IDENTIFICATION OF COPPER-INDUCIBLE GENES IN SWORDTAIL FISH (*Xiphophorus spp.)* USING DIFFERENTIAL DISPLAY

Identifikasi Gen Ikan Ekor Pedang (Xiphophorus spp.) yang Diinduksi dengan Kuprum Menggunakan Teknik Differential Display

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ABSTRACT

Differential display technique was used to identify differentially expressed gene of swordtail fish (*Xiphophorus spp*.) that induced by 1 µg/ml copper for 24 hours. From eight primers that have been used for differential display, seven of them showed the differential genes which were not present in the control. The reamplification of these fragments ranging from below 100 to 200 bp. But only two fragments were successfully cloned and sequenced, those are H-AP10 and H-AP11. Sequence analysis showed that both of the sequences revealed high similarity with Homo sapiens stress-associated endoplasmic reticulum protein, a signal generated that induces apoptosis.

Keywords: differential display, swordtail fish, copper pollution, mRNA expression, biomarker

ABSTRAK

Teknik differential display digunakan untuk mengidentifikasi gen yang berbeda yang muncul akibat pengaruh induksi kuprum sebanyak 1µg/ml pada ikan ekor pedang (Xiphophorus spp.). Dari delapan kombinasi primer yang digunakan, tujuh diantaranya menunjukkan terjadinya perbedaan gen antara ikan yang diinduksi dengan ikan yang tidak diinduksi kuprum (kontrol). Ukuran gen-gen tersebut berkisar antara 100 sampai 200 bp. Dari delapan fragmen gen tersebut hanya dua fragmen saja yang berhasil diklonkan yaitu yang menggunakan primer H-AP10 dan H-AP11. Hasil analisis sekuensnya menunjukkan bahwa kedua fragmen gen tersebut memiliki persamaan dengan gen Homo sapiens endoplasmik retikulum stres, yaitu suatu protein yang berhubungan dengan proses apoptosis.

Kata kunci: differential display, ikan ekor pedang, polusi kuprum, ekspresi mRNA, biomarker

INTRODUCTION

Pollution, especially water pollution has become a threat for human being and aquatic animals since years ago due to the discharge of industrial, agricultural, and domestic waste into the aquatic reservoirs. Fish, one part of aquatic system, are often exposed to highly contaminated water, especially in areas where the dilution rate of waste water is low. In fish, water pollution can lead to different changes ranging from biochemical alterations in single cells up to changes in whole populations (Bernet *et al.,* 1999).

Heavy metals have long been recognized as one of the most important pollutants in the coastal waters because of their toxicity and capacity to accumulate in marine organism. The main source of heavy metal pollution comes from discharge of untreated and semi treated effluents from metal-related industries such as electroplating, manufacturing of batteries, circuit boards, and car repair (Wong *et al.,* 2001). Many investigations have reported the accumulation of heavy metals in marine mammals and seabirds having a long lifespan and occupying high tropic levels in the marine food web, and showed the utility of these species as biological indicators of heavy metal pollution (Sakai *et al.,* 1995).

One kind of heavy metals, copper, is a micronutrient element which becomes toxic at elevated concentrations and is in widespread industrial use (Turner, 1990). Compare to the other heavy metals, copper is not particularly dangerous but still bring harmful effect to human. For instance, ingestion of excess copper through natural sources in the food web can cause gastrointestinal problems and the presence of 30-60 µl of copper/ml in sea water led to the exacerbation of vibriosis (Siegel, 1998). Therefore the Environmental Protection Agency's has determine the safe level of copper in drinking water for human is 1 mg/L compare with 0.018 mg/L for acute aquatic life criteria.

Genetic techniques offer a powerful approach to assess contaminant-induced changes in population, therefore molecular biology-based technology is used in this research. This technique has been known as a sensitive and reliable method, and able to monitor small changes at the molecular level, that is at the level of gene expression (Liang and Pardee, 1992). The use of molecular biomarker in the aid of monitoring environmental pollutions relies upon the ability of certain genes to be induced against specific pollutants (Baldwin *et al.*, 2003). Normally, concentrations of pollutants required to initiate changes in gene expression in a living organism are lower than those needed to cause serious environmental concerns such as damage to ecosystems and eventually death to living organisms. Based on this, gene-based assay is normally recommended as biomarker because of the advantage that it can offer to constitute an early warning system for pollution control.

MATERIALS AND METHODS

Stimulation of fish and isolation of total cellular RNA

Total cellular RNA was extracted from liver of swordtail fish that were incubated in water either untreated or treated with 1 µg/ml copper (Cu) for 24 hours using Tri Reagent (*Molecular Research* Centre) according to the protocol described by the manufacturer.

Determination of differentially expressed gene

Each RNA sample was treated with DNase-I in order to remove any traces of contaminating DNA that may present in the samples, and subsequently used for the preparation of cDNA. cDNA samples will be subjected to mRNA differential display (Liang and Pardee, 1992) by means of the polymerase chain reaction (PCR) using combination of a one-base anchored oligo-dT primer (Table 1) and a 13-mer arbitrary primer (Table 2). PCR products were then size-fractionated on a 9% (w/v) denaturing polyacrylamide gel using 1 X TBE as the electrophoresis buffer. The bands will be visualized by using silver staining method (*Promega*).

Table 1. The sequence of one-base anchored oligo-dT primer

Primer	Sequence
H-T11G	5′-AAGCTTTTTTTTTTG-3′
H-T11A	5′- AAGCTTTTTTTTTTA -3′
H-T11C	5′- AAGCTTTTTTTTTTC -3′

Table 2. The sequence of 13-mer arbitrary primers

Primer	Sequence
H-AP9	5′-AAGCTTCATTCCG-3′
H-AP10	5′-AAGCTTCCACGTA-3′
H-AP11	5′-AAGCTTCGGGTAA-3′
H-AP12	5′-AAGCTTGAGTGCT-3′
H-AP13	5′-AAGCTTCGGCATA-3′
H-AP14	5′-AAGCTTGGAGCTT-3′
H-AP15	5′-AAGCTTACGCAAC-3′
H-AP16	5′-AAGCTTTAGAGCG-3′

After staining, the differentially expressed fragments were identified, recovered from gel, reamplyfied and ligated into PCR-TRAP plasmid. The ligation products were then transformed into *E. coli* JM109, selected on LB-agar containing ampicillin and propagated. Subsequently, the recombinant plasmids were then purified and subjected to sequencing. Nucleic acid sequences were compared to other sequences in the GenBank/EMBL databases using the BLAST command (www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

Total RNA isolation showed two distinct bands (arrows) representing 18S and 28S ribosomal RNA indicating the isolated RNA samples were of high quality and intact (Figure 1).

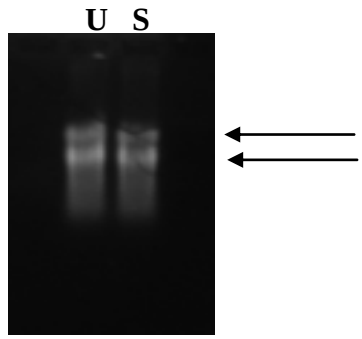

Figure 1. Total cellular RNA isolated from unstimulated (U) and copper-stimulated (S) swordtail fish (*Xiphophorus spp.*)

The PCR products from various combinations of primers generated various banding patterns as observed on 9% (w/v) denaturing polyacrylamide. In total, about 10 bands ranging from 100 bp – 1 kb were observed from each sample analysized (data not shown). Two differentially expressed band with the size of 200 bp and 180 bp was observed in copper-stimulated

sample but not in the unstimulated sample when the PCR reaction was carried out using the combination of H-AP10 and G-anchored oligo-dT primer, and, H-AP11 primer and A-anchored oligo-dT primer, respectively (Figure 2, arrow). Thus, the two fragments were found to be induced in copper-treated swordtail fish.

The identified differentially expressed fragments were successfully purified and reamplified. The fragments then ligated and transformed using *E. coli* strain JM109 cells. The transformed cells were plated out on LB process, i.e. transformed cells containing recombinant plasmid produced white colonies while cells with non-recombinant plasmid yielded blue colonies. From the colonies PCR it was found that all the colonies selected from each plate contained the correct and expected sizes of inserts indicating the success of transformation (data not shown).

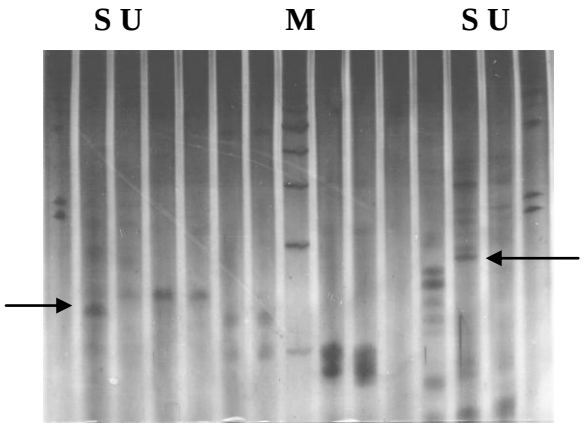

Figure 2. The PCR products from differential display reaction using the combination of H-AP10, H-AP11 primer and G, A-anchored oligo dT primer; S, copper-stimulated sample; U, unstimulated sample, M, 100bp DNA ladder

Sequence analysis by using nucleotide-nucleotide BLAST command showed that both of the sequences revealed high similarity with Homo sapiens stress-associated endoplasmic reticulum protein. Endoplasmic reticulum (RE) stress is a signal generated that induces apoptosis (Chiang *et al*., 2005), thus, it is predicted that the same pathway occurred to the swordtail fish copper-treated.

CONCLUSION

The BLAST result showed that the fragments have homologue with Homo sapiens stress-associated endoplasmic reticulum protein, a signal generated that induces apoptosis.

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