中国水产科学研究院水产种质资源与养殖技术重点开放实验室
Key Laboratory of Fisheries Genetic Resources & Aquaculture,
Chinese Academy of Fishery Sciences

研究论文集

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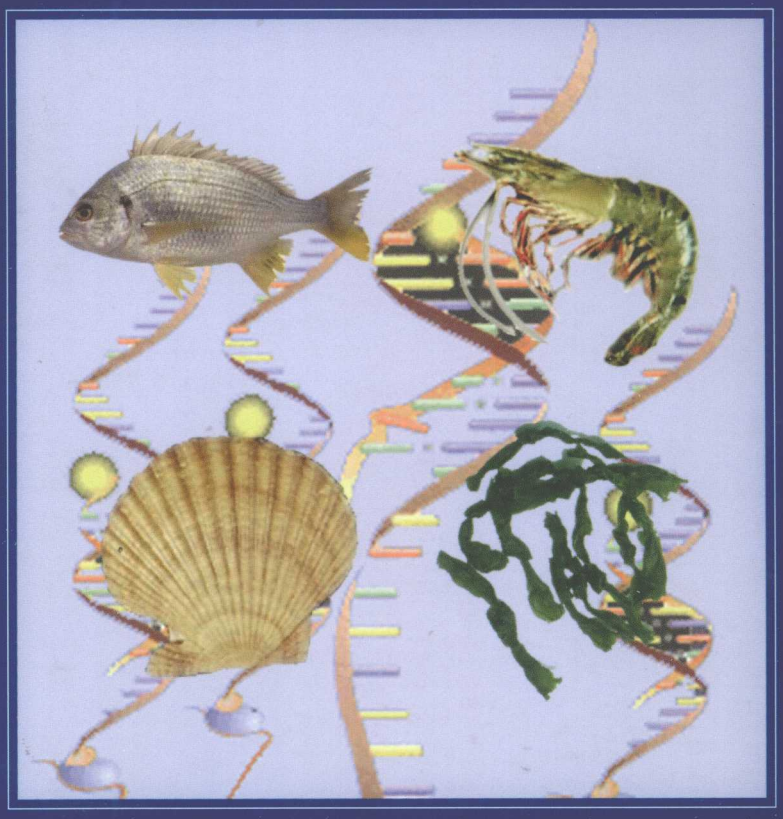

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2006年3月·广州

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前　言

2005 年，中国水产科学研究院水产种质资源与养殖技术重点开放实验室在中国水产科学研究院和依托单位中国水产科学研究院南海水产研究所的领导和支持下，各项工作按计划顺利实施，科技条件进一步改善，科技队伍建设得到加强，科技创新能力不断提高。

2005 年，本重点实验室主持和参加的各级各类科研项目共 45 项。其中，国家海洋“863”项目 4 项，国际招标项目 1 项，国家“十五”科技攻关项目 1 项，国家自然科学基金项目 3 项，科技部科技成果转化项目 1 项，省部级项目 9 项。其中延续项目共 26 项，新上项目 19 项。

2005 年，本重点实验室获得全国农业丰收奖一等奖 1 项，广东省科技进步二等奖 1 项，广东省农业技术推广一等奖 1 项，水科院科技进步三等奖 1 项，潮州市科技进步一等奖 1 项，进行科研成果鉴定 2 项，进行项目验收或评审 5 项，并获得较高的评价。在专利方面，本实验室共申请发明和实用新型专利 9 项，均已获受理，并获专利授权 4 项。在人才培养方面，新晋升研究员 1 名，副研究员 3 名；本年度本实验室到外单位攻读博士学位 4 人，与有关院校联合培养硕士生 20 人。

为了进一步加强学术交流，促进实验室建设，现将本实验室 2005 年度在各类各级期刊和学术会议上发表的 62 篇论文(其中 SCI 收录的论文有 4 篇)编辑成册。因时间仓促，水平有限，该论文集难免存在纰漏和不足之处，敬请读者指正。

同时借此机会，向一贯关心和支持本实验室建设和发展的各级领导、各位同行专家和朋友表示衷心的感谢。

编　者

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Cloning, Characterization and Expression Analysis of Interleukin-10 from the Zebrafish (*Danio rerion*)

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Cytokines are proteins produced by many different cells of the immune system and play a significant role in initiating and regulating the inflammatory process. In this research, an important cytokine, interleukin-10 (IL-10) gene, has been identified and characterized from zebrafish (*Danio rerio*) genome database. Zebrafish IL-10 is located within a 2690 bp fragment and contains five exons and four introns, sharing the same organization with mammalian IL-10 genes. An open reading frame of 543 bp was found to encode a putative 180 amino acid protein with a signal peptide of 22 amino acids, which shares 29.7-80.9% homology with amino acid sequences of other known IL-10. The signature motif of IL-10 is also conserved in zebrafish IL-10. The predicted transcript was finally confirmed by sequencing of cDNA clones. Multi-tissue reverse transcriptase PCR (RT-PCR) was performed to examine the tissue distribution and expression regulation of this gene in seven organs of normal and lipopolysaccharide (LPS) stimulation zebrafish. The results demonstrated that this gene was expressed slightly in normal kidney, gill and gut, no expression was detected in other four tissues. The expression was clearly upregulated after LPS stimulation. Using the ideal zebrafish model, further study of IL-10 characterization and function may provide insight on the understanding of the innate immune system.

Keywords: Cytokine, Genomic analysis, Interleukin, Zebrafish

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Introduction

Cytokines are proteins produced by many different cells of the immune system, which act upon other cells, and play a significant role in initiating and regulating the inflammatory process (Herve *et al.*, 2003; John *et al.*, 2003). Interleukin-10 (IL-10) is a fascinating cytokine first identified as a cytokine synthesis inhibitory factor (CSIF) in mouse (Fiorentino *et al.*, 1989; Moore *et al.*, 1990). The recent data suggest that IL-10 not only is the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cell, B cell and natural killer cell (Zdanov, 2004), but also mediates immunostimulatory properties that help to eliminate infectious and noninfectious particles with limited inflammation (Rousset *et al.*, 1992; David *et al.*, 2003; Asadullah *et al.*, 2003).

IL-10 genes have been isolated and characterized from a number of mammalian species (Goodman *et al.*, 1992; Dumoutier *et al.*, 2000; Alexander, 2004), all known mammalian IL-10 genes are composed of five exons and four introns, and contain several mRNA instability motif ATTTA sequences in the 3' untranslated regions (UTRs) (Shaw *et al.*, 1986; Brown *et al.*, 1996). The expression and regulatory mechanisms of IL-10 have been studied widely in mammal (De Wall *et al.*, 1991; Ding *et al.*, 2000). However, only a few cytokines have been cloned and characterized in the lower vertebrates. Recently, IL-10 genes have been isolated and characterized from fugu (*Fugu rubripes*) (Zou *et al.*, 2003), common carp (*Cyprinus carpio* L.) (Savan *et al.*, 2003) and rainbow trout (*Oncorhynchus mykiss*) (Inoue *et al.*, 2005) by comparative genomic analysis or by PCR-mediated homology cloning. Interestingly, unlike the mammalian IL-10, the rainbow trout IL-10 do not contain the mRNA instability motif ATTTA sequence in the 3'UTR. In normal tissues IL-10 expression patterns were different among above three kinds of fish species, but the reasons that induce this difference have been not known, to study the mechanism of this difference is

very interesting.

Zebrafish, *Danio rerio*, has recently emerged as an ideal model for the study of development, genetics and gene functions (Altmann *et al.*, 2003). In this study, IL-10 gene was identified and characterized from zebrafish using bioinformatics methods, and the open reading frame (ORF) was validated by RT-PCR. Multi-tissue RT-PCR was performed to examine the tissue distribution and expression regulation of IL-10 in seven organs of normal and LPS stimulation adult zebrafish. The results demonstrated that this gene was expressed slightly in normal kidney, gill and gut, no expression was detected in other four tissues. The expression was clearly upregulated after LPS stimulation. Further study of IL-10 characterization and function may provide insight on the understanding of the innate immune system.

Materials and Methods

Identification and characterization of zebrafish IL-10 A stretch of zebrafish genomic sequence (GenBank accession no. BX321912) was obtained directly from GenBank database at the National Center for Biotechnology Information (NCBI). Subsequently, the DNA sequence was further analyzed for predicted transcripts using GenScan program (Burge et al., 1998) (http://genes.mit.edu/GENSCAN.html). Eleven transcripts were generated by GenScan program, one of which revealed high homology with known interleukin-10 molecules in N-terminal region, but the C-terminal region had little similarity to the known IL-10 and was approximately 130 aa longer. To determine the correct reading frame of each exon, BLAST program and DNASTAR software were used to maximize the in-frame translated sequence. Finally, the individual exon sequences was compiled into the full protein encoding sequence by manual adjustment.

Putative protein and phylogenetic analysis The molecular mass and isoelectric point of the putative zebrafish IL-10 were predicted using Compute pI/Mw tool at the ExPASy molecular biology WWW server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/) and the deduced signal peptide was predicted using SignalP (Bendtsen *et al.*, 2004). Motif search was performed with eMOTIF Scan program (http://motif.stanford.edu/emotif/emotifscan.html). The homology between amino acid sequence of zebrafish IL-10 and other known IL-10s was analyzed using Clustal W (Higgins D *et al.*, 1994). Multiple sequence alignment and phylogenetic tree construct were performed using Mega3 (Kumer *et al.*, 2004). The genetic distance between species was calculated using ρ-distance method. The cladogram was generated using the neighbor-joining (NJ) method. In the analysis, the gaps were deleted, and a 1000 bootstrap procedure was used to test the robustness of the node on the trees.

cDNA cloning and muti-tissue RT-PCR RT-PCR was performed to clone and sequence zebrafish IL-10 cDNA. After LPS simulation, total RNA was extracted from kidney using Trizol reagent (Invitrogen, Carlsbad, USA) and the standard protocols recommended by the supplier. 400 ng of total RNA was used as the template for the synthesis of first strand cDNA by reverse transcriptase using AMV Reverse Transcription System (promega, Madison, USA). For PCR amplification, Primers were designed and synthesized based on the predicted cDNA sequence as follows: FW1 5'-GCTCATCTGTACATCTTCTCACTTG-3' and RV15'-CTGTCCAACCCAGCAACATCCTA-3'. PCR were carried out using 25 U Ex Taq polymerase (TaKaRa, Kyoto, Japan) in 50 μL reactions containing 250 μM of each dNTP, 0.4 μM of each primer, 5 μL 10× Taq buffer, 37.5 μL sterile H_2O and 1 μL cDNA template according to the standard protocol. PCR amplification was conducted under the following conditions: an initial cycle of denaturation step at 94°C for 2 min, 35 cycles of amplification at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. 5 μL product was size-fractioned by 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. Desired PCR products were isolated by gel elution (Qiagen, Chatsworth, USA), and ligated into pGEM-T vectors (Promega, Madison, USA). These were transformed into JM101 competent cells and plated on selective agar plates containing 100 μg/ml Ampicillin. Positive colonies were grown in overnight cultures and plasmid DNA extracted using affinity/elution method (QIA prep kit, Qiagen, Chatsworth, USA). Insert size was determined by agarose gel electrophoresis after digestion with *Eco*R 52 in comparison to the 100 bp DNA Marker. Three of cDNA colonies were sequenced on an ABI 377 instrument, respectively.

To reveal the tissue distribution and expression regulation of zebrafish IL-10, total RNA was extracted from seven organs of normal and LPS stimulation adult zebrafish respectively. The first strand cDNA was synthesized from each sample by reverse transcriptase as described previously. The gene specific primers FW2 (5'-ACGCTTCTTCTTTGCGACTG-3') and RV2 (5'-CACCATATCCCGCTTGAGTT-3') were designed to amplify 342 bp fragment of IL-10. Positive control primers were designed according to zebrafish β-actin (GenBank accession no. AF057040) as Actin-FW: 5'-CAGACTACCTGATGAAGATCCTGAC-3' and Actin-RV: 5'-GTGTTGGCATACAGGTCCTTACG-3' to amplify 336 nucleotide fragment. Multi-tissue RT-PCR was performed according to the method described by Laing (Laing *et al.*, 2001).

Results

Identification and characterization of the zebrafish IL-10 gene The genomic analysis methods was used to identify zebrafish IL-10, which has been submitted to GenBank (GenBank accession no. AY887900). Trace sequences representing exon 1 and partial exon 3 were first identified by GenScan program. Then, the other exons and the exact exon/intron boundaries were identified based on the known IL-10s using BLAST and Clustal W program. The compiled zfIL-10 gene was 2690 bp in length and contained a 543 bp ORF. Like most cytokine genes, its 3' UTR was AT (67.2%) rich and contained a typical polyadenylation signal AATAAA at nuceotide position 478 bp downstream from the translation stop codon, and four mRNA instability sequences ATTTA. Its 5' UTR contained a promoter TATA box at nucleotide position 90 bp

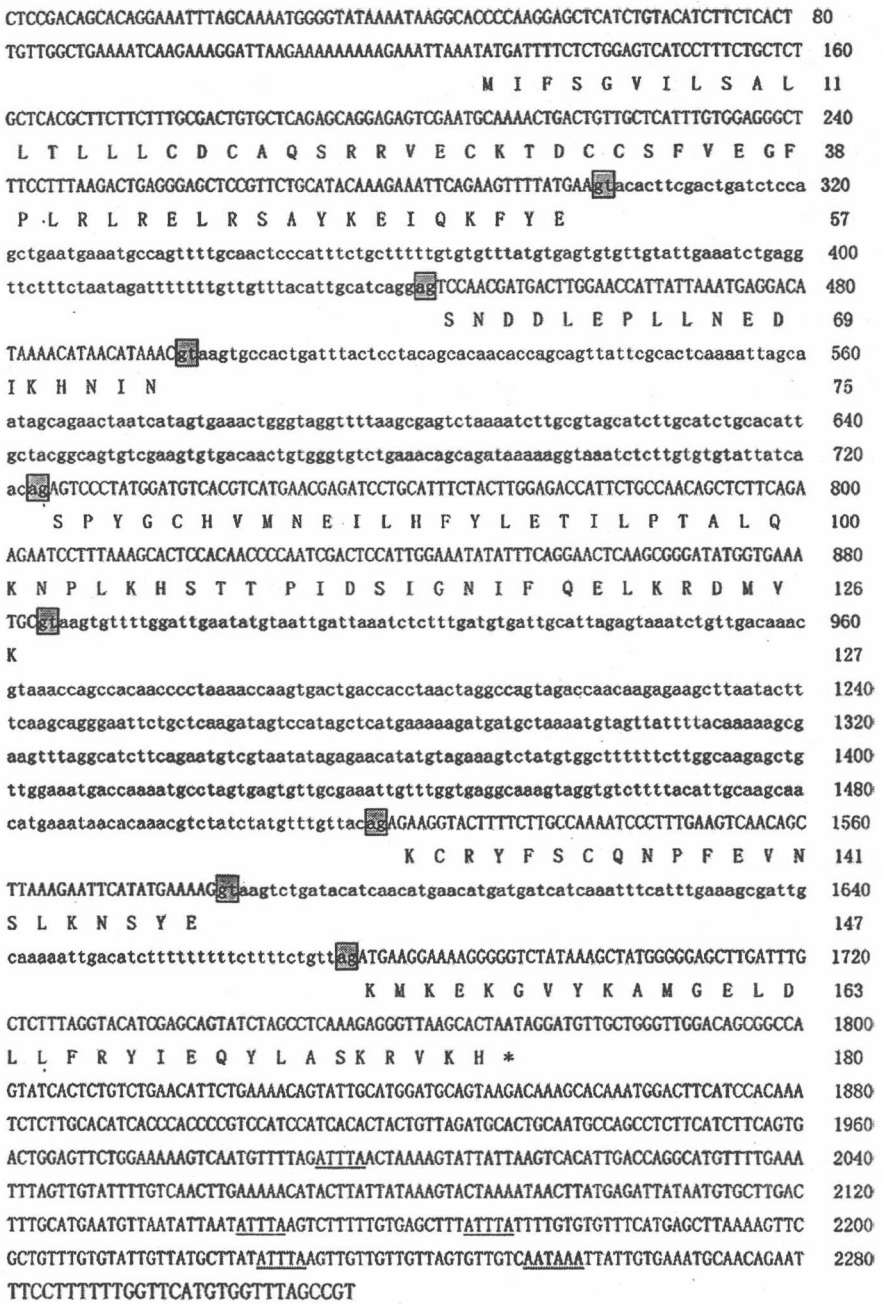

Fig. 1. The zebrafish IL-10 gene sequence and deduced amino acid sequence. The intron sequences are in *lower case* and the intron splicing consensus (gt/ag) are *boxed*. The promoter (TATA) and the poly (A) signal are in *bold* and *underlined*, whilst the mRNA instability motif in the 3' UTR is *underlined*. The deduced amino acid sequence is given *below* the nucleotides. The stop condon is represented with an *asterisk*.

upstream of the translaton initiation codon. Zebrafish IL-10 gene was composed of five exons and four introns, sharing the same exon structure with mammalian counterparts. The four introns were 144, 227, 433 bp and 94 bp in length respectively, similar to that of common carp and fugu, but more compact than that of human and rainbow trout. A typical intron splice motif (5' GT-intron-3' AG) was present at the 5' end and the 3' end of all the four introns (Fig. 1).

Analysis of the putative protein and phylogenetic tree Zebrafish IL-10 contains an open reading frame encoding 180 amino acids with a theoretical molecular weight of 20.98 kD α and the isoelectric point of 8.07 using ORF finder at NCBI and Compute pI/Mw tool at the ExPASy server. A putative signal peptide of 22-amino-acid (MIFSGVILSALLT-LLLCD

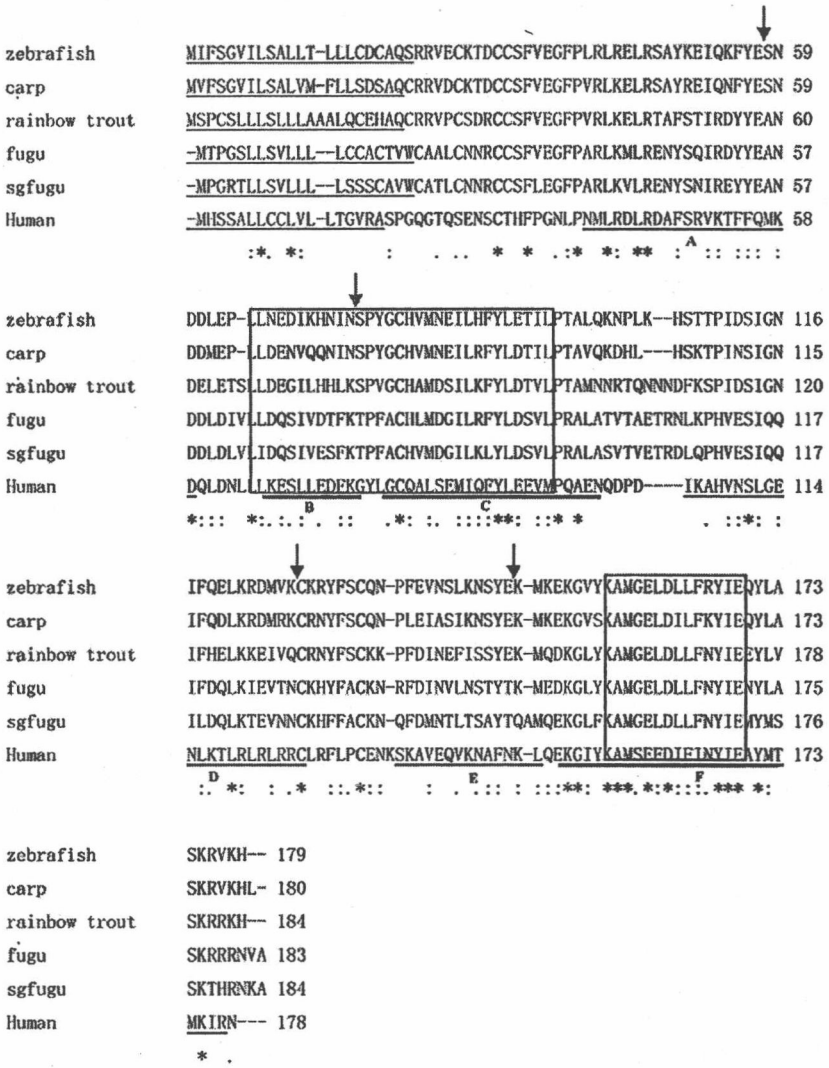

Fig. 2. Alignment of the deduced amino acid sequence of zfIL-10 with other homologues in fish and human counterparts. Identical (*) and similar (. or :) amino acid residues are indicated. The six α-helical domains predicted in human IL-10 are marked by the *bar below*. *Arrows* "↓" indicate the intron position in zfIL-10 gene. The IL-10 family signature motifs are *boxed*, and the cysteine residues involved in the formation of disulphide bridges are shown in *bold*. The signal peptide is *underlined*.

CAQS) was predicted at the N-terminus using the SignalP program. Two signature motifs were identified using the eMOTIF Scan program follows: LLNEDIKHNINSPYGCHV MNEILHFYLETIL and KAMGELDLLFRYIE belonging to the conserved IL-10 family signature sequence L-[FILMV]-X(3)-[ILV]-X(3)-[FILMV]-X(5)-C-X(5)-[ILMV]-[ILMV]-X(3)-L-X(2)-[IV]-[FILMV] and KA-X(2)-E-X-D-[ILV]-[FLY]-[FILMV]-X(2)-[ILMV]-[EKQZ] respectively. Four conserved cysteine residues in other known IL-10s were found in zebrafish IL-10. The amino acid sequence of zebrafish IL-10 was aligned (Fig. 2) and shared 29.7-80.9% homology with other known IL-10s.

Based on the genetic distances calculated from IL-10 amino acid sequence of 17 species with p-distance model, a cladogram was constructed using the N-J method with goldfish interferon (IFN) as the outgroup. As shown in Fig. 3, the in-group of 16 species was divided into two group. The first group all belonged to fish species. The second group also could be divided into two branches, one was composed of 10 species of mammal, another was avian. All the clades got very high bootstrap support values.The results implied that the phylogenetic tree based on the IL-10 gene were reliable, and generally revealed their evolutionary distances.

cDNA cloning and expression analysis RT-PCR was performed with total RNA extracted from kidney of LPS stimulation zebrafish. The predicted transcript was finally confirmed by sequencing of cDNA clones(data not shown).

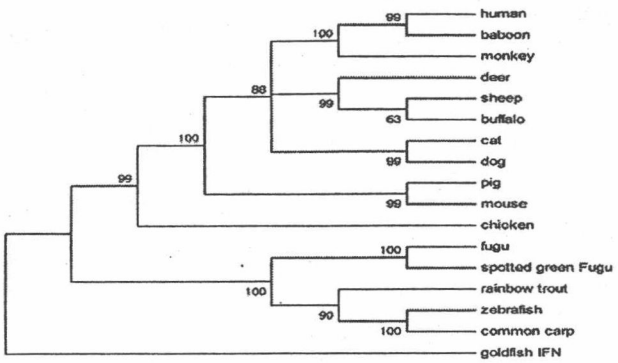

Fig. 3. A phylogenetic tree constructed by the neighbor-joining method (using Mega2 software) from the amino acid sequences of the IL-10 together with the zfIL-10 gene. The numbers indicate the bootstrap confidence values obtained for each nodes after 1000 replications. The IL-10 protein sequences were retrieved from the Swissprot database: human, CAG46825; baboon, AAV85009; monkey, P51496; deer, AAA85434; sheep, CAG38358; buffalo, AAQ94107; cat, P55029; dog, NP_001003077; pig, AAC83808; mouse, NP_034678; chicken, CAF18432; fugu, CAD62446; spotted green fugu, AAP57415; rainbow trout, BAD20648; common carp, BAC76885; goldfish interferon, AAR20886.

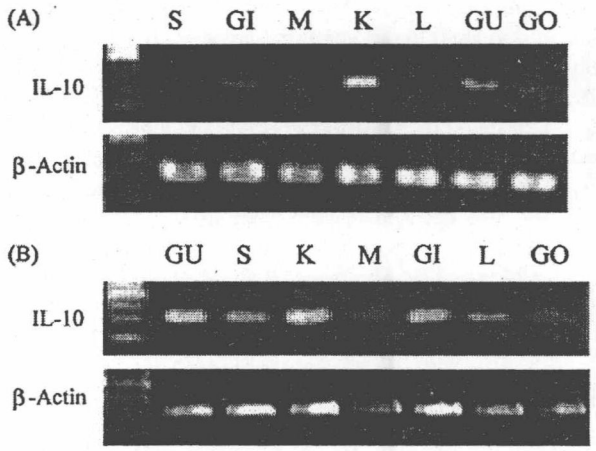

Fig. 4. Expression patterns of IL-10 gene in seven normal and LPS stimulation organs from zebrafish.Total RNA was extracted from seven organs respectively for muti-tissue RT-PCR using the IL-10 specific primers. A was normal tissues, and B was LPS stimulation tissues. Upper letters were: S. skill; GI. gill; M. muscle; K. kidney; L. liver; GU. Gut; GO. Gonad. The same samples were assayed for β-Actin mRNA expression as a positive control.

The results showed that there is only one nucleotide difference between the sequences of cDNA clones and the predicted transcript, which maybe is brought due to the difference between individuals.

Multi-tissue RT-PCR was performed to examine the tissue distribution and expression regulation of zebrafish IL-10 in seven organs of normal and LPS stimulation adult zebrafish. Slight amount of PCR product was detected using zebrafish IL-10 specific primers in normal zebrafish kidney, gill and gut. However, no visible PCR product was detected with cDNA derived from skin, muscle, gonad and liver. After 4 h following LPS stimulation, PCR product was detected in all seven organs, furthermore, the expression is clearly up-regulated in kidney, gill and gut (Fig. 4).

Discussion

IL-10 gene has been cloned in several vertebrates such as human (Vieira, 1991), mouse (Kim *et al.*, 1992), chicken (Rothwell *et al.*, 2004) and fish (Inoue *et al.*, 2005), and demonstrated that the expression patterns of fish IL-10 were different from mammalian, as well as different among fish species. To further study the characteristic and distribution of this gene, IL-10 was identified and cloned from zebrafish, a ideal experimental model. Genomic analysis, an efficient and powerful approach for identifying new gene in various organisms, was used to identify zebrafish IL-10. zebrafish IL-10 amino acid sequence shares 29.7-80.9% homology with other known IL-10s. The homology between zebrafish and common carp is the most high up to 80.9%, and our recent study demonstrated that this gene is highly conserved in fish of the same family (unpublished). Phylogenetic analysis indicated that mammalian and avian IL-10s form distinct clades to the fish IL-10s. Furthermore, pairwise comparison of amino acid and nucleotide sequence identity and similarity also indicated similar degrees of divergence between fish IL-10s and all mammalian and avian IL-10s. These results indicated that the divergence of fish IL-10s from an ancestral IL-10 occurred prior to the divergence of mammalian and avian IL-10s.

Zebrafish IL-10 don't contain NXS/T motif needed for N-terminal glycosylation, which suggests that zebrafish IL-10 is not glycosylated. Mouse IL-10 has one potential glycosylation site, but which is not required for biological activity (Moore *et al.*, 1993), so maybe the ancestor gene of IL-10 is not to be glycosylated to exert its functions. The putative mature peptide of zebrafish IL-10 contains four conserved cysteine residues (cys-31, 80, 128 and 134) which are critical to maintain the tertiary structure of IL-10s, two extra cysteine residues (cys-27 and 32) are also present in mature peptide, but it is not known that whether they are important for tertiary structure. IL-10 consists of six α-helices termed A, B, C, D, E and F, which tightly associate with the other monomer to form two interpenetrating domains (Walter *et al.*, 1995), zebrafish IL-10 also possesses all six α-helices. Zebrafish IL-10 contains two typical IL-10 signature sequence motifs, which also confirms that the predicted gene is IL-10.

The previous study indicated that the expression patterns of IL-10 were different among fish species (Inoue *et al.*, 2005). In normal tissues, common carp IL-10 was strongly expressed in head kidney and intestine, rainbow trout IL-10 was weakly

expressed in gill, puffer fugu IL-10 was weakly expressed in kidney and liver. In this study, zebrafish IL-10 was slightly in normal kidney, gill and gut tissues, which also was different from above three fish species, but why the difference induced has been not known. To further study the mechanism of this difference is interesting. After 4h LPS stimulation, zebrafish IL-10 expression was clearly up-regulated in kidney, gill and gut, and also detected in other four tissues. Therefore, it is concluded that this gene is associated with the innate immune system of zebrafish. The mechanism of expression regulation of IL-10 in zebrafish is under further research.

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cDNA cloning and mRNA expression of the translationally controlled tumor protein (TCTP) gene from Japanese sea perch (*Lateolabrax japonicus*)

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Abstract

A homologue of the lower vertebrates translationally controlled tumor protein (TCTP) was cloned from the marine fish Japanese sea perch (*Lateolabrax japonicus*) by the technology of homology cloning. The full-length cDNA sequence of the sea perch TCTP gene contained a 5' untranslated region (UTR) of 47 bp, a 3' UTR of 433 bp, and a putative open reading frame (ORF) of 510 bp encoding a polypeptide of 170 amino acids. The deduced amino acid sequence of the sea perch TCTP gene showed a high similarity to that of zebrafish, rohu, rabbit, chicken and human. Sequence analysis revealed there were a signature sequence of TCTP family, an N-glycosylation site, and five Casein kinase phosphorylation sites in the sea perch TCTP. The temporal expression of TCTP genes in healthy and lipopolysaccharide (LPS) challenged fishes was measured by semi-quantitative reverse transcription-PCR (RT-PCR). The results indicated that LPS could up-regulate the expression of sea perch TCTP in the examined tissues, including head-kidney, spleen and liver.

Key words: translationally controlled tumor protein, *Lateolabrax japonicus*, cDNA cloning, mRNA expression

1 Introduction

The translationally controlled tumor protein (TCTP) was first described as a growth-related protein in mouse *Ehrilisch ascites* tumor cells and erythroleukemia cells (Yenofsky et al., 1983). Subsequently, TCTP was founded to be present in many cells (Sanchez et al., 1997; Gross et al., 1989; Chung et al., 2000; Vercoutter-Edouart et al., 2001) except the human kidney cell (Sanchez et al., 1997; Gachet et al., 1999). Homologues of TCTP have been reported from several organisms including plants, earthworm, parasites, rabbit, shrimp, etc. (Pay et al., 1992; Sage-Ono et al., 1998; Thiele et al., 1998; Bhisutthibhan et al., 1999; Gnanasekar et al., 2002; Bangrak et al., 2004).

TCTP is a ubiquitous growth-dependent protein of ill-defined primary function. It is a house keeping and highly conserved fairly abundant protein. Exten sive characterization studies revealed that TCTP was calcium-binding, heat stable protein (Sanchez et al., 1997; Sturzenbaum et al., 1998; Kim et al., 2000) that could induce histamine release and secretion of interleukin-4 from basophils (MacDonald et al., 1995). In addition, TCTP could bind to tubulin (Gachet et al., 1999), induced intracelluar signaling (MacDonald et al., 1995) and affected cognitive function in neurodegenerative disorders (Kim et al., 2001). As indicated

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by its name, the synthesis and expression of TCTP are under translational control (Bommer et al., 1994; Xu et al., 1999). However, the expression of TCTP is also under the transcriptional control (Sturzenbaum et al., 1998; Kang et al., 1996; Sage-One et al., 1998; Mak et al., 2001). Because the expression of TCTP can be regulated by serum, PMA and IFN-γ, it is believed that TCTP can play a role in the cellular proliferation (Bohm et al., 1989; Walsh et al., 1995; Thomas and Luther, 1981; Thomas et al., 1986). Interestingly, TCTP gene was also found to be up-regulated in C6.9 rat glioma cells undergoing apoptosis in response to vitamin D3 (Baudet et al., 1998). Despite those varied potential functions, the ubiquitous distribution and high level of conservation, the primary physiological function of TCTP still remains unclear (Kakuturu et al., 2002).

Recently, many methods such as expressed sequence tagging and homology cloning have been used for the gene identification and cloning. The homology cloning method has proved to be a useful tool in isolating the genes which have been identified in the phylogenic related species. In the present study, the technology of homologue cloning was used to clone the cDNA sequence of TCTP gene from Japanese sea perch (*Lateolabrax japonicus*). And the temporal expression of TCTP gene in healthy and LPS challenged fish was measured by semi-quantitative reverse transcription-PCR (RT-PCT) in order to understand the regulation of sea perch TCTP gene and its potential role in the immune response.

2 Materials and methods

Adult healthy Japanese sea perch fishes (*Lateolabrax japonicus*) were purchased from Qingdao, Shandong Province, China, weighing about 500 g each. After being cultivated in the aerated seawater for 3 d, they were received an injection of 200 mm^3 of LPS (100 $\mu g/cm^3$) and kept 4 h before processing.

Spleen, liver and head-kidney were removed from the stimulated fishes and the total RNA was isolated using Trizol reagent following the protocol of the manufacturer. The RNA was resuspended in the DEPC-treated water and the soluble total RNA was stored at -80 ℃.

cDNA was synthesized from 2 μg of mRNA by moloney murine leukemia virus (M-MLV) reverse transcriptase at 42 ℃ for 50 min with oligo-dT adaptor primer following the protocol of the manufacturer (Table 1).The cDNA was used as template for PCR reactions.

Initially, PCR was performed using the cDNA prepared above as template, with the primers that were

Table 1. Oligonucleotide primers used in experiments

Name	Sequence (5′→3′)	Information
Oligo-dG	GGGGGGGGGGGGGGH	used for RACE-PCR
Oligo (dT)-adaptor	CTCGAGATCGATGCGGCCGCT17	used for cDNA synthesis
Adaptor primer	CTCGAGATCGATGCGGCCGC	used for RACE-PCR
F1	CATCGTCCTCAACCACAAAC	gene specific primer
F2	GTGGGACATCGTGACTACCG	gene specific primer
R1	GAGGACGATGTCTACACCAG	gene specific primer
R2	TAACATTTCTCAGCCAACAG	gene specific primer
β-actin F	ATCGTGGGGCGCCCCAGGCACC	used for RT-PCR
β-actin R	CTCCTTAATGTCACGCACGATTTC	used for RT-PCR
Fe	TAGACATCGTCCTCAACCAC	used for RT-PCR
Re	TAACATTTCTCAGCCAACAG	used for RT-PCR

designed based on conserved regions of other known TCTP gene sequences, to allow the isolation of a partial fragment from the sea perch TCTP gene. The obtained PCR product was analyzed by electrophoresis through an ethidium bromide (100 ng/cm^3) stained 1.2% agarose gel. The amplified fragment with the expected size was purified with the Concert Rapid PCR Purification System (TaKaRa) and cloned into the pMD-18T vector (TaKaRa). After being transformed into the competent cells of *Escherichia coli* JM-109, the recombinants were identified through blue-white color selection in ampicillin LB plates containing 40 mg/cm^3 of X-Gal (TaKaRa). Plasmid DNA from at least three independent clones was isolated by the Concert Rapid Plasmid Miniprep System (TaKaRa) and verified by PCR with the primers of M13 forward and reverse. The positive clones were sequenced in both directions with the primers of M13. Sequences generated were analyzed for similarity with other known sequences using BLAST programs (http://www.ncbi.nim.nih.gov/).

Based on the partially cloned sea perch TCTP sequence, the 5′ and 3′ ends of mRNA were obtained by rapid amplification of cDNA ends (RACE)-PCR, using gene-specific primers generated from the obtained sequences (see Table 1). In the 3′ RACE-PCR, cDNA was reverse transcribed from poly (A) mRNA using an oligo-dT adaptor primer. PCR was performed with a sea perch TCTP-specific forward primer F1 and the adaptor primer (see Table 1), while a second semi-nested PCR was carried out with sea perch specific primer F2 and adaptor primer. In the 5′ RACE - PCR, cDNA was tailed with poly (C) at the 3′ end with terminal deoxynucleotidyl transferase (TdT) (Promega). PCR reaction was initially performed with a sea perch TCTP-specific reverse primer R1 (see Table 1) and an oligo-dG primer, and followed by the semi-nested PCR with sea perch specific primer R2 and the oligo-dG primer.

Three overlapping fragments of TCTP gene were amplified by RT-PCR(Fig.1).The generated sequences

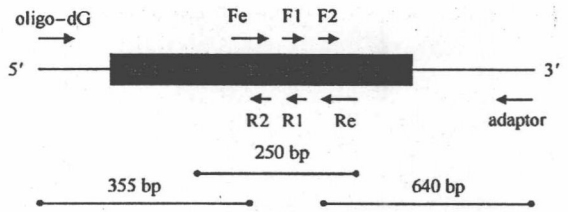

Fig. 1. Position of primers (indicated by arrows) used to amplify Japanese sea perch TCTP cDNA and the expected products. The ORF (510 bp) was indicated with a shadow box.

were analyzed for similarity to other known sequences using the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nim.nih.gov/). Multiple alignment of the sea perch TCTP was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree was constructed using Clustal X1.81 and MEGA2.0 based on the TCTP amino acid sequences.

The mRNA expression of the sea perch TCTP gene in different tissues of healthy and LPS-challenged fish was measured by semi-quantitive RT-PCR. Total RNA was isolated from the different tissues of three individuals and reverse transcribed into cDNA. A fragment of β-actin gene amplified by primer β-actin F and β-actin R was used as a positive control to verify the successful transcription and to calibrate the cDNA template for corresponding samples. A pair of TCTP-specific primers, Fe and Re, were used in RT-PCR to detect the expression of TCTP.

3 Results

The PCR product amplified by using the technology of homology cloning was about 250 bp (see Fig. 2a). TCTP-specific forward primer (TCTP F1) and the adaptor primer were used in the 3′RACE-PCR to amplify the cDNA 3′end, and the obtained PCR product was about 640 bp (see Fig. 2b). While the oligo-dG primer and sea perch TCTP-specific reverse primer (TCTP R1) were used in the 5′RACE-PCR to amplify the cDNA 5′end, the fragment amplified was about 350 bp (see Fig. 2c).

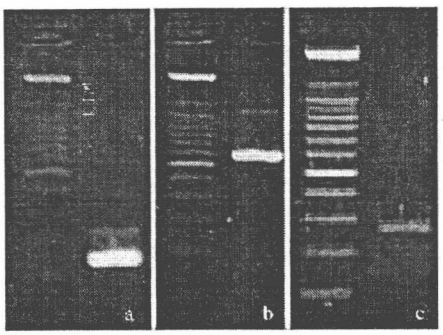

Fig. 2. The PCR products from homology cloning (a), 3′-RACE (b) and 5′-RACE (c). M represents 100 bp ladder.

A 994 bp sequence was obtained by overlapping the obtained fragments, representing the complete sea perch TCTP gene. The full-length cDNA nucleotide sequence and its encoding amino acid sequence were shown in Fig.3. It has been submitted to genbank and the accession number is AY210896. The full cDNA sequence contained a 5′ untranslated region (UTR) of 47 bp, followed by an ORF of 510 bp, a long 3′ UTR of 433 bp, a 22 bp poly $(A)^+$ tail, and a putative polyadenylation signal located 13 nucleotides upstream of the poly $(A)^+$ tail. The ORF was capable of encoding a polypeptide of 170 amino acids with an estimated molecular mass of 19.253 kDa and a predicted isoelectric point (PI) of 4.55.

The search of the amino acid sequence of sea perch TCTP with the pattern database of Prosite revealed that the sea perch TCTP contained the signature sequence (Amos, 1997). It was LKNYQFFTGENMNPEGMVGLLDY, which corresponded to the amino acid position from 127 to 149. The sea perch TCTP also contained two potential phosphorylation sites [ST]-x-[RK], corresponding to the amino acid positions at 36~38 (T-t-R) and 85~87 (S-y-K); a N-glycosylation site at 50~53 (N-A-S-A); five Casein kinase phosphorylation sites at 9~12 (S-g-d-E), 37~40 (T-r-t-D), 52~55 (S-a-e-E), 66~69 (S-g-v-D) and 79~82 (T-s-f-D); and a tyrosine sulfation site at 142~156 (g-m-v-g-l-l-d-Y-r-e-d-g-t-t-p).

Searching for sequence similarities of the sea

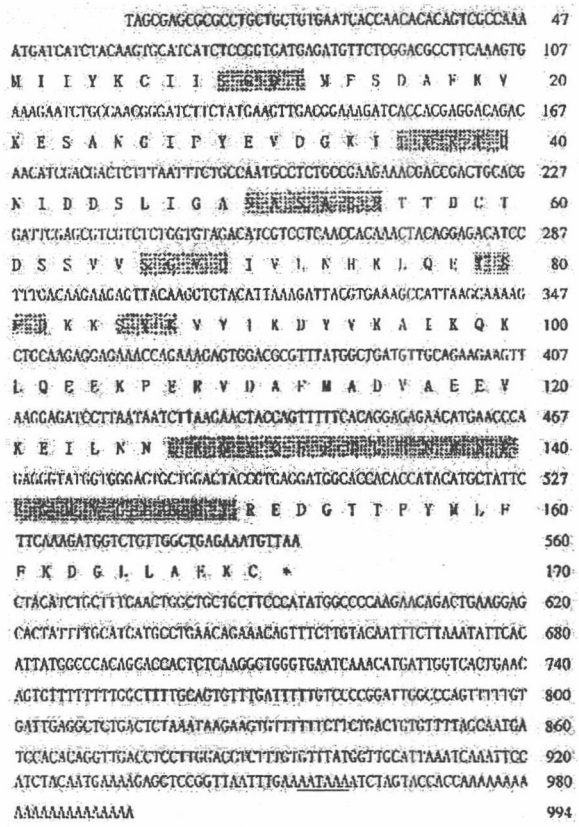

Fig. 3. The full-length cDNA sequence of Japanese sea perch TCTP. Predicted translated amino acids are shown in the lower case. Nucleotides in bold represent the start codon (position 48~50) and the stop codon (position 558~560). Signature sequences, potential phosphorylation site, N-glycosylation site, Casein kinase phosphorylation site, and tyrosine sulfation site are highlighted. The putative polyadenylation signal is underlined.

perch TCTP to other known proteins revealed close matches with highly conserved amino acid residues commonly shared by other known TCTPs (see Table 2), and it was confirmed that the isolated cDNA clone was a homologue of TCTP protein. The sea perch TCTP gene displayed high similarity to the TCTP from zebrafish (82.5%), human (81%), chicken (79%), rohu (82%), and rabbit (81%), respectively.

Multiple alignment of the sea perch TCTP with the homologous genes of other species revealed that the motif sequences were conserved in all the tested

Table 2. Homology analysis of sea perch TCTP amino acid with other known TCTP genes

Organism	Length/aa	Positives (%)	E-value	Accession number
D. rerio	171	82.5	$3e^{-59}$	AF288217
L. rohita	171	82	$2e^{-58}$	AAK27316
G. gallus	172	79	$6e^{-57}$	A38960
O. cuniculus	172	80	$4e^{-56}$	CAA12650
H. sapiens	172	81	$8e^{-57}$	AAH03352

species, indicating that the function of these motifs was also conserved (Fig. 4).

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human       MIIYRDLISHDEMFSDIYKIREIADGLCLEVEGKMVSRTEGNIDDSLIGGNASAEGPEGE 60
rabbit      MIIYRDLISHDEMFSDIYKIREIAGGLCLEVEGKMVSRTEGNIDDSLIGGNASAEGPEGE 60
chicken     MIIYRDCISQDEMFSDIYKIREVANGLCLEVEGKMVTRTEGQIDDSLIGGNASAEGPEGE 60
zebrafish   MIIYKDIITGDEMFSDIYKIKESENGMMLEVEGKMITRAEGDIDDALIGGNASAEVAD-E 59
rohu        MIIYKDIITGDEMFSDIYKIKESENGMMIEVEGKMISRAEGEIDDALIGGNASAEVQD-E 59
sea perch   MIIYKCIISQDEMFSDAFKVKESANGIFYEVDGKITTRTD-NIDDSLISANASAEETT-D 58
            ****:  *: ****** :*::* .*:  **:**: :*:: :***:**.*****   :

human       GTESTVITGVDIVMNHHLQETSFTKEAYKKYIKDYMKSIKGKLEEQRPERVKPFMTGAAE 120
rabbit      GTESTVITGVDIVMNHHLQETSFTKEAYKKYIKDYMKSIKGKLEEQRPERVKPFMTGAAE 120
chicken     GTEATVITGVDIVINHHLQETSFTKESYKKYIKDYMKAIKARLEEHKPERVKPFMTGAAE 120
zebrafish   GCDSTSVSGVDIVLNHKLQETSYDKKSYTAYIKDYMKAVKAKLQESAPNRVDPFMANAPA 119
rohu        GCESTTVSGVDIVLNHKLQETSYDKKSYTAYIKDYMKAVKAKLQEVAPDRVDPFMANAPA 119
sea perch   CTDSSVVSGVDIVLNHKLQETSFDKKSYKVYIKDYVKAIKQKLQEEKPERVDAFMADVAE 118
            ::: ::*****:**:*****: *::*. *****:*::* :*:* *:**.**:...

human       QIKHILANFKNYQFFIGENMNPDGMVALLDYREDGVTPYMIFFKDGLEMEKC 172
rabbit      QIKHILANFKNYQFYIGENMNPDGMVALLDYREDGVTPFMIFFKDGLEMEKC 172
chicken     QIKHILANFKNYQFFVGENMNPDGMVALLDFREDGVTPYMIFFKDGLEIEKC 172
zebrafish   EVKKILGNIKNFQFFTGESMNPDGMIGLLDFREDGVTPYMIFFKDGLEIEKC 171
rohu        EVKKILGNIKNFQFFTGESMNPDGMIGLLDFREDGVTPYMLFFKDGLEIEKC 171
sea perch   EVKEILNNLKNYQFFTGENMNPEGMVGLLDYREDGTTPYMLFFKDGLLAEKC 170
            ::*.** *:**:**: **.***:**:.***:****.**:*:****** ***
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Fig. 4. Multiple alignment (Clustal W) of deduced amino acid sequences of TCTP gene. Identical and similar residues identified in all the proteins are indicated by asterisks and colons. The conserved amino acid residues in the sea perch TCTP are shown in bold capitals.

A phylogenetic tree was constructed using the program of Clustal X1.81 and MEGA2.0 based on the full-length TCTP amino acid sequences (Fig.5). It clearly showed that the sea perch was clustered together with zebrafish and rohu, and formed a sister group apart from other species.

RT-PCR with specific primers Fe and Re (see

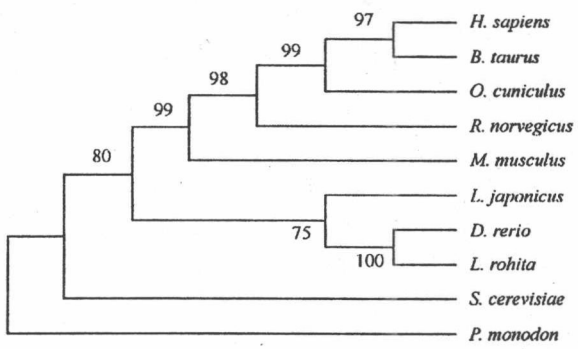

Fig. 5. Phylogenetic tree constructed based on the full-length TCTP amino acids sequences in different groups with the program Clustal X1.83 and MEGA2.1 using the neighbor-joining method. The used amino acids were from GenBank.

Table 1) that gave rise to a product of 210 bp was performed with the calibrated cDNA templates from the tissues described above. TCTP transcripts could be clearly detected in the spleen, liver and head-kidney of the control and stimulated fish, but it was very weak in the spleen of the control fish. After being stimulated with LPS, the expression of TCTP was up-regulated in all the examined tissues, especially in the spleen (Fig. 6).

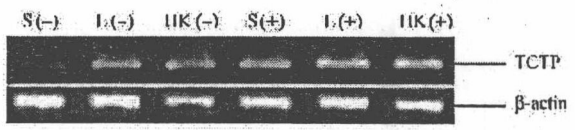

Fig. 6. The expression of TCTP in different tissues (stimulated: +; unstimulated: –) by RT-PCR. HK represents head-kidney, L liver and S spleen.

4 Discussion

Even though TCTP has been found in a wide va-

riety of organisms including mammals, plants, lower eukaryotes and prokaryotes, and a lot of TCTP genes have been cloned, zebrafish TCTP gene is the only one fish TCTP gene which has been cloned so far. The function of TCTP in fish has not been studied well, and is still not clear yet. In the present study, a TCTP homologue was cloned from the Japanese sea perch by using the technology of homology cloning and RACE-PCR. The full-length sea perch TCTP cDNA contained an ORF of 510 bp encoding a peptide of 170 amino acids, which was almost the same size as other known TCTPs. The sequence of sea perch TCTP shared high identity with and similarity to other known TCTPs, especially with zebrafish TCTP. There were 82.5% amino acids identical with zebrafish, 82% with rohu, 81% with human, 81% with rabbit, and 79% with chicken. This indicated that the sea perch TCTP had the similar primary structure to the known TCTP.

Based on the multiple alignment analysis of the sea perch TCTP with other known TCTPs, highly conserved amino acids were found in the function sites or domains (see Fig. 4). A typical TCTP family signature sequence (LKNYQFFTGENMNPEGMVGLLDY) was found in the predicted amino acid sequence at the position of 127~149. And a tyrosine sulfation site (g-m-v-g-l-l-d-Y-r-e-d-g-t-t-p) was found to be at 142~156. The other sites, such as the phosphorylation sites [ST]-x-[RK], N-glycosylation site (N-A-S-A), Casein kinase phosphorylation sites were also found to be conserved. The structure analysis suggested that the sea perch TCTP should be a homology of TCTP and should have the similar functions. In the expression assay, the sea perch TCTP transcript was detected in spleen, liver and head-kidney of the control fish, but the expression level in liver and head-kidney was higher than that in spleen. It is indicated that the sea perch TCTP was expressed constitutively in liver and head-kidney. After being stimulated with LPS, the expression of TCTP was up-regulated in spleen, liver and head-kidney. Especially, the TCTP expression in spleen was significantly enhanced after the stimulation of LPS. It is suggested that two pathways, constitutively and inductively, could be involved in the regulation of TCTP expression in fish. The inducement effect of LPS on the TCTP expression indicated that TCTP is involved in the response to the stimulation of LPS. Further work is needed to understand the role of TCTP in the immune response, and the mechanism of TCTP involving the host immune system.

Acknowledgements

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