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Characteristics of 17β-hydroxysteroid dehydrogenase 8 and its potential role in gonad of Zhikong scallop *Chlamys farreri*

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are important enzymes catalyzing steroids biosynthesis and metabolism in vertebrates. Although studies indicate steroids play a potential role in reproduction of molluscs, little is known about the presence and function of 17β-HSDs in molluscs. In the present study, a full-length cDNA encoding 17β-HSD type 8 (17β-HSD8) was identified in the Zhikong scallop *Chlamys farreri*, which is 1104 bp in length with an open reading frame of 759 bp encoding a protein of 252 amino acids. Phylogenetic analysis revealed that the *C. farreri* 17β-HSD8 (Cf-17β-HSD8) belongs to the short chain dehydrogenase/reductase family (SDR) and shares high homology with other 17β-HSD8 homologues. Catalytic activity assay *in vitro* demonstrated that the refolded Cf-17β-HSD8 expressed in *Escherichia coli* could effectively convert estradiol-17β (E2) to estrone (E1), and weakly catalyze the conversion of testosterone (T) to androstenedione (A) in the presence of NAD^+. The *Cf-17β-HSD8* mRNA was ubiquitously expressed in all tissues analyzed, including gonads. The expression levels of Cf-17β-HSD8 mRNA and protein increased with gametogenesis in both ovary and testis, and were significantly higher in testis than in ovary at growing stage and mature stage. Moreover, results of *in situ* hybridization and immunohistochemistry revealed that the mRNA and protein of Cf-17β-HSD8 were expressed in follicle cells and gametes at all stages except spermatozoa. Our findings suggest that Cf-17β-HSD8 may play an important role in regulating gametogenesis through modulating E2 levels in gonad of *C. farreri*.

1. Introduction

17β-Hydroxysteroid dehydrogenases (17β-HSDs), which are oxidoreductases, catalyze the interconversion between the active (17-hydroxy) and inactive (17-keto) forms of specific steroids in a NAD(P)(H) dependent manner [1,2]. To date, 15 types 17β-HSDs have been identified in vertebrates and most of them belong to the short chain dehydrogenase/reductase family (SDR) [3]. The only exception is 17β-HSD5 (AKR1C3), which belongs to aldo-ketoreductase (AKR) family [4,5]. Typical 17β-HSDs share SDR conserved motifs, consisting of the cofactor binding domain (TGxxxGxG), the tetrad of active site (N-S-Y-K), the structural stabilization domain NNAG, and the PGxxxT domain which determines the reaction direction [6–8]. Although 17β-HSDs are almost conserved in sequence and structure, they differ in substrate preference and reaction direction [9,10]. It is clearly established that 17β-HSDs play an essential role in the steroid biosynthesis pathway in mammals [3,11]. Nevertheless, most of 17β-HSDs are already known to be multifunctional and play important roles in different metabolic pathways [2,8,9]. Actually, 17β-HSDs have diverse substrate specificities and catalyze the conversions of other substrates than steroids, such as lipids or retinoids [12–14]. The main role of 17β-HSDs in mammals is still a subject of debate.

Several types of 17β-HSDs have been identified and demonstrated to participate in steroid metabolism in amphibians and fishes [15–20]. In protochordates and invertebrates, studies in steroids metabolism have yielded evidence of 17β-HSD activity, and several 17β-HSDs homologues have been identified [21–25]. Especially, the presence of 17β-HSD activity has also been reported in molluscs [26,27]. Recently, two types of 17β-HSDs (17β-HSD 11 and 17β-HSD 12) have been identified and characterized in abalone *Haliotis diversicolor supertexta* (Mollusc: gastropod), and demonstrated to be involved in steroid metabolism [28,29]. Whereas, expression analysis indicated that 17β-HSD 12 is likely to be involved in lipid metabolism in the neogastropod *Nucella lapillus* [30]. Information regarding 17β-HSDs involved in steroids metabolism is still fragmentary in molluscs. In this work, we focus on 17β-HSD8 to assess its molecular characteristics and physiological function in molluscs.

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Type 8 17β-HSD is also known as Ke 6 gene whose abnormal expression is related to the development of recessive polycystic kidney disease (PKD) in mouse [31–35]. In addition, 17β-HSD8 is characterized and linked to the major histocompatibility complex (MHC) class II region in human and mouse [31,36,37], whereas in zebrafish and medaka it is linked to MHC class I region [38,39]. The recombinant human and mouse 17β-HSD8 expressed in *Escherichia coli* or HEK 293 cells catalyze interconversion of estradiol-17β (E2) and estrone (E1), and weakly converts testosterone (T) into androstenedione (A) in a NAD(H)-dependent manner [40–42]. Moreover, it is shown to be expressed in various tissues including ovary and testis in human and mouse [40,42]. Similar catalytic activity and expression pattern of 17β-HSD8 are also demonstrated in Nile tilapia *Oreochromis niloticus* [16]. The cellular localization of 17β-HSD8 mRNA and protein has also been investigated in mouse by *in situ* hybridization and immunofluorescence, respectively [40,43,44]. Moreover, transcriptional expression of human *17β-HSD8* gene is induced by E2 through estrogen receptor α (ERα) and C/EBPβ [45,46]. On the other hand, human 17β-HSD8 may also be involved in the fatty acid metabolism based on the three-dimensional (3D) structure analysis and its interaction with carbonyl reductase type 4 [47,48].

Although 17β-HSD8 has been investigated in vertebrates for years, there is no relevant study on this enzyme in invertebrates. The molluscs compose the largest phylum of invertebrate animals, and many of them are important in aquaculture. Many studies have demonstrated that steroids are widely present in molluscs and play a potential role in reproduction [49–52]. However, the endogenous origin and the action mechanism of steroids are still questionable in molluscs and require further study [53–55]. To investigate the presence and physiological role of 17β-HSD8 should be helpful in understanding the endocrine system more deeply in molluscs.

The Zhikong scallop *Chlamys farreri* (Jones & Preston, 1904) is a commercially important mollusc in Northern China. The steroids in *C. farreri* are suggested to be related to reproduction, and E2 stimulates the expression of *vitellogenin* (*vtg*) gene in the ovary [56,57]. In the present work, we cloned and characterized a *17β-HSD8* cDNA in *C. farreri*, and detected catalytic activity of the recombinant protein. The distribution of *17β-HSD8* mRNA was analyzed in various tissues of *C. farreri*. Furthermore, temporal and spatial expression pattern of 17β-HSD8 in gonads of *C. farreri* during gametogenesis was investigated at mRNA and protein levels. Our aim is to reveal the characteristics and expression pattern of 17β-HSD8 during gametogenesis, and explore its biological function in steroids metabolism and gametogenesis in *C. farreri*.

2. Materials and methods

2.1. Sample collection

Healthy two-year-old scallops *C. farreri*, with mean shell height of 63.8 ± 3.3 mm, were purchased from the Xiaogang Aquatic Product Market (Qingdao, PR China). According to histological characteristics of gametogenesis described by Liao et al. [58], the gonads of *C. farreri* were grouped into four stages (the resting stage, the proliferative stage, the growing stage and the mature stage). Five male and female scallops at each stage were selected for subsequent analysis. The gonads were dissected and divided into two parts. One of them was fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) at 4 °C for 20 h, then dehydrated with serial methanol (25%, 50%, 75% and 100%) diluted in 0.01 M PBS, and stored in 100% methanol at −20 °C for *in situ* hybridization and immunohistochemistry. The remainders were frozen immediately in liquid nitrogen after removal of digestive duct and stored at −80 °C for total RNA and protein extraction. Various tissues of *C. farreri* at proliferative stage, including digestive gland, kidney, gill, adductor muscle and mantle, were also sampled for RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNAs were extracted from gonads and other tissues using Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNAs were treated with RNase-free DNase I (Takara Bio. Co. Ltd., Dalian, China) to eliminate genomic DNA, and then purified using the RNeasy mini kit (Qiagen, Hilden, Germany). Quality and quantity of the RNAs were checked by electrophoresis and spectrophotometry (NanoVue, GE Healthcare, Piscataway, NJ, USA).

First-strand cDNA was synthesized through reverse transcription (RT) according to the manufacturer's instruction of PrimeScript™ RT reagent Kit (Takara Bio. Co. Ltd., Dalian, China). Both random hexamers (5 μM) and oligo(dT) (2.5 μM) were used as primers, and 1 μg DNase I-treated total RNA was used as template in a 20-μl reaction volume. The reactions were performed at 37 °C for 30 min, terminated by heating at 85 °C for 3 min. The cDNAs were stored at −20 °C for subsequent research.

2.3. Cloning of full-length cDNA

The 3′ and 5′ RACE Ready first-strand cDNA was synthesized using SMARTer™ RACE kit according to the manufacturer's instructions (Clontech, Mountain View, CA, USA). Two specific primers designed according to a fragment of 492 bp (GenBank acc. no. DT717629.1) from EST database (NCBI), GSP-5′ (5′-AGGGAGCCACCGTAGCTGTCGTAGA-3′) and GSP-3′ (5′-CTGGCCCACATTACCAACCTTTCCAC-3′), were employed to clone 3′ and 5′ regions of target gene respectively. The 5′ and 3′ RACE products were gel-purified, subcloned and sequenced, and then assembled with SeqMan Pro (DNA STAR Inc., Madison, WI, USA).

2.4. Sequence analysis

The amino acid sequence, which was deduced from the full-length cDNA, was analyzed for its identity to other known 17β-HSD8 homologues using the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple sequence alignment of the target sequences was performed with ClustalX 2.0 program. Phylogenetic tree was constructed using the MEGA 5.05 software with 1000 bootstrap trials by the neighbor-joining (NJ) method. Molecular weight and isoelectric point (p*I*) of the target protein were predicted using DNA STAR version 5.01 (DNA STAR Inc., Madison, WI, USA).

2.5. Prokaryotic expression, purification and refolding of *C. farreri* 17β-HSD8

The open reading frame (ORF) of *Cf-17β-HSD8* cDNA was amplified with the sense primer 5′-GGATCCATGGCTTCCGCTGCAGGTCT-3′ (*Bam*H I site underlined) and the antisense primer 5′-CTCGAGAGGAAACAAACCACCTGCTACC-3′ (*Xho* I site underlined). The reaction condition was 94 °C for 5 min, 30 cycles with 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min. The PCR product was subcloned into the pMD18-T vector (Takara, Shuzo Co. Ltd., Japan) followed by digestion with *Bam*H I and *Xho* I, and then subcloned into the expression vector pET28a (Merck KGaA, Darmstadt, Germany) previously cut with the same restriction enzymes. Expressed protein would contain 6-His tags at both C-terminal and N-terminal for purification purposes on a nickel affinity column. The insert fragment was confirmed by sequencing the recombinant expression vector pET28a-17β-HSD8.