Short Communication

Increased liver protein and mRNA expression of natural killer cell-enhancing factor B (NKEF-B) in ayu (*Plecoglossus altivelis*) after *Aeromonas hydrophila* infection

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**A B S T R A C T**

Natural killer cell-enhancing factor (NKEF) may mediate cellular responses to proinflammatory molecules. The liver proteins of *Aeromonas hydrophila*-infected ayu (*Plecoglossus altivelis*) and healthy control fish were analyzed by 2DE. A protein, which increased significantly in diseased fish, was identified as NKEF-B by MALDI-TOF-MS. A full-length cDNA clone of this protein was subsequently isolated. It contains 1092 bp with an open reading frame of 591 bp, coding for 197 amino acids with MW 21.9 kDa and pI 6.38, values similar to those determined by 2DE. Ayu NKEF-B had highest similarity (93.1% amino acid identity) to those of carp and zebrafish. Phylogenetic analysis showed that ayu NKEF-B falls into the fish NKEF-B cluster and is most closely related to that of carp and zebrafish. It was determined that ayu NKEF-B mRNA expression was significantly increased in many tissues at the early stage of bacterial infection. In conclusion, the increased NKEF-B mRNA and protein expression in ayu were closely associated with *A. hydrophila* infection.

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Natural killer cell-enhancing factor (NKEF) was originally isolated and cloned from human erythroid cells and named for its ability to enhance NK cytotoxicity against tumor cells in vitro [1]. It belongs to a new class of the highly conserved peroxiredoxin (Prdx) family found in prokaryotes and eukaryotes [2]. Prdx1 and Prdx2 correspond respectively to the previously named NKEF-A and NKEF-B [3]. In fish, NKEF genes have been isolated and characterized in several species such as rainbow trout (*Oncorhynchus mykiss*) [4], carp (*Cyprinus carpio*) [5], channel catfish (*Ictalurus punctatus*) [6], turbot (*Scophthalmus maximus*) [7] and pufferfish (*Tetraodon nigroviridis*) [8]. Reports revealed relationships between fish NKEF mRNA expression and bacterial or viral infection (or LPS injection) [6–9]. Ayu (*Plecoglossus altivelis*) is an important cultured freshwater fish in Japan and China. Recently, ayu bacteremia (also known as blood poisoning or toxemia) caused by *Aeromonas hydrophila*, has been posing a serious threat to cultured ayu in China, but cellular and molecular processes involved in disease response are poorly understood. To explore these processes, we have analyzed the liver proteins by using two-dimensional gel electrophoresis (2DE) in combination with mass spectrometry (MS) [10]. The results showed that the increased ayu liver NKEF-B mRNA and protein expression were closely associated with *A. hydrophila* infection.

About 100 specimens of healthy ayu, weighing 20–25 g, were obtained from a commercial farm in Ningbo city, China. These fish were kept in freshwater tanks at 20–22 °C in a recirculating system with filtered water, fed with pelleted dry food once a day, and acclimatized to laboratory conditions for at least one month before experiments. Then fish were divided into three groups: H group, NI group and I group. Fish of H and NI groups were injected intraperitoneally with PBS, 1.1 × 10⁶ CFU *A. hydrophila* and 1.1 × 10⁶ CFU *Vibrio alginolyticus*, respectively. *V. alginolyticus* was not pathogenic to ayu, even injected with 3.86 × 10⁷ CFU in our study. At about 12 h post-injection (hpi), behavioural symptoms were observed in I group fish, including erratic swimming behavior or swimming abnormally near the surface of the water with an increased rate of respiration. These fish typically died after 36–48 h. Fish of H and NI groups, show no signs of infection, even when examined over an extended period (up to 10 days).

Liver tissues were collected from fish of H, NI and I groups at 12 hpi, samples quickly washed in a cold rinse buffer containing 0.2 mM protease inhibitor cocktail (Roche, New Jersey, USA) to remove cell debris and blood, and were then frozen by immersion in liquid nitrogen. Sample preparation and solubilization used a slight modification of the SWISS-2D PAGE sample preparation
procedure. Pooled samples are significant cost saving for comparative proteomics and can avoid the influence of individual differences [11,12]. Thus, pooled H, NI and I samples were obtained by combining equal amounts of extracted protein from the 4 fish in each treatment respectively. The serial proteomic methods were just the same to our recent study [13] and the details were not presented here again.

The 2DE and coomassie brilliant blue G-250 staining gave reproducible and reliable results for the three replications. There were many differentially expressed protein spots in the 2DE gels (Fig. 1). Challenge of ayu with nonpathogenic bacteria V. alginolyticus caused a significantly protein expression changes. For example, the expressions of many moderate and high molecular weight (MW) proteins increased (Fig. 1b). For our research interests, low MW proteins in the acidic part were selected for subsequent study. Several differently expressed protein spots were subsequently analyzed by MALDI-TOF-MS, and spots 1 and 2 were successfully identified by Mascot search. Spot 2 was down-regulated (0.30 folds), and identified as pufferfish Glucagon I (EMBL accession number CAAE01014756), with a significant (P < 0.05) expectation score (0.052). Mascot score of spot 2 was 62 and sequence coverage was 32%. Spot 1 (about 21 kDa, pl 6.4), the most significantly up-regulated protein (38.2 folds), was identified as carp (Cyprinus carpio) NKEF-B (EMBL accession number DQ324045), with a significant (P < 0.05) expectation score (0.037).

Mascot score of spot 1 was 63 and sequence coverage was 36%, suggesting that the result was believable. Liver NKEF-B protein expression level in fish of NI group was higher than that in fish of H group (3.5 folds), but remarkably lower than that in fish of I group (Fig. 1b). Since NKEF-B mRNA expression had been reported to be involved in fish immune response, it was selected for subsequent study.

A random set of 287 clones from a constructed cDNA library [13] were detected using degenerate primers dp1(+) : 5’-ATXGGXGTG GARGTXATXGCXGC-3’ and dp11(-) : 5’-TCXGTRTGAA XGCYT GXAC-3’ (A = A,T,G,C; Y = T,C; R = A,G) which were designed in accordance with the result of Mascot search, giving an expected amplification fragment of 296 bp. Totally 4 out of 287 clones were successfully amplified and partially sequenced by an ABI 3730 automated sequencer (Invitrogen, Shanghai, China). The full-length ayu NKEF-B cDNA contains 1092 bp with an open reading frame of 591 bp, coding for 197 amino acids with MW 21.9 kDa and pl 6.38, similar to the values determined by 2DE. The ATG initiating codon was at nucleotides (nts) 91–93. The 3′-UTR was 408 bp in length and an AAATATT polyadenylation signal appeared at the position 1070–1076, 16 nucleotides (nts) upstream from the site of polyadenylation. Ayu NKEF-B contains two catalytic cysteines located at amino acid positions 51 and 172.

Multiple alignment was done using ClustalW (http://clustalw.ddbj.nig.ac.jp). Phylogenetic and molecular evolutionary analyses
were conducted using MEGA version 4 [14]. Ayu NKEF-B had high similarity (93.1% amino acid identity) to carp and zebrafish NKEF-B, 85.2–87.5% to other fish NKEF-B and 77.2–79.1% to fish NKEF-A. It also had 76.5–80.3% and 71.9–73.9% amino acid identity to mammalian NKEF-A and NKEF-B respectively. Based on amino acid sequences, the phylogenetic relationship between representatives from different vertebrate lineages was evaluated and from the constructed phylogenetic tree, it appears that ayu NKEF-B is most similar to carp and zebrafish NKEF-B (Fig. 2). The NKEF-A and NKEF-B sequences fall into four different clusters: mammalian-amphibian (M-A) NKEF-A cluster, fish (F) NKEF-A cluster, F NKEF-B cluster and M-A NKEF-B cluster. The F NKEF-A cluster grouped with the M-A NKEF-A cluster and M-A NKEF-B cluster. The F NKEF-A cluster grouped with mammalian NKEF-A clusters than to the M-A NKEF-B cluster (Fig. 2). However, the F NKEF-B cluster is closer to the M-A NKEF-A and F NKEF-B clusters, suggesting an evolutionary relationship.

Fig. 2. Phylogenetic (Neighbour-joining) analysis of NKEF amino acid sequences using the MEGA4.0 program. 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. F NKEF-B and M-A NKEF-B clusters are shaded. Accession numbers of sequences used are ayu (Plecoglossus altivelis) NKEF-B, AF221841; African clawed frog (Xenopus laevis) NKEF-A, XM_001507835; mouse (Mus musculus) NKEF-A, XM_001092016 and human NKEF-B, BC000452.

Table 1. NKEF-B mRNA expression changes between ayu injected with PBS (H group), V. alginolyticus (NI group) and A. hydrophila (I group) at 12 hpi.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative intensity of RT-PCR product of NKEF-B per β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H group</td>
</tr>
<tr>
<td>Liver</td>
<td>0.53±0.15</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.75±0.20</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.35±0.09</td>
</tr>
<tr>
<td>Brain</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>Heart</td>
<td>0.36±0.11</td>
</tr>
<tr>
<td>Gill</td>
<td>0.41±0.10</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.24±0.07</td>
</tr>
</tbody>
</table>

*Significantly different from appropriate control.

TCT-3’. As an internal PCR control, pActin2(–): 5’-TCGTTGGTGA CATCAGAGAG-3’ and pActin2(–): 5’-CGCAGTCTGATGTC TGTG-3’ were used to amplify a 231 bp fragment of the housekeeping β-actin gene. Semi-quantitative RT-PCR was optimized [15] and the number of cycles that gave half-maximal amplification used for semi-quantitative PCR assay of each gene. Semi-quantitative RT-PCR conditions were: denaturing for 2 min at 94 °C, then cycles (28 for NKEF-B, 27 for β-actin) of denaturing for 30 s at 94 °C,
tissues including brain, spleen, liver, kidney, gill, muscle, heart and analysis. A Duncan (UVP, Upland, CA, USA). Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple range test considering $P \leq 0.05$ as significant followed the analysis.

At 12 hpi, NKEF-B gene transcripts were found in all tested tissues including brain, spleen, liver, kidney, gill, muscle, heart and intestine (Table 1, Fig. 3). Compared with H group, NKEF-B gene transcripts had not changed much in most tissues except intestine in NI group, but significantly increased in all tissues (2–5 folds) in I group. Ayu NKEF-B gene transcripts were highest in the spleen and moderately high in liver, kidney, heart and gill both in healthy and pathological conditions (Table 1, Fig. 3).

Changes in the protein composition of fish tissues and fluids have been associated with pathological conditions [16]. In this study, 2DE approach was employed to observe ayu liver proteins whose levels change during bacterial infection, and NKEF-B was identified as a significantly up-regulated protein (Fig. 1). The increase of NKEF-B protein expression was mainly due to ayu immune response to A. hydrophila infection, but the response of fish to exogenous proteins should also do some contribution (Fig. 1). To the best of our knowledge, it was the first report directly showing that NKEF-B protein levels are up-regulated in bacteria-infected fish.

NKEFs have been shown to enhance natural killer cell activity in mammalian [1,17]. Recent studies showed some relationships between fish NKEFs mRNA expression and bacterial or viral infection (or LPS injection). Challenge of turbot with pathogenic bacteria V. anguillarum significantly elevated NKEF-B, 60.5 °C for β-actin and extension for 30 s at 72 °C; the cycles were followed by a final extension step of 10 min at 72 °C. The products were electrophoresed on a 1.0% agarose gel and stained with ethidium bromide. After gel electrophoresis, the band intensities were quantified and analyzed with the Gel Doc-It™ Imaging system (UVP, Upland, CA, USA). Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple range test considering $P \leq 0.05$ as significant followed the analysis.

In addition to enhancing natural killer cell activity, mammalia NKEFs also have other functions such as regulating transcription activator protein [18], increasing cell resistance to oxidative stress [19], and protecting erythrocytes from further damage by oxidative stress [20,21]. Endotoxin clearly could elicit increased ROS production by macrophages in carp [22], and NKEF-B might serve as an antioxidative enzyme countering with such a condition. If expression changes and its multifaceted biological functions were taken together, NKEF-B might play a multifunctional role during the acute inflammation induced by pathogens.

However, correlation of NKEF-B mRNA and protein expression in ayu liver was not significant, possible due to technological and biological reasons [23]. Overall, gene expression at the mRNA level is generally correlated with that at the protein level, but mRNA-protein expression correlation largely varies for different genes and different gene biological categories [24,25].

The cDNA sequence of liver NKEF-B gene in ayu reported here was deposited in the EMBL/GenBank/DDBJ databases with the accession number FM200427.

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