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Short communication

Identification of α1-antitrypsin as a positive acute phase protein in ayu (*Plecoglossus altivelis*) associated with *Listonella anguillarum* infection

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ABSTRACT

α1-Antitrypsin (AAT) is implicated in the regulation of a variety of mammalian immune responses and was recently identified as a major serpin in blood plasma of some fish. However, AAT expression following bacterial infection in fish has not been well described. In this study, we cloned the full-length ayu (*Plecoglossus altivelis*) AAT gene cDNA. It contained a 1368-bp coding region, which encodes a 19-amino acids (aa) signal peptide and a 437-aa mature AAT containing the serpin's signature sequence (^{427}LKFDRPFMMLV^{437}). PNGase F digestion confirmed that the higher molecular mass of the serum AAT was caused by N-glycosylation. Phylogenetic analysis indicated that ayu AAT was closest to that of green spotted pufferfish. AAT transcripts were present in a variety of tissues, with the highest level in the liver. The real-time quantitative PCR data showed that AAT transcripts dramatically increased in various ayu tissues after *Listonella anguillarum* infection. Western blot analysis revealed that the serum AAT protein level significantly increased in response to inflammation, but displayed no significant changes after cadmium exposure or salinity challenge. This work represents the first report that identifies AAT as a positive acute-phase protein in ayu fish associated with bacterial infection, suggesting that it might play a role in fish innate immunity.

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α1-Antitrypsin (AAT), a secretory glycoprotein produced mainly in the liver and in monocytes, functions as a serine proteinase inhibitor (serpin) in human plasma [1]. It has multiple functions, including preventing endothelial cell apoptosis [2], stimulating fibroblast proliferation [3] and modulating inflammation and immune responses [4]. As an acute phase reactant in human, AAT levels in various tissues and circulating plasma increase in response to infection and accelerate resolution of the inflammatory reaction [5,6]. Since the 1980s, serum anti-protease activity has been observed in fish [7–11]. More recently, AAT and anti-thrombin III were identified as major serpins of rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) in blood plasma [12]. During the acute phase response, the anti-trypsin activity and AAT-like protein content were all down-regulated in Atlantic cod (*Gadus morhua*) and rainbow trout [13,14]. However, AAT gene expression following infection in ayu fish has not been well described. The aim of this study was to clone the corresponding ayu AAT cDNA and study the changes in mRNA and protein expression upon challenge with *Listonella anguillarum*, the causative agent of ayu vibriosis [15,16].

Sixty healthy ayu specimens weighing 20–25 g were obtained from a commercial farm in Huangtan Reservoir, Ningbo City, China. These fish were kept in three freshwater tanks at 20–22 °C in a recirculating system with filtered water and were fed dry food pellets once a day. Overnight cultures of *L. anguillarum* were diluted 1:50 in nutrient broth, grown at 28 °C with shaking and harvested in the log phase. Cells were resuspended in sterile phosphate-buffered saline (PBS) and adjusted to a final concentration of 1.0×10^6 CFU/mL [15,17]. Forty fish were intraperitoneally injected with 100 μL of *L. anguillarum* per fish [15]; the 20 remaining fish were injected with 100 μL of PBS per fish as a negative control. Each tank contained 20 infected or control fish. Approximately 8 h post-injection (hpi), the bacteria-infected fish began to show abnormal swimming and subsequent erratic movement. And about 24 hpi, obvious symptoms were observed, such as haemorrhaging in gills and fins, swollen anuses and congestive splenomegaly. About 36 hpi, all infected fish died at 36 hpi, similar to what was reported by Li et al. [15]. The control fish showed no signs of infection, even when examined over an extended period (up to 3 days). Bacteria were recovered from the liver of fish injected with *L. anguillarum* to verify the success of the experimental infections [15]. Samples of

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control and bacteria-infected fish were randomly collected at 0, 4, 8, 12 and 24 hpi, frozen in liquid nitrogen and stored at −80 °C until use.

The partial sequence of ayu AAT cDNA was obtained from a previously established cDNA library [17]. Based on this, the full-length AAT cDNA sequence was determined by using the Rapid Amplification of cDNA Ends (RACE) method [18]. The SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the cleavage site of signal peptides. The N-linked glycosylation site prediction was carried out using the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple alignment was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [19].

The full-length cDNA sequence of ayu AAT gene has been deposited in the EMBL Database with accession number FR852563. It contains an open reading frame (ORF) of 1368 nucleotides, which encodes a 456 amino acids (aa) precursor protein with a putative 19 aa signal peptide and 437 aa mature protein (48.5 kDa). A serpin signature sequence (^{427}LKFDRPFMMLV^{437}) at the C terminus may be responsible for the removal of serpin–proteinase complexes from the plasma by binding to specific receptors on cells [20]. Three N-glycosylation sites were predicted to exist at Asn81, Asn168 and Asn307 by the NetNGlyc program. Ayu AAT showed a relatively higher aa identity to that of green spotted pufferfish (*Tetraodon nigroviridis*; 54%) than to that of other fish (less than 48%). The phylogenetic tree analysis showed that ayu and other fish AAT were grouped together forming a fish cluster distinct from a mammalian cluster (Fig. 1).

At sampling time points, the fish were anaesthetised, and then the liver, spleen, head kidney, brain, heart, muscle and intestine of the infected and control fish were collected. RNA extraction, first-strand cDNA synthesis and real-time quantitative PCR were carried out as described previously [17]. Four biological replicates each and three technical replicates for each individual were performed. Primers aAATF: 5′-AGTTCGCCTTCCGCCTCTAC-3′ and aAATR: 5′-GTTTGGCCTCTTGCACC TAC-3′ were designed to amplify a 127-base pair (bp) fragment from ayu AAT cDNA. As an internal PCR control, primers pActin2(+): 5′-TCGTGCGTGACATCAAGGAG-3′ and pActin2(−): 5′- CGCACTTCATGATGCTGTTG-3′ were used to amplify a 231-bp fragment of the housekeeping β-actin gene [17]. The ayu AAT mRNA expression was normalised against β-actin expression. Relative gene expression was analysed by the comparative Ct method ($2^{-\Delta\Delta Ct}$ method) [21]. Data were expressed as the mean ± SEM and analysed by one-way analysis of variance (ANOVA) with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

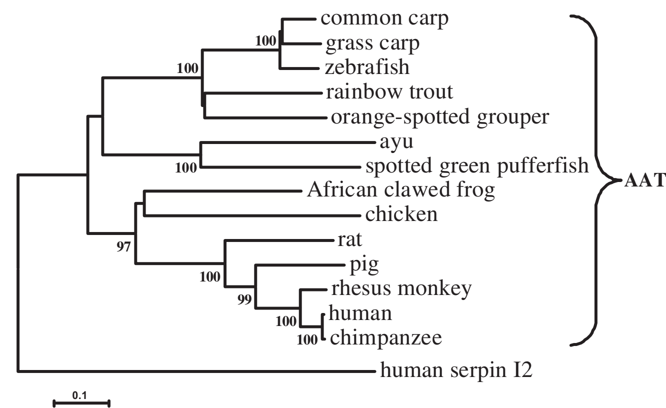

Fig. 1. Phylogenetic (Neighbor-joining) analysis of AAT amino acid sequences using the MEGA4 program, with human serpin I2 (NM_006217) as the outgroup. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when >60%). The scale bar shows the number of substitutions per base. Accession numbers of sequences used are ayu (*Plecoglossus altivelis*), FR852563; spotted green pufferfish (*Tetraodon nigroviridi*), CAAE01010097; zebrafish (*Danio rerio*), BC062869; grass carp (*Ctenopharyngodon idella*), EU621405; common carp (*Cyprinus carpio*), L27172; rainbow trout (*Oncorhynchus mykiss*), NM_001124397; orange-spotted grouper (*Epinephelus coioides*), EU867445; chicken (*Gallus gallus*), XM_421344; African clawed frog (*Xenopus tropicalis*), NM_001086802; rat (*Rattus norvegicus*), D00675; chimpanzee (*Pan troglodytes*), XM_522938; pig (*Sus scrofa*), X88780; rhesus monkey (*Macaca mulatta*), NM_001195350; human (*Homo sapiens*), BC011991.

The AAT transcripts were significantly abundant in the liver, and low in the intestine, heart, muscle, head kidney, brain and spleen of the healthy fish (Fig. 2). After challenge with *L. anguillarum*, the AAT transcripts in these tissues showed a time-dependent expression pattern. For example, the liver AAT expression immediately increased up to 1.85-fold at 4 hpi, reached the top level (4.83-fold) at 8 hpi, and went down to the control level at 24 hpi (Fig. 2).

Based on the previously determined sequence, a primer pair was designed that would amplify the complete AAT ORF (including signal peptide sequence) and included restriction sites for *Eco*RI and *Xho*I (underlined) at the 5′-ends, respectively, of the upstream (aAATexF: 5′-G<u>GAATTC</u>ATGCGGACAGCTTT GTGTCT-3′) and downstream (aAATexR: 5′-C<u>CTCGAG</u>TTATTTAGTTGGGTCC ACGA-3′) primers to facilitate directional cloning in the pET-28a. Pfu DNA Polymerase (Fermentas, Vilnius, Lithuania) was used for gene amplification according to the manufacturer's protocols. Prokaryotic overexpression and SDS-PAGE analysis were based on established protocols [18]. The purified AAT protein was used as an antigen to immunize mice to produce antiserum [18]. Western blot analysis of ayu AAT using an ECL substrate was following the method described previously [18]. Four biological replicates were used for each treatment. Sera collected from 5.0 ppm cadmium (as $CdCl_2 \cdot H_2O$)-exposed ayu [22] and 10 ppt (0.17 M NaCl) salinity-challenged ayu [23] were also used for Western blot analysis.

Recombinant AAT and ayu serum were digested with PNGase F (New England Biolabs, Beverly, MA, USA) and subsequently detected by Western blot [24]. Bands were quantitatively measured using the image analysis software Quantity One, v 4.62 (Bio-Rad Laboratories, Hercules, CA, USA). After induction with IPTG, SDS-PAGE analysis of the fusion protein of recombinant ayu AAT showed a specific band with the expected molecular weight (MW) of 54.0 kDa (Fig. 3A). Determined by Western blotting, the MW of native AAT in ayu serum was about 63.5 kDa, and it was converted to a 48.3-kDa band after PNGase F digestion (Fig. 3B). After *L. anguillarum* challenge, serum AAT level immediately increased at 4 hpi, peaked (2.62-fold) at 8 hpi and remained at a significantly high level at 24 hpi (1.75-fold) compared to that at 0 hpi (Fig. 4). In contrast, the serum AAT level did not change significantly after cadmium exposure and salinity challenge (Fig. 4).

In this study, the full-length nucleotide sequence of ayu AAT cDNA was determined. The deduced protein contained a 19-aa signal peptide and a serpin signature sequence. Phylogenetic tree analysis revealed that this ayu protein belongs to the AAT protein family. The liver was the primary organ responsible for AAT mRNA expression in ayu. Like human AAT [25], the MW of ayu AAT presented in serum was significantly larger than that calculated from the sequence, and PNGase F digestion confirmed that the higher molecular mass of the serum AAT was caused by N-glycosylation. Glycosylation reportedly appears to increase the stability of human AAT [26], but is unnecessary for its enzyme activity [27].

AAT was identified as a positive acute phase protein (APP) in humans [5]. Studies have reported that tissue and circulating

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