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Molecular and enzymatic characterization of two enzymes BmPCD and BmDHPR involving in the regeneration pathway of tetrahydrobiopterin from the silkworm *Bombyx mori*

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ABSTRACT

Tetrahydrobiopterin (BH4) is an essential cofactor of aromatic amino acid hydroxylases and nitric oxide synthase so that BH4 plays a key role in many biological processes. BH4 deficiency is associated with numerous metabolic syndromes and neuropsychological disorders. BH4 concentration in mammals is maintained through a *de novo* synthesis pathway and a regeneration pathway. Previous studies showed that the *de novo* pathway of BH4 is similar between insects and mammals. However, knowledge about the regeneration pathway of BH4 (RPB) is very limited in insects. Several mutants in the silkworm *Bombyx mori* have been approved to be associated with BH4 deficiency, which are good models to research on the RPB in insects. In this study, homologous genes encoding two enzymes, pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) involving in RPB have been cloned and identified from *B. mori*. Enzymatic activity of DHPR was found in the fat body of wild type silkworm larvae. Together with the transcription profiles, it was indicated that *BmPcd* and *BmDhpr* might normally act in the RPB of *B. mori* and the expression of *BmDhpr* was activated in the brain and sexual glands while *BmPcd* was expressed in a wider special pattern when the *de novo* pathway of BH4 was lacked in *lemon*. Biochemical analyses showed that the recombinant BmDHPR exhibited high enzymatic activity and more suitable parameters to the coenzyme of NADH *in vitro*. The results in this report give new information about the RPB in *B. mori* and help in better understanding insect BH4 biosynthetic networks.

1. Introduction

Tetrahydrobiopterin (BH4) is widespread and has importantly biochemical and physiological functions in higher eukaryotes other than plants (Thony et al., 2000). In mammals, BH4 is not only an intracellular antioxidant to scavenge reactive oxygen species (ROS) but also an essential cofactor for three aromatic amino acid hydroxylases, nitric oxide synthase (NOS, EC 1.14.13.39), and alkyl-glycerol monooxygenase (EC 1.14.16.5) (Kaufman, 1963; Thony et al., 2000). Consequently, BH4 is present in probably every cell or tissue of higher organisms and plays a key role in a number of biological processes and pathological states associated with monoamine neurotransmitter formation, cardiovascular and endothelial dysfunction, the immune response and pain sensitivity (Werner et al., 2011).

BH4 biosynthesis proceeds in a *de novo* pathway in a NADPH-dependent reaction from GTP via two intermediates, 7,8-dihydroneopterin triphosphate and 6-pyruvoyl-5,6,7,8-tetrahydropterin. The three enzymes GTP cyclohydrolase I (GTPCHI, EC 3.5.4.16) (Nar et al., 1995), 6-pyruvoyltetrahydropterin synthase (PTPS, EC 4.2.3.12) (Ploom et al., 1999) and sepiapterin reductase (SPR, EC 1.1.1.153) (Auerbach et al., 1997) are required to carry out the proper stereospecific reaction to make BH4 (Bracher et al., 1998) (Fig. 1). Besides, the concentration of BH4 *in vivo* is maintained through a regeneration pathway. It has been reported that the regeneration pathway of BH4 (RPB) is carried out by two enzymes, pterin-4a-carbinolamine dehydratase (PCD, EC 4.2.1.96) (Koster et al., 1996) and dihydropteridine reductase (DHPR, EC 1.6.99.7) in mammals (Rey et al., 1977). During the catalytic event of aromatic amino acid hydroxylation, molecular oxygen is transferred to the corresponding amino acid and BH4 is oxidized to BH4-4a-carbinolamine. In the RPB, PCD dehydrates BH4-4a-carbinolamine and converts it to quinoid dihydrobiopterin (qBH2) (Curtius et al., 1990), which could be catalyzed by DHPR and returned into BH4 in a NADH-dependent reaction (Armarego et al., 1984) (Fig. 1). By far, RPB has been found in many mammals including human (Thony et al., 1994), rats (Shen and Zhang, 1991) and sheep (Shen, 1991). Previous studies in the silkworm *Bombyx mori* and other insects showed that the *de novo* synthesis pathway of BH4 is similar between insects and mammals (Kato et al., 2006; Meng et al., 2009a; Fujii et al., 2013). Although PCD and DHPR genes have been cloned from the fruit fly *Drosophila melanogaster* (Seong et al., 1998; Park et al., 2000), knowledge about the RPB in insects is very limited.

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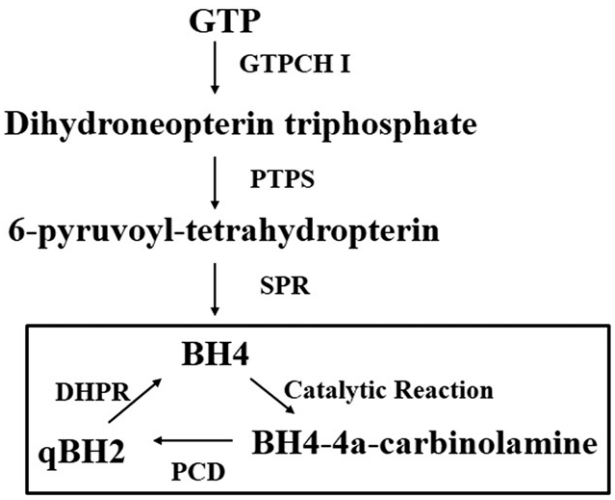

Fig. 1. *De novo* pathway and regeneration pathway of tetrahydrobiopterin biosynthesis. The regeneration pathway is boxed. Abbreviations are shown as GTP, guanosine triphosphate, GTPCHI, GTP-cyclohydrolase I, PTPS, 6-pyruvoyl tetrahydropterin synthase, SPR, sepiapterin reductase, BH4, tetrahydrobiopterin, qBH2, quinonoid dihydropteridine, PCD, pterin-4a-carbinolamine dehydratase, and DHPR, dihydropteridine reductase.

BH4 deficiency is associated with numerous metabolic syndromes and neuropsychological disorders (Blau et al., 1996a, 2001). Most of BH4 deficiencies were caused by mutations in genes encoding the enzymes involving in biosynthesis (Thony and Blau, 1997) or regeneration of BH4 (Thony et al., 1998). The mutations are all inherited in an autosomal recessive manner. BH4 deficiency has been extensively studied to understand the genetic disorder hyperphenylalaninemia (HPA) which phenylalanine accumulation leads to a syndrome that includes mental retardation (Blau et al., 1996b). It should be highlighted that more than one third of BH4 deficiency is triggered by mutations in PCD and DHPR genes (Shintaku, 2002). DHPR deficiency is the second most common reason of HPA in human (Smooker and Cotton, 1995; Blau et al., 1996a; Dianzani et al., 1998). These reports disclosed the physiological importance of RPB.

As an important lepidopteran model insect, more than 400 mutations of *B. mori* have been identified, and more than 1000 silkworm strains are maintained as genetic resources (Velu et al., 2008). Several mutant strains, including *lemon* (*lem*), *lemon lethal* (*lem*^{l}) and *albino* (*al*), are associated with BH4 deficiency at different level (Meng et al., 2009a; Fujii et al., 2013). *lem* and its lethal allele *lem*^{l} were caused by the mutations in the SPR gene (*BmSpr*). BmSPR activity in *lem*^{l} larvae was almost completely diminished, resulting in a serious synthesis defect of BH4 (Meng et al., 2009a). Similarly, mutation in BmPTPS was responsible for the mutant phenotype of *al*, in which the lethality of *al* larvae could be rescued by BH4 oral administration (Fujii et al., 2013). These studies suggest that *lem*, *lem*^{l} and *al* are good models to research on the RPB in insects.

In order to identify whether RPB plays a role in *B. mori* and it would be activated or not when the *de novo* synthesis pathway of BH4 is defective, PCD and DHPR genes of *B. mori* (*BmPcd*, *BmDhpr*) were firstly cloned and enzymatic kinetics and properties of BmDHPR were characterized in this study. In addition, expression profiles of *BmPcd* and *BmDhpr* mRNA were compared between wild and *lem* strains. The results give new insights into the two *B. mori* genes involving in RPB and help to understand RPB in insects.

2. Materials and methods

2.1. Experimental silkworm

The wild silkworm strain of p50 (Dazao) and *lem* mutant strain of ah09 were maintained in our laboratory. Mutant strains of a65 (*lem*^{l}) and a60 (*al*) were kindly presented by Dr. Yutaka Banno of Kyushu University, Japan (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp). All larvae were fed fresh mulberry leaves under normal conditions (12 h light/12 h dark, 25 °C). Homozygous larvae of a65 (*lem*^{l}/*lem*^{l}) and a60 (*al*/*al*) could not feed on leaves and die in three days after the first ecdysis.

2.2. Extraction of total proteins and detection of DHPR activity

Total proteins were extracted from different tissues on the third day of the fifth larval instar of p50 and ah09 strains and the whole bodies of lethal mutants of a65 and a60 strains, respectively. The tissues and the whole bodies were homogenized in 10 mM phosphate-buffered saline (PBS, pH 7.0) containing a mixture of proteinase inhibitors (Sangon). The supernatant of the homogenates was collected by centrifugation (12,000 rpm, 4 °C, 15 min). The concentration of total proteins in the supernatant was determined using a BCA kit (Sangon). DHPR activity was assayed according to the method reported by Lon-Fye Lye (Lye et al., 2002) and Dongkook Park (Park et al., 2000) with some modifications. The standard reaction mixture contained 100 mM Tris–HCl, pH 7.4, 20 μg of horseradish peroxidase, 10 mM H_2O_2, 10 μM dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), 100 μM NADH, and 400 μg of total proteins. All components except $DMPH_4$ were incubated for 1 min prior to initiation of reaction by addition of $DMPH_4$. The DHPR activity was determined by measuring the decrease of NADH in absorbance at 340 nm. The extinction coefficient for NADH is $\varepsilon_{340\ nm} = 6200\ M^{-1}\ cm^{-1}$. Reaction without the addition of the proteins was used as a control. The reaction mixture was kept at room temperature for 20 min. One unit of enzyme activity is the amount of enzyme that oxidizes 1 μM of NADH per min at room temperature. The data was analyzed by one-way ANOVA followed by Dunnett's test to localize the significant difference. A P-value of <0.05 was considered significant and a value <0.01 was considered extremely significant.

2.3. Preparation of total RNA and RT-PCR

The whole bodies of silkworms at different developmental stages were collected from the larvae on the third day of each instar, wandering period, pupae on the seventh day and new eclosion moths, respectively. Different tissues of silkworm larvae were collected on the third day of the fifth instar. Total RNA was extracted by phenol/chloroform method using TRIzol™ Reagent (Invitrogen) according to the instructions. The first-strand cDNA was synthesized from 1 μg of the total RNA using a TaKaRa RNA PCR Kit (TaKaRa). Expression profiles of *BmPcd* and *BmDhpr* in different strains, periods and tissues were analyzed by reverse-transcription PCR (RT-PCR). The PCR condition was 95 °C for 10 min followed by 30 cycles of 95 °C for 1 min, 51 °C for 1 min, and 72 °C for 1 min. The PCR primers used in this experiment were listed in Table 1. Transcription of *BmActin* gene was used as a

Table 1
Main primers used in this study.
Artificial *BamH*I and *Hind*III sites are underlined.

Name	Sequence (5′–3′)	Purpose
PCD-RT-F	CGTTTGCGAATGGGTAT	RT-PCR
PCD-RT-R	TTAATTATAATTTTTATCCATAA	RT-PCR
DHPR-RT-F	ATCGTCGTGTACGGTGGTCGTGG	RT-PCR
DHPR-RT-R	CAATAATTAAGTCAGTAAC	RT-PCR
Actin-RT-F	AGACGAGGCACAGAGCAA	RT-PCR
Actin-RT-R	TGTAGAAGGTATGATGCCAAA	RT-PCR
PCD-F	ATGAATAAGACAACTATGACG	Gene cloning
PCD-R	TTAATTATAATTTTTATCCATAA	Gene cloning
DHPR-F	GAAGTTGTGAACGAGAGAAGACG	Gene cloning
DHPR-R	TTACTCAATAATTAAGTCAGTAAC	Gene cloning
DHPR-pMF-F	ATAGGATCCATGGCTACCGGT	Expression in *E. coli*
DHPR-pMF-R	CCCAAGCTTTTACTCAATAATTAAG	Expression in *E. coli*