KOR. J. ENVIRON. TOXICOL.
Vol. 15, No. 4, 131~137 (2000)

Tyrosine Hydroxylase in Japanese Medaka (*Oryzias latipes*): cDNA Cloning and Molecular Monitoring of TH Gene Expression As a Biomarker

Sung-Woo Shin, Oh-Shin Kwon[1], Jung-Sang Kim[2], Tae-Soo Chon[3], Sung-Kyu Lee[4] and Sung-Cheol Koh

Division of Civil and Environmental Engineering, Korea Maritime University, Pusan, 606-791, Korea
[1] Dept. of Biochemistry, Kyungpook National University, Taegu, 702-701, Korea
[2] Dept. of Animal Engineering, Kyungpook National University, Taegu, 702-701, Korea
[3] Dept. of Biology, Pusan National University, Pusan, 609-735, Korea
[4] Toxicology Res. Center, Korea Res. Inst. of Chemical Technology, Taejon, 305-600, Korea

송사리 Tyrosine Hydroxylase: cDNA 클로닝 및 생물지표로서의 TH 유전자 발현의 분자생물학적 추적

신성우, 권오신[1], 김정상[2], 전태수[3], 이성규[4], 고성철

한국해양대학교 토목환경공학부, [1]경북대학교 생화학과, [2]경북대학교 동물공학과,
[3]부산대학교 생물학과, [4]한국화학연구소 안전성연구부

요 약

최근 독성 유해물질의 환경으로의 방출로 인해 인간 및 생태계에 대한 위해성 문제가 심각하게 제기되고 있다. 독성화학물질을 포함한 여러 환경 오염물질의 위해성평가는 화학물질의 유해성과 노출량 측정을 동시에 측정함으로써 가능한데 이 경우 생물지표(biomarker)가 최근 각광을 받고 있다.

본 연구에서는 동물의 행동에 관련된 신경전달물질의 생성에 결정적 역할을 하는 tyrosine hydroxylase (TH) 및 그 유전자가 생물지표로서 이용 가능성이 있는지를 검토하였다. Ovary cDNA library의 PCR 스크리닝을 통한 송사리 TH 유전자를 부분적으로 클론하였으며 (327 bp), DNA 염기서열 분석 결과 쥐 (rat)의 TH 유전자와 동일한 염기서열을 보였다. 그리고 다이아지논 처리구 및 무처리구에서 송사리의 머리부분 (head) 및 몸통 부분 (body)에서 추출된 총 RNA에 TH mRNA가 존재함을 RT-PCR를 통하여 확인하였다. 그러나 다이아지논의 처리효과가 송사리의 행동에 미치는 영향을 보기 위해서는 TH의 발현을 보다 정량적으로 검토할 필요가 있을 것으로 판단된다.

생물지표로서 TH의 활성 및 mRNA의 기관별 또는 조직별 검출은 독성물질에 영향을 받는 어류 신경행동 변화를 모니터링 할 수 있는 유용한 수단이 될 것이다. 나아가 환경관리에 있어서 신경화학물질과 분자생물학적 상관관계을 통한 이상반응행동의 분석은 환경 위해성평가에 상당히 기여할 것이다.

Key words : Japanese medaka (Oryzias latipes), biomarker, tyrosine hydroxylase (TH), RT-PCR

INTRODUCTION

Nowadays environmental pollutants have not only increased in quantity but also changed dramatically in quality. Furthermore the release of hazardous waste materials into the environment poses serious risks

in humans and ecosystem. In order to minimize their environmental risks caused by the wastes, the developed countries have established systems for toxicity evaluation of hazardous chemicals, legislation for their proper management plan, and their efficient administration program.[1] Ecological risk is equivalent to product of exposure and hazard of specific chemical or a mixture of chemicals. The risk assessment, therefore, requires a comprehensive measurement of exposure and hazard of the chemicals that can be achieved by toxicity evaluation using a biological system. The biological system includes biomarkers that are molecular and physiological indicators of chemical stress.

Neurotoxic chemical such as diazinon is relatively highly toxic to fish, and it is well known that it causes vertebral malformation and behavioral change of fish at relatively low concentrations.[2,3] Behavioral change caused by pesticides is most likely related to changed levels in neurotransmitters such as acetylcholine, dopamine, serotonin, and norepinephrine. Few reports have been made regarding a relationship between neurotransmitter generation and behavior responses in fish. In vertebrates, three catecholamines (CA), dopamines, noradrenaline, and adrenaline, act as major neurotransmitters in the central nervous system (CNS) and the peripheral nervous system (PNS). They are sequentially synthesized from aromatic amino acids where TH catalyzes a rate-limiting step.[4,5] In vertebrates CA are involved in the control of autonomic and neuroendocrine functions as well as several behavioral patterns including stress reactions. TH mRNA was produced in the whole rat brain from 13 days of gestation, but the largest increase of the expression was observed in the hypothalamus.[6] TH mRNA was mostly expressed in the olfactory and hypothalamic areas, whereas sparse TH expressing was observed in the telencephalic region and brainstem.[7] In rainbow trout Northern blot analysis showed that TH mRNA was expressed in the ventral brain but little signal was observed in the liver.[8] The behavioral changes in fish affected by a few neurotoxic chemicals include increase in surfacing, distance traveled, jumping, erratic movements, convulsions and opercular movements.[9] Humans exposed to narcotics or pesticides can develop Parkinson's disease. The disease is a common neurodegenerative syndrome characterized by loss of dopaminnergic neurons in the substantia nigra, formation of filamentous intraneuronal inclusions (Lewy bodies) and an extrapyramidal movement disorder.[10]

The objective of this study was to develop a molecular biological system that could allow a rapid monitoring of neurotoxicity in Japanese medaka treated with diazinon. In this study we have cloned and sequenced a partial TH gene of Japanese medaka as a molecular biomarker that could be used in toxicity assay of diazinon.

MATERIAL AND METHODS

1. Screening of a Japanese medaka cDNA library

The medaka cDNA library constructed in a λZAPII vector was kindly provided by Akira Kanamori, National Research Institute of Aquaculture Inland Station, Japan. cDNA fragments of tyrosine hydroxylase were amplified from a medaka ovary cDNA library using polymerase chain reaction (PCR). Two oligonucleotide primers used in PCR amplification were: 5′-ACA GCT GGA GGA CGT GTC-3′ (forward primer) and 5′-CAT AGC CCG AAT TCC ACA G -3′ (reverse primer) corresponding to the peptide sequences from rat TH: Gln-Leu-Glu-Asp-Val-Ser and Val-Glu-Phe-Gly-Leu-Cys, respectively. These primers were designed based upon alignments of conserved TH protein sequences available at National Center for Biological Information (NCBI). The primers were custom synthesized at Genotech, Inc. (Taejon, Korea).

2. PCR cloning of TH gene from Japanese medaka

The amplifications were carried out using a GeneAmp PCR system 2,400 according to the following procedure: 100 μl of PCR reaction mixture was made up of 5 μl of cDNA library aliquots and 100 pmol of

each primer in a PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 2 mM $MgCl_2$ and 200 μM each of dNTPs. The reaction mixture was heated for 5min and brought down to the annealing temperature 55°C. Then, 0.5 μl of AmpliTaq DNA polymerase (2.5 units/100 μl) was added to the mixture, followed by 40 cycles of extension at 72°C for 1 min, denaturation at 94°C for 1 min and annealing at 55°C for 1 minute and 30 seconds.

The PCR products were analyzed by agarose gel electrophoresis. The amplified fragment was then subcloned into the TA cloning vector (Invitrogen, Rockville, MD), and subsequent transformations were performed using F' One Shot Kit (Invitrogen, Rockville, MD). The procedures were performed according to the manufacturer's instructions, and the identity of PCR product was analyzed by nucleotide sequencing. The nucleotide sequence and protein structure were analyzed with the aid of PC GENE computer program. For DNA and protein homology search, GenBank databases were used.

3. Experimental animals and chemical exposure

Japanese medaka (*Oryzias latipes*) was obtained from Toxicology Research Center, Korea Research Institute of Chemical Technology (Taejeon, Korea). Fish were held in a square glass chamber (40 × 22 × 40 cm) containing 30-liter of dechlorinated water (pH 6.5~7.3) with aeration. The fish were subjected to diazinon treatment after starvation for 24 hr. Diazinon (purity: 99%) was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and the Japanese medaka was treated under appropriate sublethal concentrations of diazinon.

4. Total RNA extraction

For total RNA extraction the treated fish were first immediately frozen in the liquid nitrogen and preserved in a deep freezer (−70°C) until used. Total RNA was extracted according to a protocol accompanied in RNAwiz (Ambion, Texas) extraction kit. Tissues were homogenized after in a Polytron homogenizer approximately 20 mg of tissue were suspended in 1 ml of phosphate buffer (pH 8.0, 0.1 M). Total RNA was then treated with RQ1 DNase (1 U of DNase/5 μg of RNA; Promega) for 60 min at 37°C, extracted twice with phenol/chloroform, and precipitatied with ethanol.

5. Monitoring of TH gene expression in Japanese medaka through RT-PCR

The final volume for the RT-PCR was 50 μl, and amplification was carried out for 25 cycles with AMV reverse transcriptase (Promega, Wisconsin), PCR nucleotide mix, *Taq* DNA polymerase (Promega, Wisconsin), forward and reverse primers in 25 mM $MgCl_2$. The thermo cycling was as follows: reverse transcription at 65°C for 10 min; denaturation at 94°C for 1 min; annealing at 60°C for 1 min; and elongation at 70°C for 2min. The primer sequences were as follows: Forward, 5'-CAGCTGG AGGACG-TGTC-3', Reverse, 5'-CATAGCCCGA ATTCCA-CAG-3'.

RESULTS AND DISCUSSION

1. PCR cloning of Medaka TH gene

Using two oligonucleotide primers corresponding to the rat TH gene, PCR amplifications were performed to screen a medaka ovary cDNA library. The PCR products were analyzed by agarose gel electrophoresis, and only single DNA band of approximately 0.3 kb was detected. The amplified fragment was then subcloned into TA cloning vector (Invitrogen, Rockville, MD).

2. Analysis of DNA and amino acid sequences

Further results from DNA sequencing analysis revealed that the amplified fragment consists of 327 bp encoding 109 amino acid residues as shown in Fig. 1. Comparison of the amino acid sequences of medaka TH with other species enzyme is shown in Fig. 2. The amino acid sequence deduced from the cloned fragment bears a remarkable degree of homology with other species. More surprisingly, the DNA

```
A  CAGCTGGAGGACGTGTCCCGCTTCTTGAAGGAGCGGACTGGCTTCCAGCTGCGACCCGTG   61
   Q  L  E  D  V  S  R  F  L  K  E  R  T  G  F  Q  L  R  P  V

GCCGGTCTACTGTCCGCCCGTGATTTTCTGGCCAGTCTGGCCTTCCGCGTGTTTCAATGC   121
  A  G  L  L  S  A  R  D  F  L  A  S  L  A  F  R  V  F  Q  C

ACCCAGTATATCCGCCATGCCTCCTCACCTATGCATTCACCTGAGCCGGACTGCTGCCAT   181
  T  Q  Y  I  R  H  A  S  S  P  M  H  S  P  E  P  D  C  C  H

GAGCTGTTGGGACATGTACCCATGTTGGCTGACCGCACATTTGCCCAGTTCTCCCAGGAC   240
  E  L  L  G  H  V  P  M  L  A  D  R  T  F  A  Q  F  S  Q  D

ATTGGACTTGCATCTCTGGGGGCCTCAGATGAAGAAATTGAAAAACTCTCCACGGTGTAC   300
  I  G  L  A  S  L  G  A  S  D  E  E  I  E  K  L  S  T  V  Y

TGGTTCACTGTGGAATTCGGGCTA  TG   327
  W  F  T  V  E  F  G  L
```

Fig. 1. Nucleotide and deduced amino acid sequences of tyrosine hydroxylase from Japanese medaka. The predicted amino acid sequence is shown below the nucleotide sequence. The bold characters indicate the forward and reverse primers used for the PCR reaction.

```
MEDAKA   QLEDVSRFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
HUMAN    QLEDVSRFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
MOUSE    QLEDVSHFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
RAT      QLEDVSRFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
BOVINE   QLEDVSRFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
CHICKEN  QLEEVSRFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
QUAIL    QLEEVSRFLK ERTGFQLRPV RGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCCH
EEL      QLEDVSHFLK ERTGFQLRPV AGLLSARDFL ASLAFRVFQC TQYIRHASSP MHSPEPDCVH
         ***.**.*** ********** ********* ********** ********** ******** *

MEDAKA   ELLGHVPMLA DRTFAQFSQD IGLASLGASD EEIEKLSTVY WFTVEFGL
HUMAN    ELLGHVPMLA DRTFAQFSQD IGLASLGASD EEIEKLSTLS WFTVEFGL
MOUSE    ELLGHVPMLA DRTFAQFSQD IGLASLGASD EEIEKLSTVY WFTVEFGL
RAT      ELLGHVPMLA DRTFAQFSQD IGLASLGASD EEIEKLSTVY WFTVEFGL
BOVINE   ELLAHGPMLA DRTFAQFSQD IGLASLGVSD EEIEKLSTLY WFTVEFGL
CHICKEN  ELLGHVPMLA DKTFAQFSQD IGLASLGATD EEIEKLATLY WFTVEFGL
QUAIL    ELLGHVPMLA DKTFAQFSQD IGLASLGATD EEIEKLATLY WFTVEFGL
EEL      ELLGHVPMLA DRTFAQFSQN IGLASLGASE EDIEKLSTLY WFTVEFGL
         *** .*  **** *.******** ********... *.****.*.  ********
```

Fig. 2. Comparison of amino acid sequences of tyrosine hydroxylase. The sequence for medaka TH is from the present study, human from GenBank accession No. NM000360; mouse brain, M69200; rat, M10244; bovine, M36705; chicken, AJ251387; quail, M24778; European eel enzyme, AJ000731. Asterisks represent identical amino acids (86%), single dots are high similarity, and blank spaces are no homology.

sequences are completely identical to that of rat TH. An independent experiment with a RT PCR product amplified from mRNA isolated from medaka tissues confirmed that the sequence presented was correct. The isolation of a full-length clone using the cDNA fragment as a probe should help to further define the overall degree of homology between the two species. For the nucleotide sequence and protein homology search, GenBank databases were used. The greatest similarities of TH fragment observed were: 92.7% (amino acid) and 83% (nucleotide) for rainbow trout; 96.6% (amino acid) and 80% (nucleotide) for European eel. Optimum alignment of eel TH whole amino acid sequence gave 83, 85.9, and 83.1% similarity to rat, quail, and human, respectively.[7] The whole amino acid sequence information of the medaka TH will provide a reasonable similarity data.

3. Effect of diazinon on TH gene expression

The TH gene expression was monitored by RT-PCR using total RNA from body portion of Japanese medaka treated with diazinon of different concentrations or treating time. In the RT-PCR, 330 bp of mRNA was consistently amplified in all the treated samples as well as control. There were no significant differences in the TH expression level regardless of treating concentrations and time (Figs. 3 and 4). The reason appeared to be that RT-PCR was not performed using through a quantitative analysis normalized against an actin gene expression. Reserpine and cold treatments can increase TH mRNA levels in rat brain (hypothalamus and brainstem) and adrenals.[11,12]

In vertebrates, catecholamine synthesis is finely controlled by changes of TH enzyme activity. Catecholamines are involved in the regulation of autonomic and neuroendocrine functions as well as several behavior patterns including stress reactions.[13] TH activity could be inhibited by catechol compounds *in vitro* conditions.[14] In mammals, TH messenger transcription rate, mRNA stability, and translation could also be affected by biochemicals such as polypeptides[15] and sex-steroid hormones.[16] Fenitrothion, an organophophate pesticide, was found to inhibit or induce levels of dopamine and norepinephrine in the brain tissues of *Channa punctatus* that appeared to be related to physiological and neurobehavioral changes.[17] It is, therefore, plausible that diazinon, a neurotoxic pesticide, could affect mRNA transcription as well as TH activity and locomotive activity in Japanese medaka.

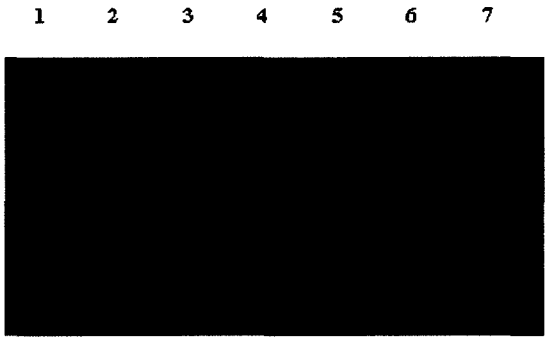

Fig. 3. Effect of diazinon concentration on the tyrosine hydroxylase (TH) gene expression in the trunk of a Japanese medaka (*Oryzias latipes*) treated with diazinon overnight. Lanes: 1, 100bp ladder; 2, plasmid carrying TH gene; 3, DMSO only; 4, 1 ppb; 5, 100 pb; 6, 5,000 ppb; 7, the sample (5,000 ppb) treated with RNase.

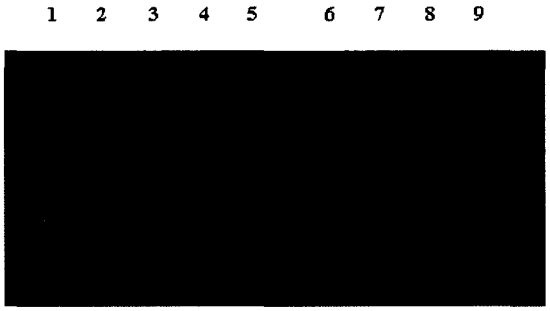

Fig. 4. Time course of tyrosine hydroxylase (TH) gene expression in the presence of diazinon (5 ppm) in the trunk of a Japanese medaka (*Oryzias latipes*). Lanes: 1, 100 bp ladder; 2, plasmid carrying TH gene; 3, 0h; 4, 1h; 5, 6 h; 6, 12 h; 7, 24 h; 8, 48 h; 9, 6 h sample treated with RNase.

Organ or tissue-specific detection of TH activity and mRNA as biomarkers will be a useful monitoring tool for neurobehavioral changes in fish influ-

enced by toxic chemicals. Furthermore, quantitative analysis of locomotive patterns and its correlation with the neurochemical and molecular data would be highly useful in measuring toxicity and hazardousness of environmental pollutants.

ABSTRACT

The release of hazardous waste materials into the environment poses serious risks in humans and ecosystems. The risk assessment of environmental pollutants including hazardous chemicals requires a comprehensive measurement of hazard and exposure of the chemicals that can be achieved by toxicity evaluation using a biological system such as biomarkers.

In this report we have tried to develop a biomarker used to elucidate a molecular basis of, and to monitor abnormal behaviors caused by diazinon in Japanese medaka (*Oryzias latipes*) as a model organism. First, an attempt was made to clone tyrosine hydroxylase gene from Japanese medaka that would be a candidate for a biomarker for neuronal modulations and behaviors. For monitoring experiments at behavioral and molecular biological levels, the fish were treated under different sublethal conditions of diazinon and their behavioral responses were observed. In this study we have successfully cloned a partial TH gene from the medaka fish through PCR screening of an ovary cDNA library. DNA sequencing analysis revealed that the amplified fragment was 327 bp encoding 109 amino acids. Comparing the DNA sequence of medaka TH with other species, TH gene revealed the DNA sequence was completely identical to that of rat TH. In the RT-PCR, 330 bp of mRNA was consistently amplified in all the treated samples including control. There were no significant differences in the TH expression level regardless of treating concentrations (1 ~ 5,000 ppb) and time (0 ~ 48 hr). The reason appeared to be that RT-PCR was not performed using through a quantitative analysis normalized against an actin gene expression.

Organ or tissue-specific detection of TH activity and mRNA as biomarkers will be a useful monitoring tool for neurobehavioral changes in fish influenced by toxic chemicals. Furthermore, quantitative analysis of locomotive patterns and its correlation with the neurochemical and molecular data would be highly useful in measuring toxicity and hazard of various environmental pollutants.

ACKNOWLEDGEMENT

This study was supported by a specialized basic research grant of Korea Science and Engineering Foundation (#98-0401-02-01-3), Republic of Korea.

REFERENCES

1. Park, J. Techniques in ecological risk assessment and case studies. In: The First International Workshop on Environmental Risk Assessment, Toxicology Research Center, Korea Research Institute of Chemical Technology. 1997 ; 3-21, Seoul, Korea.
2. Pan, G. and H.M. Dutta. The inhibition of brain acetylcholinesterase activity of juvenile largemouth bass Micropterus salmoides by sublethal concentrations of diazinon. Environ. Res. 1998 ; 79 : 133-137.
3. Dutta, H., J. Marcelino and C. Richmonds. Brain acetylcholinesterase activity and optomotor behavior in bluegills, *Lepomis macrochirus* exposed to different concentrations of diazinon. Arch. Int. Physiol. Biochim. Biophys. 1992 ; 100 : 331-334.
4. Elaine, R., G.T. Brown., G.T. Coker and L.O. Karen. Organization and evolution of the rat tyrosine hydroxylase gene. Biochemistry. 1987 ; 26 : 5208-5212.
5. Coker, G.T., L. Vinnedge and K.L. O'Malley. Characterization of rat and human tyrosine hydroxylase gene: func-tional expression of both promoters in neuronal and non-neuronal cell types. Biochemistry and Biophysical Research Communications. 1988 ; 157 : 1341-1347.
6. Coulon, J.F., N.F. Biguet., A. Cavoy., J. Dacour., J. Mallet and David. Gene Expression of Tyrosine Hydroxylase in the Developing Fetal Brain. Journal of Neurochemi-stry. 1990 ; 55 : 1412-1417.
7. Sylviane, B., F.B. Nicole., V. Bernadette., V. Michele., M. Jacques., V. Jean-Didier., D. Sylvie and V. Philippe. Tyrosine hydroxylase in the European eel (*An-*

guilla anguilla): cDNA cloning, brain distribution, and phylogenetic analysis. Journal of Neurochemistry. 1998 ; 71 : 460-470.

8. Linard, B., M.H. Pakdl and C. Saligaut. Cloning of a cDNA coding for active tyrosine hysroxylase in the rainbow trout (*Oncorhychus mykiss*): comparison with other hydroxylase and enzymatic expression. Journal of Neurochemistry. 1998 ; 71 : 920-928.
9. Murali, D.R. and G. Krishna. Neurobehavioral Changes in Freshwater Fish *Channa punctatus* Exposed to Fenitrothion: Bull. Environ. Contam. Toxicol. 1991 ; 47 : 455 -458.
10. Maggio, R., M. Riva, F. Vaglini, F. Fornai, R. Molteni, M. Armogida, G. Racagni and G.U. Corsini. Nicotine prevents experimental parkinsonism in rodents and induces striatal increase of neurotrophic factors. J Neurochem. 1998 ; 71(6) : 2439-46.
11. Faucon Biguet, N., M. Buda, A. Lamouroux, D. Samlolyk and J. Mallet. Time course of TH mRNA in rat brain and adrenal medulla after injection of reserpine. EMBO J. 1986 ; 1 : 287-291.
12. Richard, F., N. Faucon, R. Biguet, D. Labatut, J. Rollet, Mallet and M. Buda. Modulation of tyrosine hydroxylase gene expression in rat brain and adrenals by exposure to cold. J. Neurosci. Res. 1988 ; 20 : 32-37.
13. Winberg, S. and G.E. Nilsson. Roles of brain monoamine neurotransmitters in agonistic behavior and stress reactions, with particular reference to fish. Comp. Biochem. Physiol. 1993 ; 106c : 597-614.
14. Wessels-Reiker, M., R. Basinoina, A. Howlet and R. Strong. Vasoactive intestinal polypeptide-related peptides modulate tyrosine hydroxylase gene expression in PC12 cells. J. Biol. Chem. 1993 ; 266 : 9347-9350.
15. Wessels-Reiker, M., R. Basinoina, A. Howlet and R. Strong. Vasoactive intestinal polypeptide-related peptides modulate tyrosine hydroxylase gene expression in PC12 cells. J. Biol. Chem. 1993 ; 266 : 9347-9350.
16. Raab, H., C. Pilgrim and I. Reisert. Effects of sex and estrogen on tyrosine hydroxylase mRNA in cultured embryonic rat mesencephalon. Mol. Brain Res. 1995 ; 33 : 157-164.
17. Ram, M.D. and K. Gopal. Neurobehavioral changes in freshwater fish *Channa punctatus* exposed to fenithrothion. Bull. Environ. Contam. Toxicol. 1991 ; 47 : 455-458.