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Identification and characterization of a pyridoxal 5′-phosphate phosphatase in the silkworm (*Bombyx mori*)

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ABSTRACT

Vitamin B_6 comprises six interconvertible pyridine compounds, among which pyridoxal 5′-phosphate (PLP) is a coenzyme for over 140 enzymes. PLP is also a very reactive aldehyde. The most well established mechanism for maintaining low levels of free PLP is its dephosphorylation by phosphatases. A human PLP-specific phosphatase has been identified and characterized. However, very little is known about the phosphatase in other living organisms. In this study, a cDNA clone of putative PLP phosphatase was identified from *B. mori* and characterized. The cDNA encodes a polypeptide of 343 amino acid residues, and the recombinant enzyme purified from *E. coli* exhibited properties similar to that of human PLP phosphatase. *B. mori* has a single copy of the *PLPP* gene, which is located on 11th chromosome, spans a 5.7 kb region and contains five exons and four introns. PLP phosphatase transcript was detected in every larva tissue except hemolymph, and was most highly represented in Malpighian tube. We further down-regulated the gene expression of the PLP phosphatase in 5th instar larvae with the RNA interference. However, no significant changes in the gene expression of PLP biosynthetic enzymes and composition of B_6 vitamers were detected as compared with the control.

1. Introduction

Vitamin B_6 comprises six interconvertible pyridine compounds (vitamers), including pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their phosphorylated forms: pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP) and pyridoxal 5′-phosphate (PLP). Among these vitamers, PLP is a coenzyme for over 140 enzymes, which not only are involved in the synthesis, interconversion and degradation of amino acids but also play key roles in the metabolism of neurotransmitters, one-carbon units, biogenic amines, tetrapyrrolic compounds, amino sugars, modulation of steroid receptor-mediated gene expression and regulation of immune function (Di Salvo et al., 2011). In most organisms, PLP is recycled from nutrients and degraded B_6-enzymes in a salvage pathway involving phosphatases, an ATP-dependent PL kinase and a FMN-dependent PNP oxidase (McCormick and Chen, 1999). PLP is largely present in meat, associated with glycogen phosphorylase in muscles, together with smaller amounts of PMP. PN and its glucosides are the distinctive vitamers in plants. In mammals, ingested phosphorylated B_6 vitamers are first hydrolyzed to PL, PM and PN by intestinal phosphatase, while PN glucosides are hydrolyzed by a glucosidase. The absorbed vitamers mainly enter the liver, where they are phosphorylated by PL kinase to form PLP, PMP and PNP, respectively. PNP and PMP are further oxidized to be PLP by PNP oxidase. PLP reenters the circulation bound to a lysine residue of albumin (Bohney et al., 1992). Delivery of active cofactor to the tissues, however, requires hydrolysis of circulating PLP to PL by the ecto-enzyme tissue nonspecific alkaline phosphatases (Clayton, 2006). Once entered the cells, PL is rephosphorylated by PL kinase and is somehow targeted to dozens of newly synthesized apo-PLP enzymes.

The major pathways of vitamin B_6 metabolism have been established, but little is known of how PLP homeostasis is maintained in the biological tissues. PLP contains a very reactive aldehyde group at the 4′ position, which easily forms aldimines with primary and secondary amines. For this reason, non-specific binding with non-B_6 protein has been implicated in the toxic effect of vitamin B_6 (Di Salvo et al., 2011). Presumably to prevent the toxic buildup, the pool of free PLP *in vivo* is maintained at a very low level in the body. The most well established mechanism for maintaining low levels of free PLP is its dephosphorylation by phosphatases. Studies on the homeostasis have shown that there is not a single mechanism. The factors for PLP homeostasis may include catabolism of PLP by phosphatase, activities of PL kinase and PNP oxidase, degree of protein binding of the synthesized coenzyme, and transport of the precursors (Di Salvo et al., 2011, 2015).

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Alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2), both of which have broad substrate specificity for phosphomonoesters, hydrolyze PLP and PMP (Harris, 1990; Bull et al., 2002). However, a PLP-specific phosphatase has also been purified from human erythrocytes and characterized (Fonda, 1992). The molecular properties including the molecular weight, pH optimum, substrate specificity, and metal requirement indicate that the PLP phosphatase is distinct from other known phosphatases (Fonda, 1992; Fonda and Zhang, 1995). Data obtained in chemical modification studies revealed that the PLP phosphatase has essential residues such as cysteine, arginine, histidine, and a carboxylate group at or near the active site (Gao and Fonda, 1994a, 1994b, 1994c). Subsequently, a cDNA encoding the PLP phosphatase was detected in human and mouse, and the transcript was identified in all human tissues and was highly abundant in the brain (Jang et al., 2003). Interestingly, the phosphatase was also identified as chronophin, which is a novel HAD-type serine protein phosphatase regulating cofilin-dependent actin dynamics (Gohla et al., 2005). The actin dynamics may play an important role in the changes of morphological properties and excitability of the epileptic hippocampus (Kim et al., 2008). Furthermore, a PNP-specific phosphatase was purified from *Sinorhizobium meliloti* and characterized (Tazoe et al., 2006). This phosphatase was inactive on PMP and other physiologically important phosphorylated compounds, and its specific constant of *Vmax*/*Km* was nearly 2.5 times higher for PNP than for PLP.

The silkworm, *Bombyx mori*, is an important economic and model insect with high protein metabolic activity, and its PLP level displays developmental cyclic changes (Huang et al., 1998; Huang et al., 1999; Zhang and Huang, 2003). Considering the importance of studying the regulatory mechanism of its PLP levels, we have cloned the cDNAs from *B. mori* PL kinase and PNP oxidase (GENBANK accession number: NM_001043975.1 and NP_001037442.1) and characterized their corresponding enzyme products (Shi et al., 2007; Huang et al., 2009, 2011, 2012a). Our recent studies have shown that *B. mori* PL kinase and PNP oxidase are regulated at the transcription level by larva development and are responsive to hormones (Huang et al., 2016a). In this study, a cDNA encoding PLP phosphatase from *B. mori* was cloned and characterized. In addition, we down-regulated the gene expression of PLP phosphatase by body cavity injection of synthesized double-stranded small interfering RNA to 5th instar larvae, and analyzed the effect on the vitamin B_6 metabolism.

2. Experimental procedures

2.1. Identification and cloning of the PLP phosphatase cDNA from B. mori

Amino acid sequence of human PLP phosphatase (GENBANK accession number: NP_064711.1) was retrieved from GENBANK and used as query sequences to search against the expressed sequence target (EST) database of *B. mori* (http://blast.ncbi.nlm.nih.gov/Blast) and the genomic database of *B. mori* (http://silkworm.genomics. org.cn), using the Translated-BLAST program with default parameters. A pair of primers for RT-PCR was designed using the software Primer premier 5.0 (http://www.premierbiosoft.com) and DNAMAN (http://www.lynnon.com/). The sense primer is 5′-CGTGTGATTTCAATGGGCTT-3′ and the *anti*-sense is 5′-CCCTAATACTATGACGTT-3′. Strain of *B. mori* was Dazhao, reared under natural condition with mulberry leaves. Total RNA was extracted from fat body of four day larvae of 5th instar using Trizol reagent, and the cDNA was synthesized using HiFiScript cDNA Synthesis Kit (Sangon Biotech. Co. Lid., Shanghai, China). PCR reaction mix (50 μL) contained 29 μL of double distilled water, 10 μL of 5 × Buffer, 4 μL of dNTP (2.5 mM), 2 μL of each primer, 2.5 μL of cDNA template and 0.5 μL of Prim STA HS DNA Polymerase (TaKaRa Biotech. Co. Lid., Dalian, China. Amplification was performed in a thermal-cycler (MyCycler™, Biorad, USA), as follows: 4 min at 95 °C; 35 cycles of 10 s at 98 °C, 15 s at 58 °C, and 80 s at 72 °C, followed by 10 min at 72 °C. The resulting PCR product was obtained on an EB-stained 1.2% agarose gel and purified using DNA Gel Extraction kit (TransGene Biotech. Co. Lid., Beijing, China). A-tails were added to the purified DNA fragment with an A-tailing kit (Sangon Biotech. Co. Lid., Shanghai, China), and then the DNA fragment, designated as BmPLPP, was cloned into the vector of pEASY-T1, generating pEASY-T1-BmPLPP for sequencing. All the primer construction and DNA sequencing were finished by Sangon Bio-technology Company (Shanghai, China).

A Network resource (http://web.expasy.org/translate/) was used for translation of the coding area and characterization of the protein sequence. Program DNAman (http://www.lynnon.com/) and ClustalW were used for alignment of two and multiple amino acid sequences of PLP phosphatase. The *B. mori* PLP phosphatase cDNA was used as query sequences to search against the genomic database of *B. mori* for gene locating. Software SeqVISTA (http://bioinformatics-program-bu.software.informer.com/) was used to analyze the gene structure. The amino acid sequence was submitted to SWISS-MODEL server (http://swissmodel.expasy.org), and the three-dimensional structure of *B. mori* PLP phosphatase was predicted by the method of homology modeling.

2.2. Expression and purification of the recombinant B. mori PLP phosphatase

Using pEASY-T1-BmPLPP as a template, the full coding region of *BmPLPP* was obtained by PCR. The PCR had a total volume of 50 μL, containing 2.5 μL of templates, 10 μL of 5 × Buffer, 4 μL of dNTP (2.5 mM), 2 μL of each primer, 0.5 μL of Prim STA HS DNA Polymerase and ddH_2O 29 μL. To take out the terminator contained in the coding region of *BmPLPP*, primers used in the PCR were as follows: (5′-CCGGAATTCG**ATG**GGCTTGACCAAATT-3′) and *anti*-sense primer (5′-CCGCTCGAGTGACGTTACAAGTGACT-3′), which contained *EcoR I* and *Xho I* recognition sites (underlined), and initiation codon (red). Amplification was performed as follows: 5 min at 94 °C; 35 cycles of 10 s at 98 °C, 15 s at 55 °C and 90 s at 72 °C, followed by 10 min at 72 °C. The PCR product was purified using DNA Gel Extraction kit, and digested with *EcoR I* and *Xho I* restriction enzymes (Sangon Biotech. Co. Lid., Shanghai, China). The digested fragment was then purified again and ligated with T4 DNA ligase (TaKaRa Biotech. Co. Lid., Dalian, China into pET-24b(+), previously digested with the same enzymes. The resulting recombinant plasmid was named pET-24b-BmPLPP-His, which contained a T7 promoter, *B. mori PLPP* gene fused to a C-terminal hexahistidine affinity tag sequence and T7 terminator. The plasmid was used to transform the competent cells of *E. coli* Trans-T1 for sequencing.

After electrophoretic and sequencing analysis, pET-24b-BmPLPP-His was used to transform *E. coli* BL21trxB (DE3) cells for protein expression. The single bacterial colony of *E. coli* BL21trxB (DE3), harboring pET-24b-BmPLPP-His, was cultured in 10 mL Luria-Bertani (LB) medium with kanamycin (50 μg/mL) on a shaker platform, overnight, at 37 °C, and the overnight culture was then inoculated into 500 mL LB medium with kanamycin (50 μg/mL). The inoculum was grown at 37 °C with vigorous shaking until OD_{600} reached 0.8. IPTG was added to a final concentration of 0.2 mM and the cells were further incubated and shaken for 20 h at 16 °C.

Cells were harvested by centrifugation, washed, and resuspended in 20 mL of 20 mM Tris-HCl buffer, pH 7.4, containing 200 mM NaCl and 20 mM imidazole. The cell suspension was sonicated, and the lysate was cleared by centrifugation. The supernatant was then poured into the column loaded with the nickel-nitrilotriacetic acid agarose (7sea Biotech. Co. Lid., Shanghai, China), washed with Tris buffer containing 40 mM imidazole to remove un-specifically bound proteins, including *E. coli* phosphatase. Subsequently the histidine-tagged protein was eluted with 200 mM imidazole. The purity and homogeneity of the fractions and the subunit molecular weight of the PLP phosphatase were estimated by SDS-PAGE. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard.