**Molecular cloning, genomic structure, polymorphism analysis
and recombinant expression of a α 1-antitrypsin like gene from
swamp eel, *Monopterus albus***

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Abstract: Alpha-1-antitrypsin (AAT) is a highly polymorphic glycoprotein antiprotease, involved in the regulation of human immune response. Beyond some genomic characterization and a few protein characterizations, the function of teleost AAT remains uncertain. In this study we cloned an AAT-like gene from a swamp eel liver identifying four exons and three introns, and the full-length cDNA. The elucidated swamp eel AAT amino acid sequence showed high homology with known AATs from other teleosts. The swamp eel AAT was examined both in ten healthy tissues and in four bacterially-stimulated tissues resulting in up-regulation of swamp eel AAT at different times. Swamp eel AAT transcripts were ubiquitously but unevenly expressed in ten tissues. Further, the mature peptide sequence of swamp eel AAT was subcloned and transformed into *E. coli* with the recombinant proteins successfully inhibiting bovine trypsin activity. Analysis of recombinant AAT showed equimolar formation of irreversible complexes with proteinases, high stability at pH 7.0-10.0 and temperatures below 55 °C. Serum AAT protein level significantly increased in response to inflammation with AAT anti-sera, and, NF- κ B, apolipoprotein A1 and transferrin gene expression were dramatically decreased over 72 h post recombinant AAT injection. Lastly, examination of swamp eel AAT allelic polymorphism identified all alleles in both healthy and diseased stock except allele*g, found only in diseased stock, but without statistical difference between the distribution frequency of allele*g in the two stocks. These results are crucial to our ongoing study of the role of

55 teleost AAT in the innate immune system.

56 **Key words:** swamp eel; α 1-antitrypsin; genomic structure; recombinant
57 expression; polymorphism

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1. Introduction

Alpha-1 antitrypsin (AAT), a major serine proteinase inhibitor (Serpine), is synthesized primarily in the liver, but is also produced in extra-hepatic tissues and cells, including neutrophils, monocytes, alveolar macrophages, and intestinal and corneal epithelium [1]. AAT functions primarily to protect lung tissue from the destructive effects of serine proteases, such as neutrophil elastase, proteinase 3 and cathepsin G, which are released by degranulating neutrophils [2]. Although AATs are associated with maintaining protease-antiprotease homeostasis, their principle efficiency is in regulating inflammatory responses rather than inhibiting specific proteases [3]. Increasingly, observations indicate that AAT displays independent anti-inflammatory/immune-modulatory properties [4-6], as well as crucial roles in physiological processes such as preventing endothelial cell apoptosis [7], and stimulating fibroblast proliferation [8].

Circulating AAT plasma levels are usually stable, increasing correspondingly during acute phase responses, oral contraceptive therapy, and pregnancy [9,10]. As such, AAT has been defined as an important acute-phase reactant. Deficiency of AAT may be associated with a high risk of spontaneous cervical artery dissections, type II diabetes mellitus, human immunodeficiency virus type I infection, spontaneous abortions, and graft-versus-host disease [3]. AAT purified from human blood was used for modulating and/or preventing tissue injury in experimental animal models of

human diseases [3,11]. These studies suggest that the baseline concentration, the magnitude of synthesis, and the acute-phase reaction release of AAT, are important in both the acute inflammatory process and the subsequent resolution [3].

Teleost AATs have been less frequently reported than those of mammals. Although cDNA sequences encoding the ayu [12], carp [13], rainbow trout [14], and zebrafish [15] AATs have been documented, little reporting of the genomic structure of fish AAT can be found. Most studies have focused on the anti-proteinase characterizations of AAT, with few results shown on teleost immunological or anti-inflammatory functions [13,16-19]. During the acute phase response, ayu AAT expression was significantly up-regulated at the mRNA and protein level [12], but expression in Atlantic cod [20] and rainbow trout [21] were found to be down-regulated. However, precise functions and immune cell regulatory mechanism of teleost AAT are poorly described.

The medicinal and nutritional value of the swamp eel, *Monopterus albus* (Zuiew, 1793) make it an important economical freshwater fish in China and throughout Asia [22]. Although swamp eel farming has made rapid progress during the past decade, production is seriously challenged. This is due to poor bacterial resistance of farmed populations, and the decline in wild populations through large-scale application of pesticides and overfishing [23]. Bacterial hemorrhagic septicemia is one of the most destructive and common swamp eel diseases, leading to great losses at all growth stages [24,25]. Therefore,

finding protective genes involved in swamp eel defense mechanisms could provide a crucial defense against bacterial hemorrhagic septicemia. While a few studies have investigated the role of AAT in other species [12-14,20,21], the exact function in swamp eel immune system is still not fully understood. Therefore, efforts to investigate these functions and use these protective genes in marker assisted breeding are of fundamental importance to protect this valuable resource.

In this study, RACE-PCR techniques were used to clone the AAT gene and determine its structure. Swamp eel AAT expression patterns were characterized against bacterial infection and AAT was cloned into an expression vector and transformed into *Escherichia coli*. The recombinant protein was purified, and its activity verified by the inhibition of trypsin. The specific antiserum was produced and the serum AAT protein level post bacterial infection was examined. The recombinant protein was injected to fish and expression profile of NF-kB, apolipoprotein A1, and transferrin was determined. Moreover, polymorphism in swamp eel AAT was investigated. In summary, these results suggested that AATs play an important role in the fish immune system, establishing a solid foundation moving forward in our study of fish AAT function.

2. Materials and methods

2.1. Fish sampling and bacteria challenge

Healthy swamp eels (weighing 60 ± 8.4 g) were obtained from the Taihu

Fishery Farm (Jingzhou, China). The fish were acclimatized in tanks at 20 °C for 1 week before genetic analysis. Tissue samples of heart, liver, spleen, stomach, kidney, skin, intestine, muscle, brain and blood cells were collected from four fish, and preserved in liquid nitrogen for RNA extraction. Fish with classical symptoms of bacterial hemorrhagic septicemia were collected from local rice fields in 2015. The genomic DNA of these diseased fish were immediately extracted from the livers post mortem and stored at -80 °C for further study.

The healthy swamp eels were infected with *A. hydrophila* described by Li et al. [26]. Briefly, the pathogenic bacteria (*A. hydrophila*) were cultured at 28 °C to mid-logarithmic growth in Luria-Bertani medium. The bacteria were then collected by centrifugation at 4,500 rpm for 5 min, and re-suspended at approximately 2.0×10^7 CFU/mL in phosphate-buffered saline. The fish were anesthetized by immersion in MS222 and injected intraperitoneally with 20 µL of bacterial suspension or with the same volume of phosphate buffered saline (controls). Infected and control fish were sacrificed 3, 6, 12, 24, 48 and 72 h after injection. Then, the kidney, liver, spleen, intestine and blood pooled from three fish at each time point were mixed as one sample; Four such pooled samples were collected after infection and preserved for qRT-PCR (N=4). Blood samples were kept at 4 °C overnight, then centrifuged at 5000 g for 5 min to collect the sera. All the sera samples were kept at -80 °C for dynamic analysis of the serum AAT concentrations after bacterial infection. Other

184 samples were stored in liquid nitrogen until RNA extraction.

185 2.2. DNA, RNA isolation and cDNA synthesis

186 The genomic DNA was isolated from the swamp eel livers using a DNA
187 isolation kit (Solarbio, Shanghai, China) per the manufacturer's instructions.
188 Total RNA was isolated using Trizol reagent (Invitrogen, USA) following the
189 manufacturer's protocol. RNA samples were treated with PrimeScript RT
190 reagent kit with gDNA eraser (Takara, Dalian, China) to remove any
191 contaminating genomic DNA. The First strand cDNA was synthesized from
192 total RNA by M-MLV reverse transcriptase (Promega, USA) according to the
193 instructions of the manufacturer.

194 2.3. Primer synthesis, cDNA cloning of swamp eel α 1-antitrypsin

195 Degenerate primer pairs, de-F/R (Table 1), were designed according to
196 the putative AAT genes obtained by retrieving the GenBank database, such as
197 orange-spotted grouper (GenBank: EU867445), fugu rubripes
198 (XM_011611856), half-smooth tongue sole (XM_008312789), rainbow trout
199 (NM_001124397) and zebrafish (BC062869). Rapid amplification of cDNA
200 ends (RACE) was performed to isolate the full-length cDNA of AAT.
201 Gene-specific primers, GSP5 and GSP3 (Table 1) synthesized based on the
202 cDNA fragment obtained, were used as 5'-RACE primer and 3'-RACE primer,
203 respectively. RACE-PCR amplification was performed using a Smart Race
204 cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the
205 manufacturer's instructions. Touchdown PCR was used for RACE-PCR. The

sequence was: 94 °C for 3min, 94 °C for 1min, 70 °C for 45 s, 72 °C for 2 min, for 5 cycles. This was followed by 94 °C for 1 min, 65 °C for 50 s, 72 °C for 1 min, for 30 cycles; and 72 °C for 10 min for elongation. The PCR products were purified using the Zymoclean Gel DNA Recovery kit (ZYMO, Irvine, CA, USA). The purified cDNA fragments were ligated to pEASY-T1 (Transgen, Beijing, China) vectors and cloned into DH5α competent cells. At least three clones were sequenced using the ABI3730 automated sequencer with M13F primer.

2.4. Introns amplification and genomic structure determination

Two pairs of primers were synthesized to amplify the introns to determine the genomic structure of eel AAT (Table 1). Primer pairs intr1-F/R and intr2n3-F/R were used for amplification of intron1 and intron2/3, respectively. Exon-intron junctions were deduced according to the human AAT.

2.5. Amino acid sequence analysis, multiple sequence alignment and phylogenetic tree analysis

Sequence homology analysis was performed using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The determined amino acid sequences were analyzed with the Expert Protein Analysis System (EXPASY) (<http://www.expasy.org/>), and the protein domain features were predicted by Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>). The signal peptides were predicted with SignalP4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). Multiple alignments of amino acid sequences of swamp eel AAT with other species were performed

using Clustal-W [27], and a phylogenetic tree of AAT proteins was constructed using deduced amino acid sequences using the neighbor-joining method [28]. Bootstrap tests were replicated 10,000 times to derive the confidence values for the phylogeny analysis.

2.6. Expression and purification of recombinant proteins

Fusion forms of GST- α 1AT were obtained by over-expression in BL21 (DE3) *E. coli* cells as reported previously [29]. The mature peptide sequence of the α 1-antitrypsin gene was amplified using the forward primer rc-F (Table 1) with an *EcoR* I site, and the reverse primer rc-R (Table 1) with an *Xho*I site. PCR products were double digested and ligated into the same restriction sites of pGEX-4T-1 (Amersham Biosciences). The recombinant plasmid (pGEX- α 1AT) was transferred into the *E. coli* BL21 (DE3), screened and confirmed by DNA sequencing. Confirmed positive colonies were cultured in Luria-Bertani medium containing 50 μ g/ml ampicillin at 37 °C for 4 h, and inoculated (1/100, v/v) into another fresh Luria-Bertani medium with ampicillin. When the OD₆₀₀ of the culture reached 0.6-0.8, the isopropyl- β -thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM and incubated for an additional 16 h at 22 °C. The cells were harvested by centrifugation, suspended in lysis buffer (40 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, pH 8.0), then sonicated on ice. The cell lysate was spun to pellet the debris, and the supernatant was added to GST Sepharose 4B affinity column (Amersham Biosciences) for

purification according to the instructions of the manufacturer. After washing out contaminated proteins, fusion proteins were eluted with reduced glutathione in 40 mM Tris-HCl, pH 8.0. The purified fusion protein was digested with thrombin (1 U/μl) (GE Healthcare) at 22 °C for 20 h to remove the GST tag completely. Partially digested fusion protein was examined to determine the molecular weight of the recombinant protein. Then the purity of the expressed protein was verified by 12% SDS-PAGE. *E. coli* BL21 (DE3) harboring pGEX-4T-1 after induction with IPTG was used as negative control; *E. coli* BL21 (DE3) harboring recombinant plasmid without induction was used as positive control. Protein concentrations were determined via Bradford assay, with bovine serum albumin used as a standard. The purified enzyme was kept at -80 °C until enzymatic activity measurements.

2.7. Western blotting analysis of recombinant proteins

Western blotting was performed as described by Lin et al. [30] with slight modifications. Briefly, the cell lysates of controls and recombinant proteins were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), after which the membrane was blocked with 5% (W/V) non-fat powdered milk in PBS for 2 h. Mouse anti-GST (Proteintech, Chicago, IL, USA) was used as the primary antibody (1:1000), and once bound, was detected with horseradish peroxidase conjugated goat anti-mouse IgG antibody (1:1000) (Proteintech, Chicago, IL, USA). Finally, 3,3'-diaminobenzidine and H₂O₂ were used to visualize the immunoblotting.

2.8. Complex formation between the swamp eel α 1AT and serine proteinases

Complex formation was performed as previously published [18] with slight modifications. Purified swamp eel α 1AT was diluted to 100 μ g/ml with 20 mM Tris-HCl buffer (pH 7.4), then incubated on ice for 1 min with porcine elastase (Sigma- Aldrich, St. Louis, MO), bovine trypsin and chymotrypsin (Amresco, Solon, OH) at a molar ratio 1: 1.5. The binding reaction was stopped with the addition of 0.1 M PMSF and loading buffer (5% SDS, 0.25 M Tris-HCl, 20% glycerol and 0.002% bromophenol blue, pH 7.0) then boiled for 5 min. The reaction products were then analyzed by SDS-PAGE and CBB staining.

2.9. Trypsin inhibitory activity

The trypsin inhibitory activity was measured using a previously established assay [31] with minor modifications. Bovine trypsin (Sigma, Aldrich, St. Louis, MO) and N α -benzoyl-DL-arginine-pnitroanilide (BAPNA, Sigma, Aldrich, St. Louis, MO) were used as the proteinase and the substrate, respectively, then incubated with 80 μ l of purified recombinant protein (α 1AT) for 15 min at 37 °C with 54 μ M of bovine trypsin in 0.2 M Tris-HCl, pH 8.0 in a total volume of 100 μ l. The residual activity was monitored at 405 nm after adding 160 μ l 2.8 mM BAPNA. The reaction was run for 15 min at 37 °C, and then stopped with the addition of 40 μ l 50% acetic acid. The change in absorbance due to the release of p-nitroanilide was used to measure trypsin activity in a 96-well microtiter plate. The proteolytic activity was measured by

monitoring the changes in absorbance with a Bio-RAD iMark microplate reader. One unit of specific activity was defined as the amount of recombinant protein necessary to reduce change of absorbance of the test wells to 2% of the negative control well.

2.10. Thermal, pH stability and the mean 50% inhibition concentration (IC₅₀) determination

Thermal stability of the purified recombinant protein (α 1AT) was determined by pre-incubating the protein in assay mixtures (pH 8.0) for 15 min at temperatures ranging from 5 to 75 °C. Subsequently, samples were cooled on ice for 2 min. The residual trypsin inhibitory activity was determined at 37°C for 15 min as described above. Remaining inhibitory activity was expressed as the relative inhibitory activity, compared with that of the untreated sample.

The effect of pH on enzyme activity was determined using 0.2 M Tris-HCl buffer (pH 7.0-10.0). The residual enzyme activity was assayed under standard conditions.

To determine the mean 50% inhibitory concentration (IC₅₀) of purified recombinant protein (α 1AT), increasing amounts α 1AT, ranging from 0 to 0.16 mg/ml, were added into the standard reaction system. The residual activities were examined at 25 °C in 0.2 M Tris-HCl buffer (pH, 8.5), and those without recombinant protein were defined as 100% activity. The linear regression equation was formulated by SPSS version 20.0 based on the percent of residual activities obtained, with the IC₅₀ value was calculated according to the

equation.

2.11. Production of rabbit polyclonal antisera against swamp eel AAT

Purified α 1AT was used to produce antibodies in three New Zealand white rabbits (specific pathogen-free, 2.0 kg each). The sera were collected from each rabbit before the first immunization as a negative control. The rabbits were immunized subcutaneously first with 500 μ g of the α 1AT emulsified in Freund's complete adjuvant (Sigma, St. Louis, USA) followed by 4 booster immunizations at one week intervals using the same dose of the protein emulsified with Freund's incomplete adjuvant (Sigma, St. Louis, USA). The rabbits were bled from the marginal ear vein 10 days after the last injection, and the presence of antibodies was determined by Western blot analysis. The rabbits were then anesthetized before bleeding; the collected blood was allowed to clot at 4 °C overnight. The blood serum was extracted by centrifuging at 5000 g for 5 min, then purified using a protein G column (GE Healthcare, Little Chalfont, UK), and stored at -70 °C for further study.

2.12. Dynamic analysis of serum AAT protein level after bacterial infection

Aliquots of the sera samples collected at different times after bacterial infection, and controls, were measured by Bradford assay using bovine serum albumin as a standard. The sera samples were subjected to SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked with 5% (W/V) non-fat powdered milk in PBS for 2 h and then probed with purified

rabbit polyclonal (500 ng/ml). The membranes were washed three times with PBS-T and then incubated for 1 h with HRP-conjugated goat anti-rabbit IgG (1:1000) (Proteintech, Chicago, IL, USA). Finally, 3,3'-diaminobenzidine and H₂O₂ were used to examine the immunoblotting. The relative band intensity was measured with Quantity One v4.62. The data were analyzed by one-way ANOVA followed by Duncan multiple comparison tests using SPSS 20.0. Significance was set at p<0.05.

2.13. Protein injection

The protein concentration was determined using a Bradford protein assay kit (Tiangen, Beijing, China). Seventy-two healthy individuals (weighing 55±4.6 g) were randomly divided into three groups. Twenty-four eels were injected intraperitoneally with 100 µl active α1AT (0.5 mg/ml). The same dosage of heat-killed protein (100 °C for 8 min) and PBS (control) were injected into another two groups, respectively. Livers were collected at seven time points (0, 3, 6, 12, 24, 48, and 72 h) for RNA isolation, with n=3 in each group.

2.14 Real-time PCR analysis of AAT mRNA expression in vivo and expression of NF-κB, apolipoprotein A1 and transferrin gene after protein injection

The expression of AAT mRNA was measured in heart, liver, spleen, stomach, kidney, skin, intestine, muscle, brain tissues and blood cells. Also, the temporal expression was assessed in the liver, spleen, intestine, and kidney upon challenge with *A. hydrophila*. Gene expression of NF-κB,

apolipoprotein A1, and transferrin were detected after the AAT injection.

RT-PCR was performed in a CFX96 Real-time Detection System (Bio-RAD, USA). The housekeeping gene β -actin was used as an internal control for cDNA normalization. Primer pairs rt-F/R, kap-F/R, apo-A1-F/R and Tf-F/R (Table 1) were used to amplify the gene fragments of AAT, NF- κ B, apolipoprotein A1, and transferrin, respectively. The amplification was performed in a total volume of 20 μ l, containing 10 μ l of SYBR Premix Ex TaqTM (Takara, Dalian, China), 1 μ l of the diluted cDNA, 0.4 μ l of ROX reference dye II, 0.4 μ l of each primer and 7.8 μ l of sterile H₂O. The real-time PCR program was 95°C for 1 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 1 min. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm amplification and detection of the special PCR product. The comparative C_T method ($2^{-\Delta\Delta C_T}$ method) was used to analyze the expression level of the previously listed 4 genes [32]. SPSS 20.0 was used for the significance test between the experimental group and the control.

2.15. Polymorphism analysis of swamp eel α 1-antitrypsin alleles

In order to isolate the alleles of swamp eel AAT, genomic DNA from 40 healthy and 21 sick individuals were extracted. A pair of gene-specific primer alle-F/R was synthesized to amplify the complete exon 1 of swamp eel AAT. All PCR products were purified and cloned into pEASY-T1 (Transgen, Beijing, China) and transferred into DH5 α competent cells. Five to seven positive clones per individual were sequenced. Multiple alignments of swamp eel AAT

alleles were performed using Clustal-W [27]. Transition and transversion were analyzed using DnaSP 4.0. χ^2 was used to test the significance of the allele frequency in both healthy and diseased stock.

3. Results

3.1 cDNA and genomic sequence of swamp eel α 1-antitrypsin

The nucleotide sequence of the 2481 bp cDNA, isolated from the swamp eel liver and coding for the full length AAT, is presented in Figure. 1A. Swamp eel AAT cDNA (GenBank accession No. KU866459) contains a 5' noncoding region of 620 bp, a coding region of 1239 bp, a stop codon, a 3' noncoding region of 619 bp and a poly(A)⁺ tail of 13 bp. The 1239bp encoding region was found to code a protein with 413 amino acid residues. This suggests that the signal sequence may be subject to post-translational cleavage after the Ala residue at position 20 by prediction with SignalP 4.1. Genomic DNA (GenBank accession No. KX371911) containing the intron-exon structure resulting in a 2993 bp fragment is shown in Figure. 1B. The genomic DNA consists of four exons and three introns, which is very similar to human ATT.

The secondary structure of the putative AAT amino acid residues was analyzed and detected 4 potential N-linked glycosylation sites (N-X-S/T). A motif search of the sequence revealed that there are a serpin signature, a serpin motif, and a reactive center loop in the exon 4. Moreover, a histidine-rich region was found at the N-terminal region (Figure 2). Surprisingly, the two residues (Val at position P1 and Met at position P1') in the serpin reactive

center sites were distinctive from those of the other AATs, which are usually highly conserved (Met-Ser at the P1-PI' positions) (Figure 2).

3.2 Multiple alignment and phylogenetic analysis

Alignments of the deduced amino acid sequence of swamp eel AAT compared with those of other vertebrates showed a great similarity with other fishes. The amino acid sequence of swamp eel AAT has 66.1%, 61.4%, 59.9%, 57.7%, 54.1%, 53.9%, and 53.8% identity with that of orange-spotted grouper (EU867445.1), half-smooth tongue sole (XM_008312789.1), Japanese medaka (XM_011491746.1), Fugu rubripes (XM_011611856.1), rainbow trout (NM_001124397.1), Atlantic salmon (XM_014205830.1), and zebrafish (BC062869.1), respectively. By comparison, lower similarity was seen with those of mammals, birds and amphibian (Figure 2).

The phylogenetic tree further exhibited a close relationship with other teleost. The swamp eel AAT clustered together with those from teleost, forming a group independent from the mammalian, avian and amphibian AAT genes (Figure 3).

3.3 Tissue expression profiles of swamp eel α 1-antitrypsin

The gene expression of swamp eel AAT was examined in ten tissues, including heart, liver, spleen, stomach, kidney, skin, intestine, muscle, brain and blood cells from four healthy fish using qRT-PCR. The results showed swamp eel AAT expression in all examined tissues, but with distinctly different expression levels among the various tissues. Expression results showed that

high levels of transcripts of AAT were detected in liver, moderate levels were detected in blood cells, stomach, kidney and intestine, and low expression in other tissues (Figure 4).

3.4 Expressional analysis of swamp eel α 1-antitrypsin in response to pathogenic bacteria infection

To further study the transcriptional changes of the AAT gene with stimulation of pathogenic bacteria *A. hydrophila*, we used relative quantitative real-time PCR to detect the mRNA levels of AAT in liver, spleen, intestine and kidney at 0, 3, 6, 12, 24, 48 and 72 h after infection. The results demonstrated that the AAT mRNA level in the four tissues were up-regulated, while levels of AAT in the negative control were not increased (Figure 5). Bacterial infection resulted in a marked AAT expressional pattern change in the liver, with expression levels initially up-regulated from 0 h to 12 h, then acutely decreased between 12 and 24 h, followed by a recovery to normal levels after 72 h. Expression peaked at 3 h post-treatment and was 2.9-fold higher than the control (Figure 5A). The splenic expression showed a slight up-regulation during the early stages of infection from 3 h to 12 h, followed by a drastic increase then a dramatic decrease of expression level of AAT from 12 h to 72 h, with peak expression noted after 24 h (3.6-fold > control) (Figure 5B). However, the expression level of AAT in the kidney was almost unchanged up to 6 h post infection, followed by a slight increase from 12 h to 48 h, with the highest expressional level reached at 48 h post-treatment (1.7-fold > control; Figure

5C), which resolved to normal levels after 72 h. Infection with bacteria stimulated high intestinal AAT, with levels peaking at 3 h (46-fold > control; Figure 5D) and then sharply declining from 3 h to 72 h.

3.5 Fusion expression in *E.coli*, recombinant protein purification and Western blot analysis

Compared with the negative and positive controls, the recombinant fusion protein (GST- α 1AT) was highly expressed in IPTG induced *E. coli* BL21 (DE3). The predicted weight of the fusion protein was 69 kDa, with the GST-tag adding ~26 kDa to the N terminus of the recombinant protein (α 1AT). Following SDS-PAGE, the detected fusion protein band was consistent with the predicted molecular weight (Supplementary Figure S1). The fusion protein was successfully purified by affinity chromatography. The partially digested fusion protein exhibited three bands (69kDa for GST- α 1AT, 26 kDa for GST and 43 kDa for α 1AT, respectively) (Supplementary Figure S1). After thrombin digestion, the recombinant protein (α 1AT) showed a single 43 kDa band, suggesting a purity sufficient for protein characterization (Supplementary Figure S1).

Western blot analysis showed that both the post-induction purified fusion protein, and the total protein from pGEX- α 1AT harboring strains post-induction, reacted with the mouse anti-GST antibody displaying an expected ~69 kDa band, while the same band was not detected in either the negative (i.e *E. coli* BL21 (DE3) harboring pGEX-4T-1 after induction) or positive (i.e *E. coli* BL21

(DE3) harboring pGEX- α 1AT without induction) controls (Supplementary Figure S1). However, negative control showed an expected ~26 kDa band. Moreover, the partially digested fusion protein displayed two expected bands, representing the fusion protein and the GST tag, respectively. The target protein exhibited no bands at 43 kDa (Supplementary Figure S1). These results suggest that the pGEX- α 1AT expression vector was successfully constructed and that the recombinant protein could be used for further analysis.

3.6 Complex formation examination of the recombinant protein

In order to demonstrate the activity of the recombinant α 1AT, its ability to form serine-protease complexes was examined. Figure 6 shows that the recombinant protein forms SDS-stable complexes not only with elastase but also with chymotrypsin and trypsin.

3.7 Characterization of swamp eel α 1AT

The trypsin inhibition assay showed that the purified protein displayed clear inhibitory activity to bovine trypsin. Specific activity of swamp eel α 1AT was determined to be 82.87 U/mg of protein in the standard reaction system (Supplementary Table S1).

The effect of temperature on recombinant α 1AT protein was examined at various temperatures. These studies showed that the recombinant α 1AT protein retained almost full inhibitory activity up to 55°C and more than 70% remained after incubation at 65°C for 15 min (Figure 7A).

The effect of pH on recombinant α 1AT protein was examined using 0.2 M Tris-HCl buffer at 25 °C. After exposure to pH values ranging from 7.0 to 10.0 for 15 minutes, the recombinant protein was found to be most stable at pH 8.5. Moreover, it retained more than half of its maximum activity at pH 7.0 to 10.0 (Figure 7B).

Percentage of inhibition was determined by linear regression analysis. The linear regression equation was calculated as $Y=102.522-402.917X$, $R^2=0.987$. Based on this equation, the mean 50% inhibitory concentration (IC_{50}) required to inactivate 54 μ M bovine trypsin was determined to be 0.131 mg/ml (~30 μ M) (Figure 7C). This indicates a molar ratio close to 1:1 during combination of this inhibitor with proteinase.

3.8 Dynamic analysis of serum AAT protein level after bacterial infection

Western blot analysis using specific antiserum showed that bacterial infection significantly increased the AAT protein level in sera (Figure 8). The serum AAT level increased dramatically from 0 h to 12 h after infection, followed by a slight decrease, but continued at a significantly high level from 12 h to 72 h, peaking overall at 12 h post-treatment (4.1-fold>control) (Figure 8).

3.9 Expression analysis of NF- κ B, apolipoprotein A1 and transferrin after protein injection

In general, injection of the AAT protein resulted in significant decrease of the expression of NF- κ B, apolipoprotein A1, and transferrin gene from the time of injection to 72 h (Figure 9). Compared to the PBS control, both heat-killed

AAT and active AAT provoked similar changes in the expression level of these three genes. However, the active AAT was somewhat more efficient than heat-killed AAT (Figure 9). From 0 h to 24 h after AAT injection, the NF- κ B expression was continually lessened to a significantly low level, with expression reaching a stable level from 24 h to 72 h (<one-fortieth of control) (Figure 9A). The expression of apolipoprotein A1 showed an initial decrease after injection from 0 h to 6 h, followed by a sharp increase after 12 h, and then a dramatic reduction from 12 h to 72 h after infection (<One twentieth of control) (Figure 9B). The expression of the transferrin gene was severely decreased from 0 h to 24 h after infection. This was followed by a dramatic increase at 48 h, and then a sharp reduction at 72 h after injection. Heat-killed AAT and active AAT decreased the expression of transferrin to 3 percent and 0.3 percent of the control at 72 h, respectively (Figure 9C).

3.10 Polymorphism analysis of swamp eel α 1-antitrypsin alleles

The genomic DNA from 40 healthy and 21 diseased eels was amplified via PCR using the primer pair alle-F/R. Five to seven positive clones per individual were sequenced. A total of 359 nucleotide sequences were obtained, and nine different genotypes were identified and named as different alleles of swamp eel AAT. Further sequence alignment displayed that allele*a, b, d, e and f shared identical amino acid sequences, although there were several positions of base transition and transversion among them. In contrast to the other alleles, allele*g and allele*h contains a "DHHHH" repeat sequence and an "H" amino

acid insertion, respectively, as well as simple amino acid substitution. In addition, allele*i exhibited a Ser165Phe single amino acid mutation alone (Figure 10).

Distribution frequency between the healthy and diseased stock were examined to evaluate the relationship between AAT alleles polymorphism and disease susceptibility. Almost all alleles were found in both healthy and diseased stock except allele*g, which is detected only in diseased individuals (9.5%) (Figure11). χ^2 test results indicate no significant difference between distribution frequencies of allele*a, b, c, d, e, f, h and i in healthy and diseased stock. Surprisingly, little difference was found between the distribution frequencies of allele*g in the two stocks ($P < 0.05$) (Figure 11).

4. Discussion

Alpha1-antitrypsin (AAT) is a major anti-protease whose circulation concentration rises in response to excessive levels of circulating proteases, especially neutrophil elastase released during the course of inflammation [33]. Human AAT is not only an inhibitor, maintaining protease-antiprotease homeostasis, but also a multifunctional protein with anti-inflammatory, cytoprotective and immunoregulatory properties [3,4]. Moreover, human AAT suppressed LPS-stimulated release of TNF- α and IL-1 β , and enhanced the production of anti-inflammatory IL-10 in monocytes [34]. Efforts to find counterparts from teleost have assumed that fish AAT would be acute phase reactants [12,20,21]. Despite all this, fish AAT gene resources are scarce and

the immunological role of teleost AATs need to be revealed. Although innate immunity constitutes the first line of defense in all vertebrates, for lower vertebrates, such as fish, it is critical because their acquired immunity is less developed than in mammals [35]. Therefore, it is of vital importance to isolate new AAT-like genes from teleosts and to discuss their functions in fish innate immune system.

This study was performed to isolate and characterize swamp eel AAT-like genes. Sequence analysis showed that swamp eel AAT consists of four exons and three introns. The structure of human AAT is organized into three non-coding exons (1A, 1B and 1C), four coding exons (2–5) and six introns [36] and therefore the primary transcript of the coding region of human ATT also contains four exons and three introns. Further comparison showed that all the three introns of swamp eel AAT were much shorter than those of human ATT. However, the length of exon 2 (271 bp) and exon 3 (148 bp) of swamp eel AAT was same as that of exon 3 and 4 (i.e exon 2 and 3 without considering the non-coding exons) from human AAT, respectively (Figure 1B). These results further support that SerpinA genes have similarities in their structure and characteristically consist of four exons with identical positioning and phasing of the intron–exon boundaries [37].

The swamp eel AAT shared higher similarity to teleost AATs. In addition, putative conserved serpin domains have been identified by BLAST program (Figure 2). In general, the sequence within the reactive center of serine

protease inhibitors is highly conserved [38]. In the present study, 80% of strictly-conserved amino acids in the serpin motif are found. Moreover, almost half of (P17-P4') residues in the reactive center loop are conserved (Figure 2). This strongly suggests that swamp eel AAT belongs to serpin superfamily. However, the most conserved Met-Ser active inhibitory site of AAT has been converted to Val-Met in this new AAT gene, which could change its inhibitory specificity from those of other fish AAT.

The most remarkable feature of a serpin is the ability to form irreversible and electrophoretically stable complexes with its serine proteinases at an equimolar ratio during inactivation [13,14,16,18,39]. In the present study, the swamp eel recombinant protein could form SDS-stable complexes with porcine elastase, bovine trypsin and chymotrypsin. And IC_{50} value suggested that the inhibitor combines with trypsin at a molar ratio of close to 1:1. AAT from teleost varied with species in their abilities to recognize and bind individual serine proteinases [13,14,16,18]. In this study, swamp eel recombinant AAT exhibited obvious inhibitory activity to bovine trypsin. Further, the purified recombinant protein exhibited marked stability over broad ranges of pH (7.0 to 10.0) and temperatures below 55°C. The common carp muscle AAT retained the full inhibitory activity up to 60°C. Eighty percent of inhibitory activity remained after incubation at 70°C for 10 min [19]. Comparatively, carp serum AAT showed maximum thermal stability at 50°C and largely lost stability above 60°C [16]. The results suggest that the biochemical characteristics of swamp AAT are

very similar to other known AATs though it contains of a distinctive active center.

In mammals, AAT is primarily synthesized by the liver and released into the blood. Additional sources of human AAT production include intestinal epithelial cells, pulmonary alveolar macrophages, neutrophils, blood monocytes and dendritic cells [1, 40]. Within tissues, AAT was found in kidney, stomach, small intestine, pancreas, spleen, thymus, adrenal glands, ovaries, and testes [3]. AAT expression displayed some degree of substrate and/or autoregulation with enhanced synthesis after exposure to elastase [41]. Increased literatures reports that AAT-like proteins from seminal plasma, blood plasma, muscle, and serum of various fish exhibited similar biochemical characteristics [16-19]. Ayu AAT transcripts were present in a variety of tissues, with the highest level in the liver [12]. These results suggest that teleost AAT-like genes have diverse tissue distribution with swamp eel AAT found in all tested tissues, with the most abundant mRNA found in the liver. In addition, the AAT expression was significantly up-regulated in liver, kidney, spleen and intestine after infection with *A. hydrophilia*. Further, the serum AAT protein level was increased significantly after bacterial infection, peaked at 12 h (4.1-fold) remaining at a significantly high level compared to that in controls (Figure 8). This phenomenon was consistent with reports by Lü et al. [12]. The ayu AAT gene was dramatically increased in various tissues after *Listonella anguillarum* infection. Moreover, the serum AAT protein level significantly increased in

response to inflammation without displaying significant change in response to abiotic stress [12]. From these results it may be inferred that fish AAT would be an important acute phase protein like those in mammals.

The vertebrate transcription factor NF- κ B plays a pivotal role in regulation of immune responses and inflammation [42] and mediates a variety of cellular processes, such as cell growth and apoptosis [43]. Target genes of NF- κ B include inflammatory cytokines, chemokines, immunoreceptors, cell adhesion molecules as well as many acute phase proteins [44]. Human alpha-1 antitrypsin (hAAT) has protective effects in type 1 diabetes, rheumatoid arthritis, and lupusprone mouse models as well as other experimental models of human disease [3]. Human AAT treatment significantly inhibited the production of inflammatory cytokines including IFN- γ , TNF- α and IL-1 β in MRL/lpr mice [4]. Intraperitoneal injection of native or recombinant AAT in diabetic mice resulted in decreased inflammatory cytokine expression, and increased insulin release [45]. Intraperitoneal injection of native AAT or recombinant AAT in elastase-deficient mice resulted in significant reductions in infiltrating neutrophils, lavage fluid levels of TNF- α , and the neutrophil chemokine KC (CXCL1) [46]. Moreover, exogenous AAT stimulation of blood cells inhibited IL-8, IL-6, TNF- α , and IL-1 β significantly but did not suppress induction of the anti-inflammatory cytokines IL-1Ra and IL-10 [47]. Found downstream of the NF- κ B signaling pathway, the decrease of these proinflammatory cytokines may be due to inhibition of NF- κ B activity. While the detailed molecular

mechanism remains to be revealed, it has been shown that AAT suppresses NF- κ B activation [48]. Aldonyte et al. reported that it was the concentration of AAT rather than its conformation that affected proinflammatory responses in human monocytes. AAT treatments at high concentrations suppress the activity of NF- κ B as well as some cytokines [49]. Therefore, it is reasonable to hypothesize that injection of AAT would lead to decreased swamp eel NF- κ B gene expression. In the present study, recombinant AAT (50 μ g per individual) was injected to swamp eel, analysis of the gene expression of NF- κ B showed that both the heat-killed and active AAT significantly reduced its expression in the liver. This result inferred that AAT would be implicated in the NF- κ B-mediated proinflammatory cytokine production in fish. However, further study is needed to elucidate the mechanism.

AAT, apolipoprotein A1 and transferrin are inflammation inhibitors and play a positive anti-inflammatory role in human plasma [50]. Teleost transferrin was reported not only to play a critical role in iron transport, but also in innate immune, by depleting iron from invading pathogens [51,52]. Fish apolipoprotein A1 the major protein component of high density lipoprotein (HDL), plays important roles in lipid metabolism and immunity [53, 54]. Our study demonstrates that injection of recombinant AAT significantly decreased the expression of apolipoprotein A1 and transferrin gene. Although the underlying mechanism of the phenomenon remains uncertain, it is reasonable to suggest by these data that AAT plays a role in sustaining a balance between

the inflammatory and anti-inflammatory molecules, reducing resulting inflammation.

More than 90 genetic variants of AAT have been identified and are strictly associated with specific AAT plasma levels in a co-dominantly inherited fashion [1, 55]. Multiple isoforms of AAT in vertebrates may be formed due to the presence of multiple alleles or alternative splicing [56]. The presence of two isoforms in carp perimeningeal fluid, seminal plasma and sarcoplasmic was also observed [13,18,19]. These results suggest that multiple subforms of AAT in teleost would be common. In the present study, nine alleles were isolated from 61 individuals with almost all alleles were detected in both healthy and sick stock, except allele*g. χ^2 test results suggested that there is few difference between distribution frequency of allele*g in the two stocks ($P < 0.05$). However, without more samples studied, we cannot conclude that there is a definitive association between allele*g and susceptibility to pathogenic bacteria. To that end, further detailed studies on the precise functions, gene expression regulatory mechanism and allele frequency in numerous samples of swamp eel AAT are in progress.

In conclusion, swamp eel AAT full-length cDNA and gDNA was cloned and the expression was shown to be up-regulated in response to bacterial infection at different times post-treatment. Furthermore, recombinant swamp eel AAT exhibited similar biochemical characteristics to known AATs. Intraperitoneal injection of the AAT protein significantly decreased the expression of NF- κ B,

apolipoprotein A1, and transferrin gene. Moreover, 9 alleles were isolated and the distribution frequencies of these alleles were analyzed. Overall, these results established a solid foundation for our study on the role of fish AAT function.

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Competing interest statement

The authors declare no conflict of interest.

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Figures and captions.

Li et al., Figure 1. A: Nucleotide sequence of genomic DNA and deduced amino acid sequence of swamp eel α 1-antitrypsin. cDNA of the α 1-antitrypsin gene is indicated in uppercase character; introns are given in lowercase letters. The signal peptide sequence is shadowed. N-linked glycosylation sites are represented with boxes. The ploy (A)⁺ signal sequence AATAAA is underlined. B: Comparison of swamp eel AAT genomic DNA (top) with human AAT genomic DNA (bottom); the boxes represents exons, the lines between the exons represent introns.

Li et al., Figure 3. Phylogenetic analysis of swamp eel AAT and other vertebrates. Constructions are performed on the basis of the homology sequences calculated from the complete amino acid sequence of the AATs. The tree was constructed using a neighbour-joining method. Gaps are completely deleted. The scale bar is 0.05, which refers to percentage of divergence.

Li et al., Figure 4. Expression of α 1-antitrypsin gene in different tissues of swamp eel, determined by quantitative real-time PCR and analyzed by the comparative CT method. Bars represent the mean $\pm$ SD (n=4). Bars with different letters are significantly different ($p < 0.05$).

Li et al., **Figure 5.** Relative expression of the α 1-antitrypsin gene in A) liver, B) spleen, C) kidney, and D) intestine of swamp eel after challenge with *A. hydropholia*. Vertical bars represent the mean $\pm$ SD (n=4). Values with asterisks are significantly different ($p<0.05$).

Li et al., Figure 6. Formation of SDS-stable complexes between purified α 1AT and porcine elastase, bovine trypsin and chymotrypsin. 1, purified swamp eel α 1AT; 2, complex porcine elastase/ α 1AT; 3, porcine elastase; 4, complex bovine trypsin/ α 1AT; 5, bovine trypsin; 6, complex bovine chymotrypsin/ α 1AT; 7, bovine chymotrypsin.

Li et al., Figure 7. Biochemical characterizations of α 1AT. A): Stability of purified swamp eel α 1AT towards temperature. The activity was measured in 0.2 M Tris-HCl buffer (pH 8.0) at 37 °C. The experiments were done in triplicate and the error bar represents the percentage error in each set of reading. B): Effect of pH on purified swamp eel α 1AT. The activity was measured in 0.2 M Tris-HCl buffer (pH 7.0–10.0) at 25 °C. The activity at pH 8.5 corresponds to 100% activity. The experiments were done in triplicate and the error bar represents the percentage error in each set of reading. C): Linear regression analysis of trypsin inhibitory activity of swamp eel α 1AT. The mean 50% inhibitory concentration (IC₅₀) was calculated based on the linear regression equation ($Y=102.522-402.917X$, $R^2=0.987$) formulated by SPSS 20.0. Residual activity was examined at 25 °C in 0.2 M Tris-HCl buffer (pH 8.5).

Li et al., Figure 8. Dynamic analysis of serum AAT protein level after bacterial infection. Western blot analysis of serum AAT via rabbit anti-AAT sera (top). Histogram displaying the changes in relative band intensity of AAT (bottom). The relative band intensity was evaluated by Quantity One v4.62. Each bar represents the mean $\pm$ SD of the results from four samples. Bars with different letters are significantly different ($p < 0.05$).

Li et al., Figure 9. Expression of NF-kB (A), Apolipoprotein A1 (B) and Transferrin (C) gene after injection purified AAT at different time points. Bars represent the mean $\pm$ SD (n=3). Bars with different letters are significantly different (p<0.05).

Li et al., Figure 10. A: Different genotypes of swamp eel α 1-antitrypsin nucleotide sequences based on exon 1; B: Sequence comparison of putative amino acids from different genotypes based on Clustal W. Dots indicate identity with the consensus sequence, dashes indicate gaps.

Li et al., Figure 11. Distribution of swamp eel α 1-antitrypsin alleles in diseased stock (white bars) and healthy stock (black bars). P values are marked on the top of the bars. Asterisk (*) indicates significant difference with χ^2 test ($P < 0.05$).

Tables and captions.

Li et al., Table 1. Primers used in this study.

Primer name	Primers sequences (5'-3')	Utility in this study
de-F	GTCTGAAKGC <u>S</u> ARGACTGCT	cDNA fragment amplification
de-R	GATCTTGCCCATGAAGAGRATGCT	cDNA fragment amplification
NUP	CTAATACGACTCACTATAGGG	RACE amplification
GSP5	GTGGAGATGCCAGCGGTGAATAGAAGATG	5' RACE amplification
GSP3	AGCATCCTCTTCATGGGCAAGATC	3' RACE amplification
intr1-F	AGATGCGTGGTGTCTTTGCTAG	Intron 1 amplification
intr1-R	TCGAAGGGTTTCTCCCACTGT	Intron 1 amplification
intr2n3-F	ACAGTGGGAGAAACCCTTCGA	Intron 2 and 3 amplification
intr2n3-R	TGATCTTGCCCATGAAGAGGATG	Intron 2 and 3 amplification
ORF-F	AGATGCGTGGTGTCTTTGCTAG	ORF amplification
ORF-R	TTACTTGGCTGTAGGGTTGTTGAT	ORF amplification
alle-F	ATGCGTGGTGTCTTTGCTAGATG	Allele amplification
alle-R	CGCTTCTTACCTTTGAAGTAGAC	Allele amplification
rt-F	GTGTCCCACCAGGCTGTG	RT-PCR analysis of AAT
rt-R	TGATCTTGCCCATGAAGAGGATG	RT-PCR analysis of AAT
kap-F	GTAGAGATGGTGAAGGTATCGCAG	RT-PCR analysis of NF-κB
kap-R	TCACAAGCTCATATCGTCCCAC	RT-PCR analysis of NF-κB
apo-A1-F	CTGAGCGTGTGGAGCAACTG	RT-PCR analysis of
apo-A1-R	TCTGCTACCTTCACCCCA	Apolipoprotein A1
Tf-F	GA CT CGGCAACAAGGTGGTC	RT-PCR analysis of transferrin
Tf-R	TGACAGGTATGGGAAGTGCAT	RT-PCR analysis of transferrin
β-actin-F	GCTGTGCTGTCCCTGTA	Expression analysis of β-actin
β-actin-R	GAGTAGCCACGCTCTGTC	Expression analysis of β-actin
rc-F	GCCGAATTCATGGACCACCACCATCAC	Recombinant expression
rc-R	CCGCTCGAGTTACTTGGCTGTAGGGTTG	Recombinant expression

Notes: F, forward primer; R, reverse primer; Degeneracy bases in primers, K=G or T; S=C or G; R=A or G. ORF, open reading frame. Both the *EcoR* I and *Xho* I restriction enzyme cutting sites are underlined.