Comparative Biochemistry and Physiology, Part B 167 (2014) 8–15

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

# Cloning and expression of selenoprotein W from pearl mussels *Cristaria plicata*

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ARTICLE INFO

*Article history:*
Received 20 January 2013
Received in revised form 23 September 2013
Accepted 23 September 2013
Available online 2 October 2013

*Keywords:*
*Cristaria plicata*
Selenoprotein W (SelW)
Gene cloning
mRNA expression

ABSTRACT

Selenoprotein W (SelW) is a selenocysteine containing protein with redox activity involved in the antioxidant response. In this study, a selenoprotein W was cloned from pearl mussel *Cristaria plicata* (designated as CpSelW), and the expression patterns were characterized in tissues after *Aeromonas hydrophila* challenged. The full-length cDNA of cpSelW was of 858 bp, containing a 5′ untranslated region (UTR) of 145 bp, a 3′ UTR of 455 bp with a poly (A) tail, and an open reading frame (ORF) of 258 bp encoding a polypeptide of 85 amino acids with the predicted molecular mass of 9.277 kDa, which shared 61% identity with SelW from *Gallus gallus*. A tertiary structure model generated for the CpSelW displayed a β-α-β-β-β-α secondary structure pattern, which was similar to mouse SelW protein 3D structure. The mRNA of CpSelW was constitutively expressed in tested tissues of healthy mussel, including mantle, gill, hemocytes, muscle, and hepatopancreas, and it was highly expressed in hepatopancreas. After mussels were stimulated by *A. hydrophila*, the mRNA expression of CpSelW in hemocytes at 6, 12 and 24 h, in gill at 12 h and in hepatopancreas at 24 h was significantly down-regulated.



## 1. Introduction

Selenium (Se) is an essential trace element of animals, and several diseases of humans and animals are related to selenium deficiency, such as white muscle disease in livestock and Keshan disease in humans (Rayman, 2000). Se is incorporated into proteins as selenocysteine (Sec), designated the 21st amino acid, which is specified in the genetic code by the UGA codon, typically serving as stop codon. Selective incorporation of Sec into selenoproteins requires the involvement of complex translational machinery (Korotkov et al., 2002; Lescure et al., 2002; Krol, 2003). Firstly, a specialized mRNA secondary structure (selenocysteine insertion sequence, SECIS element) is required, and the Sec insertion sequence (SECIS) element locates in the 3′-untranslated region (3′UTR) of selenoprotein mRNAs at considerable distances from the UGA codon (Hatfield and Gladyshev, 2002). Moreover, multiple cellular factors, include a Sec-tRNA specific elongation factor (eEFsec), SECIS-binding protein (SBP2), ribosomal protein L30, soluble liver antigen/liver protein and SECp43 are involved in the selenoprotein synthesis (Hatfield and Gladyshev, 2002; Chavatte et al., 2005; Xu et al., 2005; Small-Howard et al., 2006).

The encoding genes of twenty five selenoprotein have been identified in mammals, and have a variety of biological roles (Kryukov et al., 2003). Selenoprotein W (SelW) is a small selenoprotein that contains one selenocysteine residue, and it was first known in sheep suffering from selenium deficiency (Sun et al., 2001), and is major expressed in muscle and heart in mammals (Whanger, 2001; Schweizer et al., 2004). SelW is capable of binding reduced glutathione (GSH) (Jeong et al., 2002), and overexpression of SelW experiment suggested that it may be an important component of the cellular defense system against oxidative stress (Sun et al., 2001). SelW is involved in redox regulation through its interactions with 14-3-3 proteins, it is suggested that SelW is a member of the thioredoxin family (Aachmann et al., 2007; Dikiy et al., 2007). Recently, SelW gene has been cloned from humans, monkeys, rats, mice, pig, sheep (Whanger, 2009), chicken (Ou et al., 2011), and fish (Kryukov and Gladyshev, 2000). However, relatively little is known about SelW genes from molluscan.

The freshwater mussel *Cristaria plicata* (Bivalvia: Eulamellibranchia: Unionidae), which is of great economical importance and known as "pearl bivalves" in the aquaculture industry of China, has been suffering serious problems due to the outbreak of diseases, especially wound infection caused by nucleus inserting operation that leads to high mortality in the process of pearl production (Wen et al., 2006). Thus, understanding of the immunity of freshwater mussel is crucial for diseases management and development of sustainable mussel culture and pearl production. Identification and cloning of the genes involved in mussel immune response will help us to understand the molecular basis of the innate immune response to the pathogen infection and environment stress.

Herein, the cDNA encoding putative SelW gene was identified and isolated from the *C. plicata*. Furthermore, the expression levels of CpSelW mRNA in various tissues of health adult mussel and in hemocytes, gills

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http://dx.doi.org/10.1016/j.cbpb.2013.09.008

and hepatopancreas of stimulated mussels by *Aeromonas hydrophila* were evaluated.

## 2. Materials and methods

### 2.1. Pearl mussels and immune challenge

A total of 92 adult pearl mussels *C. plicata* (shell length 18–25 cm, body mass 1.5–2.0 kg) were obtained from Poyang Lake in Jiangxi Province, China, and maintained at 25 ± 2 °C in freshwater tanks at room temperature under natural photoperiod for a week. They were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*. Pearl mussels were injected into the adductor muscle 0.1 mL of PBS (Phosphate Buffered Saline) as control group or 0.1 mL bacterial suspension (*A. hydrophila*, dissolved in PBS, $10^9$ cells $mL^{-1}$) as challenged group. Gill, hemolymph and hepatopancreas were collected from four pearl mussels at 0, 6, 12, 24 and 48 h post-injection and immediately stored in liquid nitrogen until used. Unchallenged pearl mussel's hemolymph, gill, hepatopancreas, mantle and muscle were collected and stored in liquid nitrogen.

### 2.2. cDNA library construction and EST analysis

Hemolymph was withdrawn 48 h after the injection from the posterior adductor muscle with a 25 G needle into a syringe. The hemolymph was immediately centrifuged at 3000 *g* at 4 °C for 10 min to separate the hemocytes from the plasma. About 10 mL hemolymph were taken from the two mussels (about 5 mL/each mussel) and were used to prepare total RNA by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The mRNA was then isolated from the total RNA using the PolyATract Isolation System (Promega). To construct a cDNA library with full-length insertion, the SMART cDNA library construction kit (Clontech) was used following the manufacturer's instructions with minor modification. The synthesized ds cDNA by Long-Distance PCR were directly ligated into a pGEM®-T Easy Vector (Promega) and transformed into *Escherichia coli* DH5α cells. The clones were picked out randomly, and were stored at −80 °C for further analysis. Random sequencing of the library using T7 primer yielded 534 successful sequencing reactions. BLAST analysis of all the EST sequences revealed that an EST of 931 bp (EST no. Z003-005_C03) was homologous to the SelW of *Gallus gallus* (ACX47065) and *Equus caballus* (NP_001163893). This EST was found to contain a start codon. This suggested that its 5′-end was complete, and was selected for further cloning of the CpSelW gene of *C. plicata*.

### 2.3. RNA extraction and first-strand cDNA synthesis

Total RNA samples were extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and quality of total RNA were estimated by spectrophotometry at an absorbance of 260 nm and agarose-gel electrophoresis, respectively. The Smart cDNA was synthesized and was amplified using a Clontech SMART PCR cDNA Synthesis Kit (Clontech) by following the supplier's protocol. The synthesis reactions were performed at 65 °C for 5 min, 42 °C for 1 h, and terminated by heating at 70 °C for 15 min, the cDNA mix was stored at −80 °C for Race-PCR and quantitative real-time PCR.

### 2.4. Cloning the full-length cDNA of CpSelW

Based on the identified EST sequence, two gene-specific primers CpSelW-F1: ACTGGGTTTTTTGAGGTGGAAGTGGA and CpSelW-F2: GCA CTCAAAGAAGGGTGGAGATGGAT, were designed to amplify 3′-end of the CpSelW cDNA by rapid amplification of cDNA ends (RACE) and nested PCR technique. The first round reaction was performed by using primers CpSelW-F1 and 10 × Universal Primer A Mix (UPM) in a 25 μL of reaction volume, containing 2.5 μL of 10 × Ex Taq Buffer, 2.0 μL of dNTP Mixture (2.5 Mm each), 0.6 μL of each primer, 0.6 μL of cDNA, 0.3 μL of Ex Taq polymerase (5 U/μL) (Takara, Japan) and 18.4 μL of double-distilled water. The cycle condition was one initial denaturation cycle of 94 °C for 5 min, then 35 PCR cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The second round reaction was performed using the CpSelW-F2 and UPM primer under the same PCR conditions. The product of the first round was diluted 50× as template. The PCR products were separated by agarose gel (1.2%) electrophoresis, and then the bands were excised and purified using a DNA Gel Extraction Kit (KeLi, China). Finally, the purified DNA fragments were cloned into the pMD18-T vector (TaKaRa) and sequenced.

### 2.5. Sequence analysis of cpSelW

The CpSelW gene sequence was analyzed using the BLAST algorithm at web servers of the National Center for Biotechnology information, and the deduced amino acid sequence and open reading frame (ORF) were analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The similarity and identity of CpSelW with known SelW from other organisms were calculated by the online program (http://www.biosoft.net/sms/index.html). The SECIS element of CpSelW was analyzed using the SECISearch 2.19 program (http://www.genome.unl.edu/SECISearch.html). The presumed three dimensional structures was established for the CpSelW using the SWISS-MODEL prediction algorithm (http://www.swissmodel.expasy.org/), and displayed by DeepView/Swiss-Pdb Viewer version 4.0.1. Multiple alignments were generated by the CLUSTAL 1.8 program within DNASTAR. The phylogenetic tree was constructed with MEGA program version 4.1 based on amino acid sequences alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

### 2.6. CpSelW mRNA expression in different tissues and the response to A. hydrophila challenge

The expressions of CpSelW in different tissues of healthy and challenged mussels by *A. hydrophila* were determined by SYBR Green quantitative RT-PCR. To study the tissue-specific expression of CpSelW in the tissues of healthy mussels, including hemocytes, hepatopancreas, mantle, gill and muscle were collected from four healthy individuals. In bacteria challenge experiment, fifty mussels were randomly divided into 2 groups as challenged group and control group, respectively, and each group contained 25 animals. Animal treatment, RNA extraction and first-strand cDNA were described in Sections 2.1 and 2.3. cDNA mix was diluted 100 times with RNA-free water for next step. A 160 bp DNA fragment of CpSelW was amplified using specific primer pair of CpSelW-F (5′-ATGAGGCTACGGTGCTAAGTT-3′) and CpSelW-R (5′-TCCATCTCCACCCTTCTTTG-3′). A housekeeping gene, β actin was used as the internal control to verify the RT-PCR reaction and adjust the cDNA templates. A 116 bp product of β-actin gene was amplified by specific primers (actin-F: 5′-TGTGCTGTCTGGCGGTTCA-3′; actin-R 5′-TCCTCTCTGGTGGAGCGATG-3′). Quantitative RT-PCR was carried out on a quantitative thermal cycler (Mastercycler ep Realplex2 PCR, Eppendorf) in a 25 mL of reaction volume, containing 12.5 μL of 2 × SYBR Green Premix ExTaq (Takara, Japan), 0.4 μL of each primer, 1.0 μL cDNA template, and 10.7 μL of RNA-free water. The cycle condition was one initial denaturation cycle of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min. To compare with amplification efficiency of target gene and reference gene, we constituted a dilution series using a template (1, 0.1, 0.01, 0.001, 0.0001), and target gene and reference gene were amplified and a curve was drawn using ΔCt value of two genes in each dilution gradient, the consistency of two gene amplification efficiency is represented by the slope of the curve. Meanwhile, dissociation curve analysis of amplification products was performed at the