso1), three alternatively spliced mRNAs encoding DilaCASP1iso2, DilaCASP1iso3, and DilaCASP1iso4 were identified (Figs. 1 and S1B). Contrary to some of the human isoforms, where the prodomain (isoform γ) or both the prodomain and the region containing the active site (isoforms δ and ϵ) have been deleted [71], all sea bass isoforms contain the pro- and p20 domains, including the fully conserved active site QACRG sequence. The differences introduced by the alternative splicing events in sea bass caspase-1 isoforms are all located downstream from the active site, with DilaCASP1iso2 and DilaCASP1iso3 displaying a shorter p10 domain with a different amino acid sequence at the C-terminal end. DilaCASP1iso4 has a longer p20 domain but no p10 (Fig. S1B). The presence of alternative transcript variants of caspase-1 in sea bass and humans [72], [73] suggests that their existence has been conserved throughout evolution and therefore they are likely to play a functional role, as described for other caspase isoforms (see below).

Similarly to what has been described for human caspase-1 [17], [74], [56], [57], DilaCASP1iso1 is autocatalytically converted *in vitro* into two active heterodimeric complexes consisting of p10 paired either with p24 or p20 (Figs. 2A and 3). Strikingly, as for *Drosophila melanogaster* DRONC [75], sea bass caspase-1 p24 is generated by cleavage after a glutamate residue (P1 position) instead of the aspartate described in mammalian caspases as an absolute requirement for enzyme specificity [13], [76], [17]. Conversion of sea bass recombinant p45 to p20/p10 and p24/p10 heterodimers occurs in a time-dependent manner through a series of intermediates resembling the auto-processing of the human caspase-1 precursor, in which the first step is the cleavage of p10 and linker from the p45 form, followed by proteolytic conversion of the resulting p36 into smaller and more active p20 and p24 forms [56], [57]. Apparently, processing of procaspase-1 through several intermediates has been evolutionarily maintained at least from teleost fish to humans.

Sea bass caspase-1 isoforms 2 and 3 display some auto-processing activity, although in a much lesser degree than isoform 1. Of notice is that none of the fragments resulting from auto-processing of isoforms 2 and 3 correspond to p24, p20 or p10 (Fig. 4B and C), explaining why no enzymatic activity could be detected. However, contrary to the human isoforms [71], both sea bass isoforms 2 and 3 are processed into their p36, p20 and shorter p10 subunits by isoform 1. These data do not allow to conclude whether isoforms 2 and 3 participate in IL-1 β processing or isoform 1 activation *in vivo*. On the other hand, being largely inactive, they may participate in a direct inhibitory mechanism of caspase-1, together with DilaCASP1iso4, by competing through CARD-CARD interactions for the inflammasome complex that activates caspase-1, as has been proposed for other CARD-containing proteins such as COP, INCA, ICEBERG and caspase-12 (reviewed in [77]). Alternative isoforms have been implicated in the inhibition of caspase-9 [78], [79], [80], and more recently also of caspase-3, where a short isoform (caspase-3S) antagonizes its apoptotic activity [81], thereby playing a physiological role in regulating cell death. Therefore, sea bass caspase-1 isoforms could

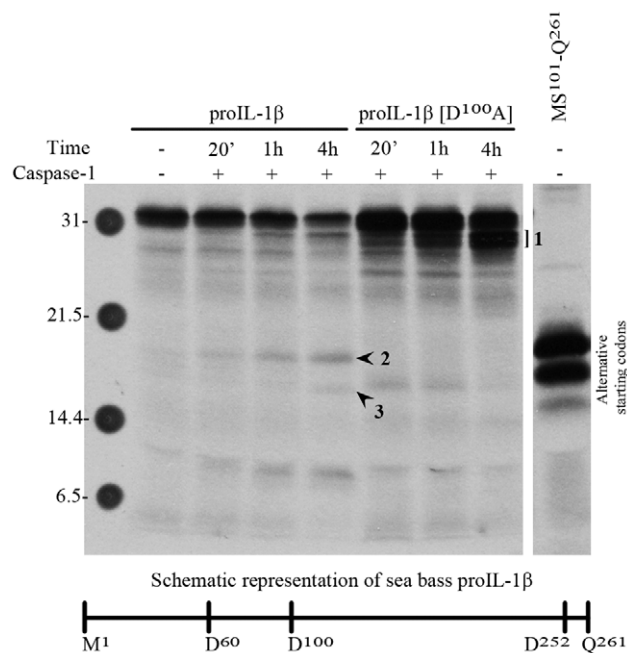


Figure 5. Cleavage of *in vitro* translated bass proIL-1 β and proIL1 β [D¹⁰⁰A] by sea bass caspase-1. The same volume of *in vitro* synthesized proIL1 β and proIL1 β [D¹⁰⁰A] were used and loaded on the gel. Fragments 1, 2 and 3 are labeled. Putative mature *in vitro* synthesized sea bass IL1 β (MS¹⁰¹-Q²⁶¹) has been loaded as control. Numbers on the left indicate the mass of the molecular weight markers in kDa. The double bands corresponding to the highest molecular weight form (fragment 1) suggested cleavage at the C-terminal end of proIL-1 β . This interpretation was supported by N-terminal sequencing of fragment 1' (Fig. S3B), which revealed that this fragment has the N-terminal sequence of proIL-1 β (M¹ESEMKC). Furthermore, mutation of D²⁵² results in a proIL-1 β protein that no longer yields fragment 1 upon incubation with caspase-1 (Fig S3C).
doi:10.1371/journal.pone.0050450.g005

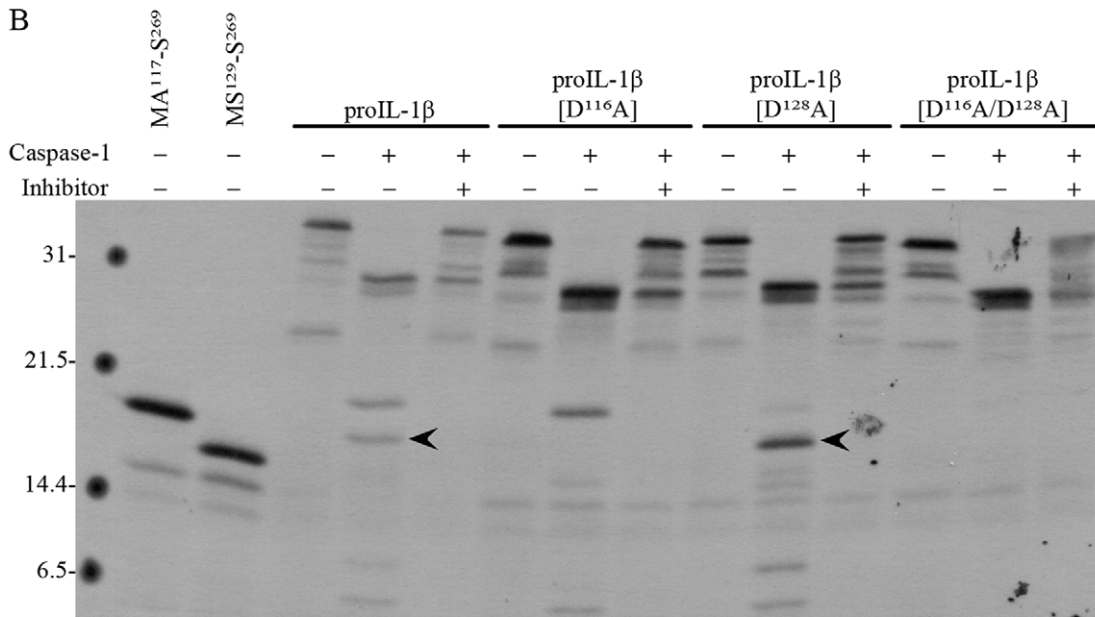
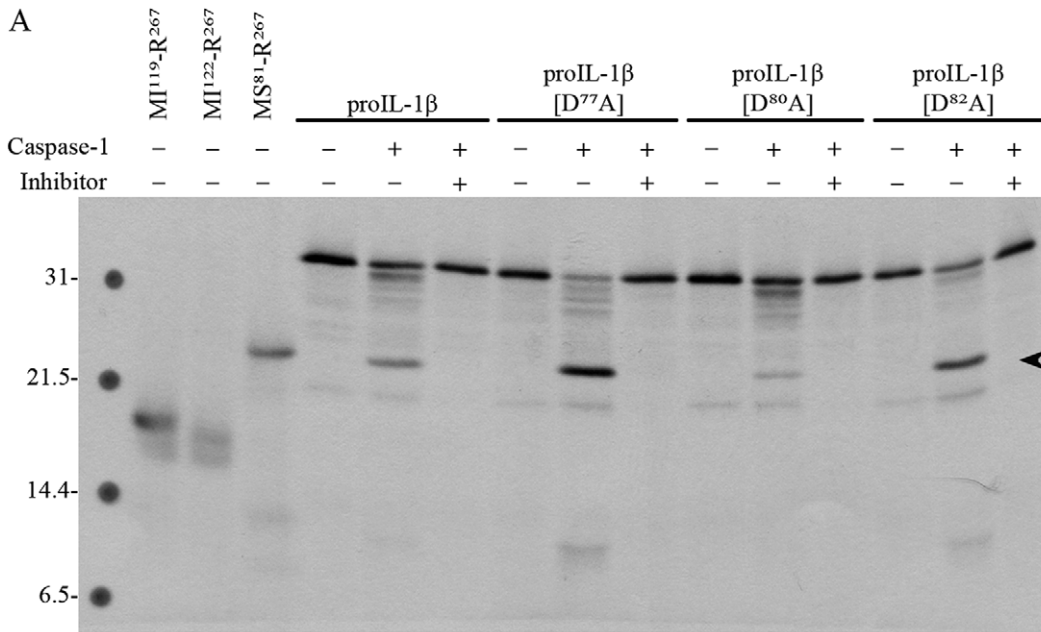


Figure 6. Processing of *in vitro* translated chicken and human proIL-1 β by sea bass caspase-1. (A) Processing of *in vitro* translated chicken proIL-1 β and mutants by sea bass caspase-1. The same volume of *in vitro* translated chicken proIL-1 β , proIL1 β [D⁷⁷A], proIL1 β [D⁸⁰A] and proIL1 β [D⁸²A] were loaded on the gel. *In vitro* translated putative mature chicken IL-1 β forms (MI¹¹⁹-R²⁶⁷, MI¹²²-R²⁶⁷ and MS⁸¹-R²⁶⁷) were loaded as controls. (B) Processing of *in vitro* translated human proIL-1 β and mutants by sea bass caspase-1. (C) Processing of *in vitro* translated human proIL-1 β and mutants by human caspase-1. The same volume of *in vitro* translated proIL-1 β , proIL1 β [D¹¹⁶A], proIL1 β [D¹²⁸A] and proIL1 β [D¹¹⁶A/D¹²⁸A] was loaded on the gel. *In vitro* translated mature human IL-1 β (MA¹¹⁷-S²⁶⁹) and human IL-1 β form (MS¹²⁹-S²⁶⁹) starting at S¹²⁹, homologue to sea bass S¹⁰¹, were loaded as controls. Mature forms are highlighted by arrow heads. Numbers on the left indicate the mass of the molecular weight markers in kDa. doi:10.1371/journal.pone.0050450.g006

also play a role in the regulation of the inflammatory response. Moreover, processing of isoforms 2 and 3 by isoform 1 released shorter p10 fragments, which could compete with normal p10 to generate inhibitory p20/short p10 heterodimers, as suggested for human ICE ϵ [71].

In mammals, IL-1 β is a pro-inflammatory cytokine produced as a 31 kDa inactive precursor [9], [10] that is processed by caspase-1 cleavage after D¹¹⁶ in humans and D¹¹⁷ in mice, yielding a C-terminal secreted active form of 17.5 kDa [14], [15], [11], [12], [16], [17], [18]. The primary structure of fish proIL-1 β is similar to that of the mammalian cytokine [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30]. However, the caspase-1 cleavage site present in all mammalian proIL-1 β molecules is absent in the proIL-1 β sequences reported so far for non-mammalian vertebrates (Fig. S4 and [39], [40], [41]), and the actual cleavage site has been greatly debated since the first non-mammalian IL-1 β was sequenced [22]. Cleavage of *in vitro* translated and recombinant sea bass proIL-1 β by caspase-1 suggests that mature sea bass IL-1 β results from processing of its pro-form at D¹⁰⁰ giving rise to an 18 kDa polypeptide, in accordance with the form detected *in vivo* (Fig. 7). Future studies on the activity of this polypeptide will clarify

whether it corresponds to the biologically active IL-1 β or to an inactive fragment.

As aspartate residues corresponding to sea bass D¹⁰⁰ are present in all vertebrate IL-1 β sequences (Fig. S4), it would be tempting to speculate that non-mammalian proIL-1 β would be cleaved at this site and, thus, the mammalian IL-1 β cleavage site would constitute the exception rather than the norm. Particularly in chicken, this position could be the likely activation site, rather than the alanine residue that was considered for the putative mature protein that was structurally characterized [36], as that constitutes an improbable caspase-1 cleavage site. However, the results presented here suggest that avian proIL-1 β s may not be cleaved at the equivalent sea bass D¹⁰⁰, but instead preferentially cleaved after D⁸⁰ (Fig. 6 and S5B), present in another proIL-1 β stretch where aspartates are phylogenetically well conserved (Fig. S4). In other fish species, fragments with different molecular mass have been proposed as putative active IL-1 β [82], [37], [38], [31], [83], [31], and aspartate D⁶⁰ in sea bream, equivalent to chicken D⁸⁰, has even been suggested as a possible cleavage site, because processing at D⁶⁰ would generate a fragment with a molecular mass consistent with the apparent size (22 kDa) of the IL-1 β fragment detected by Western blotting in that species [31]. However, not only none of the reported studies included amino acid sequencing of the processed IL-1 β fragments, but mutation of D⁶⁰ did not affect at all sea bass IL-1 β processing (Fig. S3A), in opposition to mutation of D¹⁰⁰ where IL-1 β processing to an 18 kDa form was abrogated (Fig. 6, S3A,C). To which extent processing of proIL-1 β at this position is the norm among fish remains to be elucidated. The most obvious implication of sea bass proIL-1 β processing at D¹⁰⁰ is that the resulting mature form will be necessarily different from a structural viewpoint from the mammalian and chicken counterparts. In homology models of sea bass IL-1 β produced using the available experimental structures (PDB entries 1IOB (human), 8I1B (mouse) or 2WRY (chicken)) as templates (not shown), residue D¹⁰⁰ is located in a beta sheet-forming N-terminal segment. Therefore, structural conservation in this region would implicate the ablation of the N-terminal beta strand (which in the mammalian and chicken IL-1 β s has an important structural function) and concomitant destabilization of the other C-terminal beta sheet-forming segments of the molecule upon cleavage of sea bass IL-1 β at D¹⁰⁰. Notably, in the mammalian proIL-1 β caspase-1 cleavage site the P1' position is almost always occupied by a small hydrophobic amino acid, which has been described as one of the preferential features of the peptide substrates of mammalian caspase-1 [13], [17]. On the contrary, most of the aspartates in the putative caspase-1 cleavage sites of non-mammalian vertebrates, sequence-wise to sea bass D¹⁰⁰ or chicken D⁸⁰, are not followed by a small hydrophobic amino acid. Also of notice is the fact that a small hydrophobic amino acid is present in the P1' position after the phylogenetically conserved sea bass caspase-1 cleavage sites in monkeys (A¹²⁹ in P1'), raising questions about the evolutionary driving force that resulted in the appearance of the mammalian cleavage site, as the

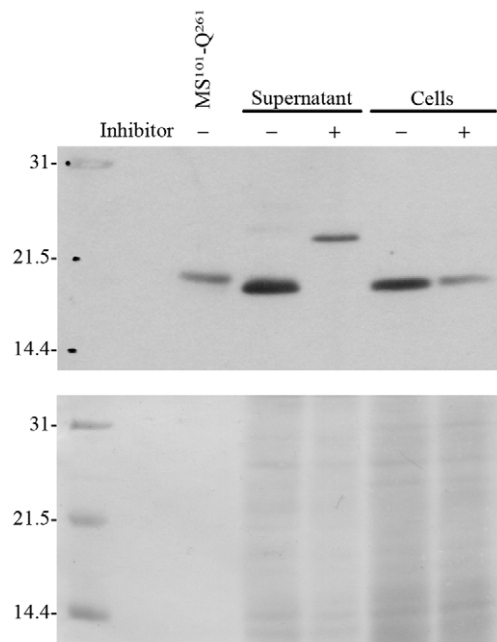


Figure 7. Sea bass IL-1 β processing *in vivo*. (A) Western blotting using the anti-sea bass IL-1 β polyclonal antibody. (B) Ponceau staining showing the protein loading. Inhibitor: specific caspase-1 inhibitor AC-YVAD-CHO. Recombinant putative mature sea bass IL-1 β (MS¹⁰¹-Q²⁶¹) was loaded as control. Supernatant and Cells denote TCA precipitates of the supernatants and cell pellet derived from 1 ml of cell suspension. Numbers on the left indicate the mass of the molecular weight markers in kDa. doi:10.1371/journal.pone.0050450.g007

mammalian aspartate at position P1 is only conserved in mammalian IL-1 β sequences.

In conclusion, the present work shows that in a teleost fish, pro-caspase-1 auto-processing occurs through a series of intermediates, yielding active p24/p10 and p20/p10 heterodimers in a similar way to that described previously for human caspase-1. Moreover, the existence of alternative spliced variants of caspase-1 in sea bass is reported, suggesting that caspase-1 isoforms have been evolutionarily maintained and therefore likely play a regulatory role in the inflammatory response, as shown for isoforms of other caspases. Finally, it is shown that sea bass IL-1 β is cleaved by caspase-1 at a phylogenetically conserved aspartic acid present in all known IL-1 β sequences. However, in avian proIL-1 β s another well phylogenetically conserved aspartate, only absent in humans, chimpanzees and zebrafish, may be apparently the cleavage site, suggesting that IL-1 β processing may be class and/or even species specific.

Supporting Information

Figure S1 Caspase-1 is encoded by a single gene in sea bass that encodes at least four different transcripts. **(A)** Southern blotting analysis. **(B)** Multiple sequence alignment of the predicted primary structures of sea bass caspase-1 isoforms and human caspase-1 α (HosaCASPIalpha). Sea bass caspase-1 comprises a pro-domain (Met¹-Asp¹⁰³), followed by a p20 domain (Gln¹⁰⁹-Asp²⁷⁸), a short connecting peptide (Ser²⁷⁹-Asp³⁰⁴), and a p10 domain (Thr³⁰⁵-Leu³⁹⁴). The active-site pentapeptide QACRG is boxed. The aspartic acid cleavage sites are shaded in gray. Asterisks and “:” and “.” denote identity and chemical similarity between amino acids according to the default CLUSTAL W scoring matrix. The amino acid residues in the sea bass caspase-1 isoforms 2, 3 and 4 that differ from the isoform 1 are in bold. DilaCASP1iso1 (GenBank accession number: DQ198376) has 1663 bp including, as in human and mouse caspase-1 mRNA [18,68], two polyadenylation signals within the 3'-UTR. The ORF encodes a 394 aa long protein that, as in the human and mouse sequences, has no hydrophobic signal sequence. Sequence analysis revealed that the sea bass protein contains both caspase family p20 and p10 domain profiles and a caspase family active site signature (K²⁵⁷PKIIIHQACRG). A prodomain containing a caspase recruitment domain (CARD), typical of pro-inflammatory caspases, is also present. DilaCASP1iso2 (GenBank accession number: HQ398873) results from an alternative splicing event involving the utilization of an alternative splicing site located within intron 7, which leads to the retention of 31 nucleotides from this intron (Fig. 1). This creates a putative caspase-1 isoform containing the complete pro- and p20 domains but having a shorter p10 domain (72 aa compared to 90 aa of the full length p10). DilaCASP1iso3 (GenBank accession numbers: HQ398874) is generated by an exon skipping event, in which exon 7 is lost (Fig. 1). This creates a putative caspase-1 isoform containing the complete pro- and p20 domains but having a much shorter p10 domain (25 aa compared to 90 aa of the full length p10). DilaCASP1iso4 (GenBank accession number: HQ398875) results from two alternative splicing events. One results in the retention of intron 5, generating a stop codon located 62 nucleotides downstream the beginning of this intron. The other is generated by an exon skipping event, in which exon 7 is spliced out of the transcript, as in DilaCASP1iso3 (Fig. 1). This creates a putative caspase-1 isoform containing a complete pro-domain; however, the splicing originates a shift in the reading frame that generates a different coding region after the active center. Although there is an

aspartate residue within this alternative coding region, it is apparently not used as cleavage site, as this isoform is not auto-processed nor processed by active DilaCASP1iso1 (see below), therefore rendering a longer p20 domain (11 aa longer and 21 aa different) and no p10. **(C)** Expression analysis of caspase-1 isoforms in sea bass spleen.

(TIF)

Figure S2 Neighbour-joining tree (MEGA version 3.1) [63] with p-distance and complete deletion of gaps of inflammatory (caspase-1, -4, -5, -11 and -12) and apoptogenic (caspase-3 and -9) caspases. The amino acid sequences were aligned with CLUSTAL W [62] using the default parameters. The numbers in branching nodes denote the bootstrap percentages for 1000 replicates. The different branches are supported by high bootstrap values. GenBank accession numbers: for caspase-1 see Table S2; for caspase-1/4: ABX79372 (*Canis lupus familiaris*); for caspase-3: ABC70996 (*Dicentrarchus labrax*), AAM43816 (*Takifugu rubripes*), BAB32409 (*Danio rerio*), CAC88866 (*Homo sapiens*), AAH81854 (*Rattus norvegicus*), AAH38825 (*Mus musculus*); for caspase-4: NP_001216 (*H. sapiens*), NP_788811 (*Bos taurus*), NP_446188 (*R. norvegicus*); for caspase-5: ABB58698 (*H. sapiens*), XP_001100375 (*Macaca mulatta*); for caspase-9: BAA87905 (*H. sapiens*), AAK26235 (*R. norvegicus*), AAH56447 (*M. musculus*), ABC70998 (*D. labrax*); the sequence of *Tetraodon nigroviridis* putative caspase-9 (CAG01765) was obtained by Blast search of the Genbank database with the sequence of sea bass caspase-9 as query; for caspase-11: CAA73531 (*M. musculus*), AAK38735 (*R. norvegicus*); for caspase-12: AAT91067 (*M. musculus*), EDL78548 (*R. norvegicus*), ABG21363 (*M. mulatta*), NP001070704 (*C. lupus familiaris*), ABX79369 (*Felis catus*).

(DOC)

Figure S3 Processing of *in vitro* synthesized and recombinant sea bass proIL-1 β by caspase-1. **(A)** Time-course processing of *in vitro* synthesized sea bass proIL-1 β proIL1 β [D⁶⁰A] and proIL1 β [D¹⁰⁰A] by sea bass caspase-1, in the presence or absence of caspase-1 inhibitor Ac-YVAD-CHO, using caspase-1 buffer. *In vitro* synthesized putative mature sea bass IL-1 β (MS¹⁰¹-Q²⁶¹) was loaded as control. **(B)** Western blotting showing the time-course processing of recombinant sea bass proIL-1 β by caspase-1 using caspase-1 buffer. proIL-1 β [His] (proIL-1 β) and DilaCASP1iso1 (Casp1) were loaded as controls. On the right, a schematic illustration of the fragments obtained, as concluded from N-terminal sequencing, is shown; capital letters denote amino acid residues and the superscript numbers the amino acid position within the sea bass IL-1 β sequence. **(C)** Time-course processing of *in vitro* synthesized sea bass proIL-1 β proIL1 β [D⁶⁰A] and proIL1 β [D¹⁰⁰A] using caspase-1 buffer. *In vitro* synthesized mature sea bass IL-1 β (MS¹⁰¹-Q²⁶¹) was loaded as control. **(D)** Processing of *in vitro* synthesized sea bass proIL-1 β with or without caspase-1 buffer and with different amounts of caspase-1 (relative amount of caspase-1 added to the reaction). *In vitro* synthesized mature sea bass IL-1 β (MS¹⁰¹-Q²⁶¹) was loaded as control. ProIL-1 β fragment 1, 2 and 3 are indicated in (C) and (D). Numbers on the left (right in D) indicate the mass of the molecular weight markers in kDa. The same volume of each *in vitro* synthesized proIL-1 β forms were used and loaded on the gel.

(TIF)

Figure S4 Alignment of IL-1 β amino acid sequences from vertebrates belonging to different Classes. The regions having aspartate cleavage sites are shown (aspartates are shaded black). The sequences were aligned with CLUSTAL W [62] using the default parameters. GenBank accession numbers are shown within brackets. Aspartate residues are blue color and bold type. The

caspase-1 cleavage site in mammalian IL-1 β sequences is shaded yellow. The conserved aspartic acids homologous to the caspase-1 cleavage site in sea bass IL-1 β sequence are shaded green. The conserved aspartic acids homologous to the putative caspase-1 cleavage site in avian IL-1 β sequence are shaded red. Mutated aspartate residue in the sea bass and chicken IL-1 β sequences are shaded black.
(DOC)

Figure S5 Processing of *in vitro* translated sea bass and chicken proIL-1 β by human and sea bass caspase-1, respectively. **(A)** Chicken proIL-1 β is processed by human caspase-1. Putative mature chicken IL-1 β forms (MS⁸¹R²⁶⁷, MI¹¹⁹R²⁶⁷ and MI¹²²R²⁶⁷) were loaded as controls. **(B)** Processing of *in vitro* synthesized chicken, duck, goose and turkey proIL-1 β by sea bass caspase-1. Putative mature chicken IL-1 β forms (MS⁸¹R²⁶⁷, MI¹¹⁹R²⁶⁷ and MI¹²²R²⁶⁷) were loaded as controls. **(C)** Sea bass proIL-1 β is not processed by human caspase-1. Numbers on the left indicate the mass of the molecular weight markers in kDa. The same volume of *in vitro* synthesized proIL-1 β forms for each species was used and loaded on the gel.
(TIF)

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Table S1 Primer sequences and application.
(DOC)

Table S2 Amino acid sequence conservation for caspase-1 of different species.
(DOC)

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Author Contributions

Conceived and designed the experiments: MIRR AdV NMSdS. Performed the experiments: MIRR. Analyzed the data: MIRR PJPB JEA AdV NMSdS. Wrote the paper: MIRR AdV NMSdS.

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