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Molecular cloning and characterisation of scavenger receptor class B in pearl oyster *Pinctada fuctada martensii*

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Background: Molluscs can accumulate carotenoids in their body tissues by predominantly feeding on aquatic plant sources. Carotenoid transport and absorption are determined by the regulation of various proteins such as Scavenger receptor class B(*SR-BI*). We report the identification and characterisation of pearl oyster *Pinctada fuctada martensii SR-BI* (*PmSR-BI*). The correlation between total carotenoid content (TCC) and gene expression was also estimated.

Results: The full-length cDNA of *PmSR-BI* was 1828 bp, including an open-reading frame encoding of 1518 bp with a *pI* value of 5.83. PmSR-BI protein contains a hydrophobic CD36 domain and four centrally clustered cysteine residues for the arrangement of disulphide bridges. The deduced amino acid sequence had an identity of 30% to 60% with the SR-B of other organisms. Reverse transcription polymerase chain reaction analysis showed that mRNA transcripts were expressed in multiple tissues of adult pearl oyster. A higher expression of *PmSR-BI* gene was observed in the hepatopancreas than in the adductor muscle, gill and mantle. The TCC and gene expression of *PmSR-BI* were significantly correlated ($P < 0.05$), with a correlation coefficient of 0.978.

Conclusions: The results suggested that *PmSR-BI* is involved in the absorption of carotenoids in the pearl oyster *P. fuctada martensii.*

1. Introduction

Carotenoids are yellow to red C40 hydrophobic isoprenoid pigments and are widely distributed in nature. More than 600 structural distinct carotenoids have been isolated [1]. Carotenoid pigments play important physiological functions in many organisms. In higher plants and photosynthetic microorganisms, they protect the tissues and cells against photosensitised oxidation, in addition to their function as accessory pigments in light harvesting [2,3]. Moreover, carotenoids are considered precursors of phytohormone and abscisic acid [3]. In animals, the pigments are the precursors (provitamins) for the formation of vitamin A [4,5]; and are active oxygen quenchers with potential anti-cancer activities [6,7]. Carotenoids are beneficial for the prevention of coronary heart diseases, certain kinds of cancer, and age-related macular degeneration in humans [8].

Plants, fungi and bacteria can synthesise carotenoids. However, carotenoids cannot be synthesised de novo by animals, except for aphids and spider mites [9]. Therefore, carotenoids in many animals are a result of carotenoid accumulation from the diet or from specific chemical modifications by metabolic reactions [10]. Marine shellfish, such as scallops, mussels and abalones, can accumulate a variety of carotenoids [9]. The principal carotenoids in marine shellfish are β-carotene, lutein A, zeaxanthin, diatoxanthin, pectenolone, pectenol and mytiloxanthin. Like other animals, marine shellfish species must obtain carotenoids from food and subsequently transport them to the cells of target tissues.

The delivery of carotenoids to cells can be divided into three categories: enzyme-mediated processes, receptor-mediated endocytosis and selective lipid transport [11]. Carotenoid transport and absorption are determined by the regulation of various proteins involved in the process [12] that are mainly involved in ATP-binding cassette A1 (ABCA1), scavenger receptor class B type I (SR-BI) and cluster-determinant 36 (CD36) [13]. SR-BI and CD36 belong to the B class scavenger receptor family (SR-B). SR-B is a type III transmembrane receptor with two transmembrane domains, an extracellular loop with multiple glycosylation sites and two short intracellular tails [14]. As a scavenger receptor, the highly glycosylated extracellular domain has numerous substrate binding sites [15] for mediating the cellular uptake of carotenoids [7], those involved in immune response defence [16], those that participate in signal transduction and apoptosis and phagocytosis of apoptotic cells [17,18].

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The pearl oyster *Pinctada fuctada martensii* is naturally distributed along the coast of southern China. The species is one of the most important commercial shellfish in the south of China and is mainly cultured for its round nucleated pearls. Moreover, the shellfish is edible and highly nutritious [19]. Basing on the transportisome dataset of the pearl oyster mantle [20], we screened and characterised lipid metabolism-related genes, such as apolipoprotein and SR-B. Herein, we describe a *P. fuctada martensii* SR-BI gene, termed *PmSR-BI*, which shares a high structural and functional homology with the SR-BI, to understand the carotenoid metabolism in pearl oyster.

2. Material and methods

2.1. Experimental animals and sample collection

Adult pearl oysters were obtained from a stock farmed in Leizhou (Zhanjiang, Guangdong Province, China) and preconditioned for 2 days at 25°C to 30°C in a 1000 L tank with circulating seawater. Various tissues, including adductor muscle, gill, hepatopancreas and mantle, were collected and frozen in liquid nitrogen for the subsequent studies.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from the mantle tissue using the TRIzol reagent (Invitrogen). The cDNA first-strand synthesis was performed based on M-MLV RT usage information (Promega) using RQI DNase (Promega)-treated total RNA as template. cDNA mix was diluted to 1:50 and stored at -80°C for subsequent fluorescent real-time PCR.

2.3. Cloning the full-length cDNA of PmSR-BI

PmSR-BI cDNA was obtained using reverse transcription PCR (RT-PCR) and RACE technique. Degenerate primers were designed based on SR-B unigenes, which were selected from the transcriptome dataset of our library [20]. The intermediate fragment PCR reaction was implemented in a total volume of 10 μL, including 5 μL of Premix Taq, 0.4 μL of template cDNA, 0.4 μL of each primer (10 μmol L^{-1}) and 3.8 μL of double-distilled water. The PCR temperature profile was 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min and a final extension step at 72°C for 10 min. The band of the expected size (1200 bp) was excised and purified by agarose gel DNA fragment recovery kit (TaKaRa), subcloned into pMD-18 T vector (TaKaRa) and transformed into competent *Escherichia coli* cells DH5α. Bacteria were grown in ampicillin-containing Luria–Bertani plates, and the recombinants were selected and sequenced using the blue–white colour selection and screened with M13 forward and reverse primers from Sangon (Shanghai, China) [21].

The 5′- and 3′-ends of the *PmSR-BI* cDNA were obtained by RACE technique. The 5′-end and PCR reaction were implemented in a total volume of 10 μL, including 5 μL of Premix Taq, 0.4 μL of template cDNA, 0.4 μL of each primer (*PmSR-BI*-5′ outer and UPM) and 3.8 μL of water. The reaction was performed at 94°C for 5 min, 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 2 min and 72°C for 10 min, with storage at 4°C. A nested PCR was performed using NUP and *PmSR-BI*-5′ inner. The amplification reactions of the 3′-end, *PmSR-BI*-5′ outer and *PmSR-BI*-5′ inner changed to *PmSR-BI*-3′ outer and *PmSR-BI*-3′ inner. The reaction procedure was followed. Table 1 shows the primer sequence used in the cloning and real-time PCR of *PmSR-BI* gene.

2.4. Sequence analysis of PmSR-BI

The *PmSR-BI* gene cDNA sequence was analysed by the BLAST algorithm at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequence was analysed with the Expert Protein Analysis System (http://www.expasy.org/). Characteristic domains or motifs were identified using the PROSITE profile database. The Clustal W program (http://www.ebi.ac.uk/clustalw/) was used for multiple alignments of SR-BI. An unrooted phylogenetic tree was constructed according to amino acid sequences of the selected SR-BI using the neighbour-joining algorithm embedded in the MEGA6.0 program. The bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

Table 1
Primer sequence used in the cloning and real-time PCR of *PmSR-BI* gene.

Primer name	Primer sequence	Action
5′-inner	GGTCTCACGATACGCAATGGTTC	5′RACE
5′-outer	GTTGCTATCACCGCCTATGTCTA	5′RACE
3′-inner	TCCATCCAGACCCTGAGCAACAT	3′RACE
3′-outer	TTTCTCAAGTAATGAAGGACCCG	3′RACE
UPM	CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE
S	TGATGTCATAAATCCAGAGGAAGTA	Middle fragment PCR
A	ATTTCACAACCTCTTCATCATCCTC	Middle fragment PCR
GAPDH-S	CACTCGCCAAGATAATCAACG	Reference genes
GAPDH-A	CCATTCCTGTCAACTTCCCAT	Reference genes
M13F(-47)	CGCCAGGGTTTTCCCAGTCACGAC	Colony PCR
M13R(-48)	AGCGGATAACAATTTCACACAGGA	Colony PCR
RT-1s	AACTGAAAAAGCAGCCAACGAT	Real-time PCR
RT-1a	ACAGATGAGAATAAAAGCACCGA	Real-time PCR

2.5. Quantitative analysis of PmSR-BI mRNA expression

PmSR-BI mRNA expression was determined by quantitative real-time RT-PCR (qRT-PCR) with GAPDH as a reference gene. qRT-PCR was performed in a total volume of 10 μL, containing 5 μL of SYBR Green Master Mix (Rox), 0.4 μL pf cDNA, 0.4 μL of each primer (10 mM) and 3.8 μL of laboratory-grade water. The qRT-PCR program was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s according to the manufacturer's instructions [22]. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyse the expression level of the candidate genes.

2.6. PmSR-BI gene expression in yellow- and white-coloured strains

Expression levels of selected transcripts were investigated in the adductor muscle tissues of 10 yellow-coloured and 10 white-coloured pearl oysters. All oysters used in this experiment were sampled from the third-generation selected lines [23]. Total RNA was extracted and quality and quantity were determined using a Nanodrop spectrophotometer. A 1 μg of mRNA was used to synthesise cDNA by PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). qRT-PCR was conducted in a LightCycler®480 System using the SYBR Premix Ex Taq II qRT-PCR Kit (TaKaRa). Each assay was performed with GAPDH mRNA as the internal control.

2.7. Total carotenoid contents of yellow- and white-coloured strains

The protocols for total carotenoid content extraction were detailed by Lei et al. [24]. Total carotenoid content (μg/g) $= D_{480} \times 10^4 \times V / (E \times m)$, where D_{480} indicates the absorbance at 480 nm. V represents extracting liquid (mL), E represents molar extinction coefficient (2500) and m represents sample quality.

2.8. Statistical analyses

All data were expressed as mean ± standard deviation. Total carotenoid content (TCC) between yellow- and white-coloured strains was compared by *T*-test. The correlation between *PmSR-BI* gene

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