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Tissue Expression of the 6-pyruvoyl tetrahydropterin Synthase Gene in Adult Zebrafish, *Danio rerio*

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Introduction

Teleost is one of the poikilotherms that can display different kind of colours on their body. This is due to the presence of different types of chromatophores, a group of pigment containing cells that is distributed in the epidermal layer of skin and scales. Unlike mammals, teleost possess several types of pigment cells such as erythrophores, xanthophores, iridophores, leucophores besides the well studied melanophore. Pteridine is the main pigment found in teleost chromatophores (Marmol & Beermann, 1996; Ben *et al.*, 2003). The *de novo* pathway leading to the formation of the deposition of this pigment has been extensively proposed by Ziegler *et al.*, (2000). The key enzymes responsible for this pathway have also been identified. The 6-pyruvoyl tetrahydropterin synthase (PTPS) is the enzyme which catalyses the conversion of dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin in the second step of the biosynthesis of tetrahydrobiopterin (BH_4) pathway enroute to pteridine formation (Ploom *et al.*, 1999; Ikemoto *et al.*, 2002). Besides pteridine formation, BH_4 is also essential for involved in various physiological processes including nitric oxide synthesis, monoamine neurotransmitter biosynthesis and mammal melanogenesis (Geller *et al.*, 2000; Seong *et al.*, 2000). Using zebrafish as a model, we hope to understand the developmental and physiological regulation of the PTPS gene. As a pre-requisite, we report here the cloning of a early first PTPS fragment and its subsequent use in analyzing PTPS expression in different zebrafish tissue using the RT-PCR method.

Materials and Methods

Animals

Wild type zebrafish, *Danio rerio*, were maintained in aquarium with dechlorinated water. The fish were fed twice per day with bloodworm or *Artemia*. Only adult zebrafish were used in this experiment.

Detection of 6-pyruvoyl tetrahydropterin synthase gene expressions by semiquantitative RT-PCR analysis

Total RNA of brain, caudal fin, eye, gill, intestine, liver, muscle, skin and swim bladder tissues were isolated from adult zebrafish using TriReagent® (Molecular Research Center, Inc.) according to the manufacturer's protocol. The concentrations of total RNA were determined by A_{260}/A_{280} measurements and 1 µg of total RNA was treated with RQ1 RNase-Free Dnase (Promega). The treated RNA was then reverse transcribed by M-MLV Reverse Transcriptase (Promega) with Random Primers following the manufacturer's instructions to synthesis first-strand cDNA.

Prior to the PCR, the specific primers were designed using on-line programme, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/) from zebrafish EST of *6-pyruvoyl tetrahydropterin synthase* in GenBank (accession number: CB366525). The PCR was performed in a volume of 25 µl containing *Taq* DNA Polymerase (Promega), 1X Thermophilic DNA Polymerase Buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 7 pmol of each primer and 2.5 µl of first-strand cDNA sample. The amplification cycle was carried out at 94 °C for 5 min as initial denaturing step, 35 cycles at 94.0 °C for 30 s, 58.4 °C for 30 s, 72 °C for 1 min and extension step at 72 °C for 10 min. 5 µl of amplified sample was then analyzed using 1.5 % agarose gel stained with ethidium bromide.

The PCR product was purified from ethidium bromide stained agarose gel using QIAquick® Gel Extraction Kit (QIAGEN) and ligated into pGEM®-T Easy Vector (Promega). Subsequently, the recombinant plasmid was then transformed into *E. coli* DH5α competent cell and sequenced.

To conduct the semi-quantitative RT-PCR analysis, same PCR condition as above were performed using total RNA from different type of zebrafish tissues. Besides, a house keeping gene, β-Actin was used as internal control for the RT-PCR analysis. The sense (5' ccg tga cat caa gga gaa gct 3') and antisense (5' tcg tgg ata ccg caa gat tcc 3') primers were used for RT-PCR amplification (Lam *et al.*, 2003).

Results and Discussion

RT-PCR was performed as mentioned above using liver tissue and a single band with predicted size 430 bp was detected on 1.5% ethidium bromide stained agarose gel (Fig 1).

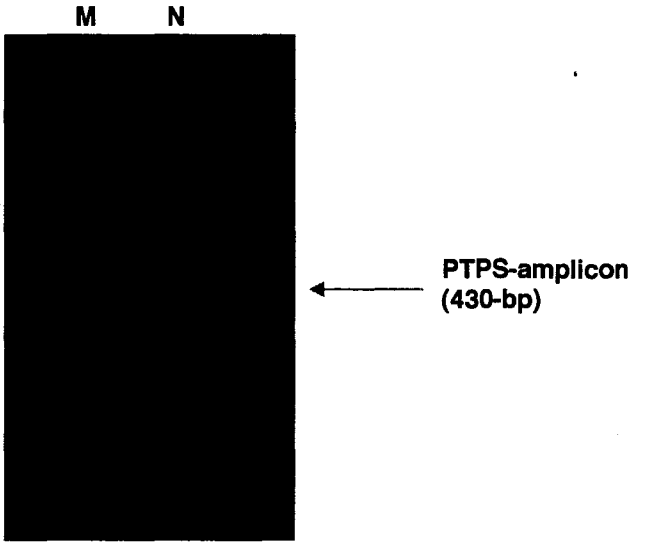

Figure 3: A predicted 430 bp products from the RT-PCR amplification were analyzed on 1.5% (w/v) agarose gel electrophoresis. N: PCR control, PCR performed in the absence of cDNA; M: 100 bp DNA ladder marker (Promega).

To ensure that the specific primers can amplify the correct fragment of PTPS cDNA, the approximately 430 bp amplicons were ligated into pGEM®-T Easy Vector (Promega), transformed into *E. coli* DH5α competent cell and sequenced. The nucleotide sequence was then compared and analyzed using the discontiguous Mega BLAST from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The BLAST result (Fig. 2) showed 99 % identities to published EST of zebrafish PTPS gene (GenBank accession number: CB366525). Thus, it can be conclude that the primers specifically amplified the zebrafish PTPS gene.

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Query: 1   atagaggagaggaggaggatgagtgaacgcgtcgccttcatcacgcgcgtctgcagcttc 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 27  atagaggagaggaggaggatgagtgaacgcgtcgccttcatcacgcgcgtctgcagcttc 86

Query: 61  agcgcctgccacagactacacagtaaatgtttgagtgatgaggagaataagagaacgttt 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 87  agcgcctgccacagactacacagtaaatgtttgagtgatgaggagaataagagaacgttt 146

Query: 121 gggaaatgcaacaatcctaatggtcatggccataactatacagttgaagtgacggtgcgt 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 147 gggaaatgcaacaatcctaatggtcatggccataactatacagttgaagtgacggtgcgt 206

Query: 181 ggaaagatcgacaagaacacaggaatggtgatgaacctcactgatctgaaggagtttatt 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 207 ggaaagatcgacaagaacacaggaatggtgatgaacctcactgatctgaaggagtttatt 266

Query: 241 gaggaggccgtcatgaagcctcttgaccataaaaacctggacctggatgtgccgtatttt 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 267 gaggaggccgtcatgaagcctcttgaccataaaaacctggacctggatgtgccgtatttt 326

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Query: 361 ctgctgcctcacgacagcctgtacgagatcaaagtctacgagacggccaaaaacatcgtg 420
           |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 387 ctgctgccgcacgacagcctgtacgagatcaaagtctacgagacggccaaaaacatcgtg 446

Query: 421 atctacagag 430
           ||||||||||
Sbjct: 447 atctacagag 456
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Figure 2: Sequence of EST of zebrafish PTPS gene. The sequence of both the forward (PTPS F1) and reverse (PTPS R1) primers were shown in bold.

Semi-quantitative RT-PCR analysis was performed using the above mentioned specific primers, PTPS F1 and PTPS R1 for this purpose. The agarose gel image showed that PTPS expression was highest in liver and gill, followed by moderate expression in brain, eye, intestine and swim bladder and low expression in caudal fin, muscle and skin (Fig. 3).

This current result coincides with know PTPS function in various parts of the teleost organ and tissues. The high expression in liver for example was in tandem with reports in guppy fish (Ben *et al.*, 2003). High expression of PTPS in liver is due to the biosynthesis of BH_4 which is the essential cofactor for the aromatic amino acid hydroxylases, such as hydroxylation of phenylalanine to the tyrosine by phenyalanine hydroxylase (Kaufman, 1970; Citron *et al.*, 1990). Similary, another aromatic amino acid, tryptophan hydroxylase which catalyses the tryptophan to serotonin is also present in the brain (Auerbach *et al.*, 1997; Shintaku *et al.*, 2000). Furthermore, the presence of PTPS in brain is probably due to the biosynthesis of nitric oxide which is important for nerontransmission (Klatt *et al.*, 1993). The presence of PTPS in skin is not surprising since this enzyme is responsible for formation of pteridine pigment in zebrafish (Ziegler 2000).

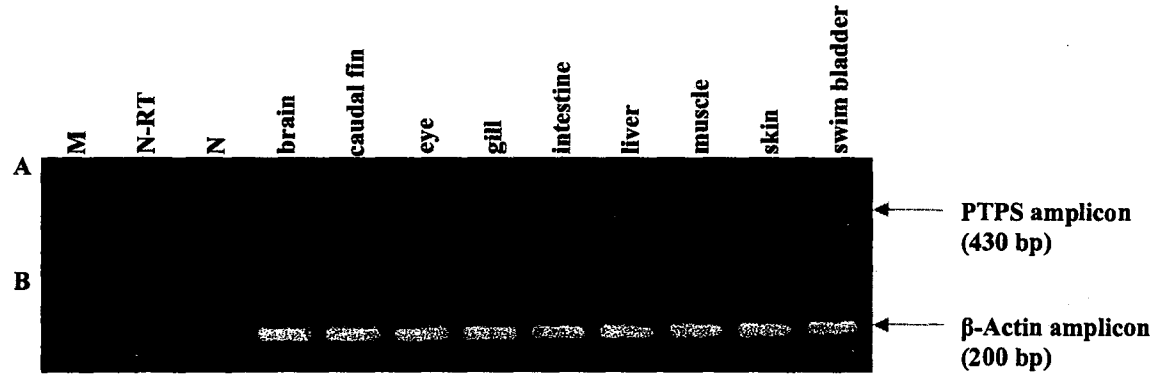

Figure 3: Gene expression profile by RT-PCR analysis of (a) *β-Actin* and (b) *ptps*, from brain, caudal fin, eye, gill, intestine, liver, muscle, skin and swim bladder tissues of zebrafish. Equivalent of each amplification reaction were analyzed on 1.5% (w/v) agarose gel electrophoresis. N-RT: RT control, reverse transcription performed in the absence of RNA prior to PCR amplification; N: PCR control, PCR performed in the absence of cDNA; M: 100 bp DNA ladder marker (Promega).

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