Int. J. Indust. Entomol. Vol. 23, No. 2, 2011, pp. 223~229

International Journal of *Industrial Entomology*

Cloning and Characterization of *Bombyx mori* Cyclophilin A

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(Received 22 September 2011; Accepted 19 November 2011)

Cyclophilins are originally identified as cytosolic binding protein of the immunosuppressive drug cyclosporine A. They have an activity of peptidyl prolyl cis/trans-isomerases (PPIase), which may play important roles in protein folding, trafficking, assembly and cell signaling. In this study, we report the cloning and characterization of a *Bombyx mori* cyclophilin A (*b*CypA) cDNA. The full-length cDNA of *b*CypA consist of 947 nucleotides with a polyadenylation signal sequence AATAAA and contain an open reading frame of 498 nucleotides encoding a polypeptide of 166 amino acids. The deduced amino acid sequence of *b*CypA shares a central peptidyl prolyl cis/trans-isomerase and a cyclosporin-A-binding domain with other cyclophilin sequences. Relative quantification real-time (RT) PCR analysis shows that mRNA transcripts of *b*CypA are detected in all the investigated tissues and highest expression level in the skin of 3-day-old 5 instar larva. Also, *b*CypA had PPIase activity on the proline-containing peptides. Accordingly, we suggest that *b*CypA is a new member of the cyclophilin A (CyPA) family and will be useful for quality control of bioactivity recombinant proteins with proline-containing peptides.

Keywords: *Bombyx mori*, Peptidyl prolyl cis/trans-isomerases (PPI), Cyclophilin A

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Introduction

Cyclophilins (Cyps) belong to a family of proteins that have peptidyl-prolyl *cis-trans* isomerase (PPIase) activity which catalyze the isomerization of proline imidic peptide bonds (Fischer *et al*., 1989). The Cyps catalysis of folding protein in vitro and in vivo (Takahashi *et al*., 1989), make cyclophilins play important roles in the regulation of cellular processes (Schmid *et al*., 1993; Schonbrunner *et al*., 1991; Steinmann *et al*., 1991), including protein trafficking (Price *et al*., 1991; Uittenbogaard *et al*., 1998), the nucleolytic degradation of the genome (Montague *et al*., 1997) and regulation of the inflammatory response (Arora *et al*., 2005). They have been found in a wide range of species from prokaryotes and eukaryotes through to yeast and animals. There are 7 major Cyps (*h*CypA, *h*CypB, *h*CypC, *h*CypD, *h*CypE, *h*Cyp40 and *h*CypNK) and 16 unique Cyps in humans (Galat *et al*., 2003; Waldmeier *et al*., 2003). *Drosophila* has at least 9 Cyps (Waldmeier *et al*., 2003) and the plant *Arabidopsis thaliana* has 29 putative Cyps (He *et al*., 2004), whereas 8 Cyps have been found in *Saccharomyces cerevisiae* (Arevalo-Rodriguez *et al*., 2004). The first member of the Cyps to be identified in mammals, cyclophilin A (CypA), is an 18 kDa protein and localized in the cytoplasm. CypA are also known as a cytosolic receptor for the immunosuppressive drugs cyclosporine A (CsA) and immunophilins, such as the parvulines and the FK-506-binding proteins (FKBPs) (Handschumacher *et al*., 1984). Additional, CsA-CypA complex can bind intracellular proteins and play important roles in the immune system of mammals. For example, the immunosuppressive action of CsA is exerted via a ternary complex between CypA, CsA and calcineurin. The CsA-CypA complex binds to inhibits calcineurin, a calcium-calmodulin-activated serine/threonine specific protein phosphatase. Inhibition of calcineurin blocks the translocation of nuclear factor of activated T cells (NF-AT) from the

cytosol to the nucleus (Liu *et al*., 1991). Recent studies have suggested a novel role for CypA in *S. cerevisiae*. The pathogenic yeast *C. neoformans* has two similar CypA related proteins, Cpa1 and Cpa2. They are required for growth of *C. neoformans* at elevated temperature and for full expression of fungal virulence. These roles suggest a novel role for cyclophilin A homologs in the growth and virulence of this pathogen (Wang *et al*., 2001).

In this paper, we report the cloning and characterization of a cDNA encoding a *Bombyx mori* Cyclophilin A (*b*CypA, GenBank accession no.: AY 966877). In a RT-PCR analysis, *b*CypA mRNA transcript is found universally distribution in all investigated tissues and development stages of *B. mori*. Assay of PPIase activity of this enzyme was also performed.

Materials and Methods

Experimental cells

Bm5 cells (derived from *Bombyx mori* pupal ovary) were cultured at 27ºC in TC-100 medium containing 10% (v/v) heat-inactivated fetal bovine serum.

Construction of subtraction suppression hybridization (SSH) cDNA libraries

Bm5 cells were divided into two groups. The first group was incubated in TC-100 medium containing 10% (v/v) heat-inactivated fetal bovine serum. The second group was incubated in media containing tunicamycin at 5 μg/ml. Each group of Bm5 cells was incubated for 5 hours and harvested. Total RNA of each group was prepared using a TRI REAGENT (Molecular Research center) according to the manufacture's protocol. The quality and the quantity of each sample were analysed by agarose gel electrophoresis and spectrophotometry. PolyA$^+$ RNA was prepared from total RNA using a Micro-Fast Track 2.0 mRNA isolation kit (Invitrogen) according to the manufacture's protocol. SSH cDNA libraries were generated using the PCR-Select cDNA Subtraction kit (Clontech): cDNAs were prepared from 2 μg of each group of mRNA. The cDNAs from group 1 was used as the driver. The cDNAs from group 2 was used as the tester. The subtraction procedure was progressed according to the manufacture's protocol. SSH cDNA libraries were cloned into pGEM-T easy vector and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) according to the manufacture's protocol. Analysis of the sequences was carried out using DNA Sequencing Analysis Software (Perkin-Elmer) and BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.Gov/Blast.cgi). Alignments were performed using program ClustalW (http://align.genome.jp).

cDNA cloning

cDNA synthesis was performed using a PolyA$^+$ RNA from the Bm5 cells treated with tunicamycin and Marathon cDNA Amplification kit (Clontech). The full-length cDNA of *b*Cyp A was amplified by PCR with a set of primers: AP1 adaptor primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') and gene specific primer (5'-CCATCCTAATACGACTCACTATAGGGCTCG-3'). PCR was run using the following thermocycler program: 95ºC for 2 min, followed by 30 cycles at 95ºC for 30 s, 54ºC for 30 s and 72ºC for 1 min and a final step at 72°C for 5 min. PCR product was subcloned into the pGEM-T easy vector (Promega) and sequenced. Sequencing data was analyzed with the DNASTAR LaserGene program. A databank search for homologous genes and similarity analysis were carried out via BLAST program.

RNA isolation and Relative quantification real-time PCR

Total RNA of all development stages extracted from eggs (1-day-old), larva (from 1 instar 1-day-old to 5 instar 1-day-old), pupa (5-day-old) and imago (0-day-old female). Fifth instar larvae of *B.mori* were dissected under a stereomicroscope (Leica, Germany). Organs such as the fat body, midgut, malpighian tube, silk gland, ovary and testis were harvested and washed twice with PBS. Total RNA was prepared using by TRI REAGENT according to the manufacture's protocol (Molecular Research Center). Total RNA (2 μg) was amplified by a three-step protocol using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA mix was diluted to 1:5 and stored at -20°C for subsequent RT-PCR. Tow bCypA gene specific primers (forward, 5'-GGACTTCACCAACCATAAC-3'; reverse, 5'-GTATCAGCACCAGCATTAG-3') were used to amplify a product of 110 bp. A constitutive expression gene, the *B. mori* Actin 3 gene, was used as endogenous control to verify the Relative quantification RT-PCR reaction. Two primers (forward, 5'-TTGGCATCATACCTTCTACA-3'; reverse, 5'-CTGGGTCATCTTCTCTCTG-3') were used to amplify a 112 bp fragment of *B. mori* Actin 3 gene. The RT-PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosysems). The amplifications were performed in a 25 μl reation volume containing 12.5 μl of 2 × SYBR green master mix (TaKaRa). The thermal profile for RT-PCR was 95ºC for 15 s followed by 40 cycles at 95ºC for 15 s and 46ºC for 32 s and a final step at 95ºC for 15 s, 46ºC for 32 min and 95ºC for 15 s. Each sample was run in triplicate and data analysis was performed with the SDS software V2.0 (Applied Biosysems).

Bioinformatics analysis

In order to illustrate the genomic organization, the cDNA

sequence was blasted to contigs of the *B.mori* genome in the GeneBank and the Silkworm Genome Research Program (http://sgp.dna.affrc.go.jp/KAIKObase/), and DNASTAR LaserGene 7.1 program was used to search for intron. The sequence comparison was conducted by BLAST program (http://www.ncbi.nlm.nih.gov). The location of the gene on *B.mori* chromosomes was carried out via Silkworm Genome Research Program (http://sgp.dna.affrc.go.jp/KAIKObase). The deduced amino acid sequence of *b*CypA was aligned with the corresponding various CypA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) using the program Clustal W (http://align. genome.jp). Base on this alignment, phylogenetic tree was constructed with Geneious pro 4.7 program by using the neighbor-joining method (Saitou *et al*., 1987).

Expression of *B.mori* cyclophilin A using baculovirus expression vector system

The cDNA fragment of the mature *b*CypA was amplified by PCR with a set of primers: N-terminal primer 5'-GGAATTCCAAAATGTCTCTACCACGAGTA-3' and c-terminal primer 5'-CCGCTCGAGCGGGGCAATCT-GACCACA-3'. The amplified cDNA was ligated into pGEM-T easy vector (Promega). The cDNA fragment in the plasmid was digested with restriction enzymes *Eco*RI and *Xho*I. The fragment was purified by agarose gel electrophoresis and ligated into pBac-1 vector (Novagen). The pBac-*b*CypA construct was in the preparation of the recombinant baculovirus according to instruction manual of BacVector system (Novagen). Recombinant *b*CypA was expressed by Sf9 cells. A monolayer culture was infected with the recombinant baculovirus and incubated in a 25 cm^3 culture flask at 27ºC for 4 days. The purification of *b*CypA fused with six-histidine was carried out with MagExtractor His-taq kit according to the manufacture's protocol (TOYOBO). The purification steps were monitored by 12% SDS-PAGE.

Western blot analysis

Cell extracts were separated by SDS-PAGE and transferred onto Polyvinylidene Difluoride (PVDF) membrane (Amersham Biosciences). The membrane was incubated with primer antibody followed by incubation with a second antibody according to the manufacturer's protocols. ECL-plus chemiluminescence (Amersham Biosciences) and X-ray film were used for detection. Anti-CypA monoclonal antibody (Abcam) was used 1:2,000 dilution. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G anti serum (Abcam) was used 1:4,000 dilution.

Enzymatic activity assay

The enzymatic activity was assayed with the protease coupled method, using α-chymotrypsin (Sigma) and succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Sigma) as substrate. Purified *human* CypA was used as a control. The method is based on the spectrophotometric determination at 395 nm of p-nitroaniline (Sigma), which is released by α-chymotrypsin from the synthetic substrate only when the prolyl peptide bond is in the trans configuration. Assays were performed in 35 mM HEPES buffer, pH 7.8, at 10ºC (Heitman *et al*., 1993).

Results and Discussion

Sequence analysis of *b*Cyp A cDNA

We obtained 40 UPR-related genes through the Gene Ontology Consortium database search (Yun *et al*., 2011). Among the rest, clone TT8-6 was homologous to the Cyclophilin A gene from *Blattella germanica*. The full length cDNA sequence of *b*CypA contained a 5′-teminal untranslated region (UTR) of 91 bp, a 3′ UTR of 359 bp with polyadenylation consensus signal (AATAAA) and poly A tail, and an open reading fream of 498 bp encoding a protein of 166 amino acids with a predicted molecular

Fig. 1. The nucleotide and deduced amino acid sequences of *B. mori* Cyclophilin A cDNA (accession number in GenBank: AY 966877). The length 5'-UTR is 91 bp, *b*Cyp A ORF is 498 bp, 3'-UTR is 359 bp.

Fig. 2. Genomic organization of *b*CypA. It is based on contig 10795 (GenBank accession no.: BABH01010795). The length of intron 1 (I1) is 525 bp, exon 1 (E1) is 69 bp, exon 2 (E2) is 429 bp.

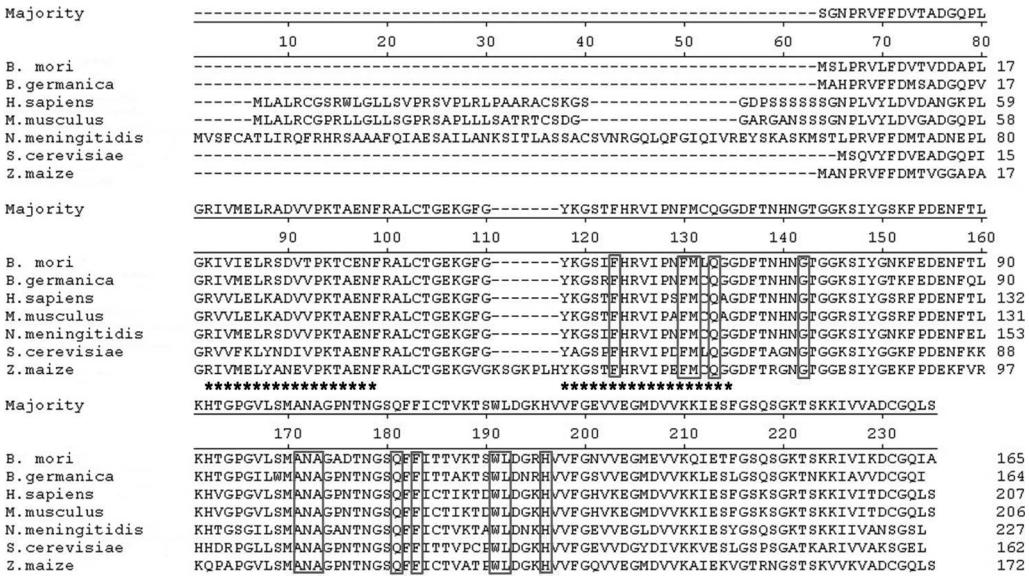

Fig. 3. Multiple alignment analysis of the deduced amino acid sequences of *B.mori* cyclophilin A with six other related cyclophilin A. Identical residues are marked with an asterisk. The 13 residues that constitute the CsA-binding site of human CypA, conserved in all sequences, are marked with the box. '*' indicates Cyp-type peptidyl-prolyl *cis-trans* isomerase signatures. Sequences were obtained from the SwissProt data bank with the following accession numbers: CAA60869, *B. germanica*; P30405, *H. sapiens*; P2507, *N. meningitidis*; Q99KR7, *M. musculus*; P14832*, S. cerevisiae*; P21569, *Zea maize*.

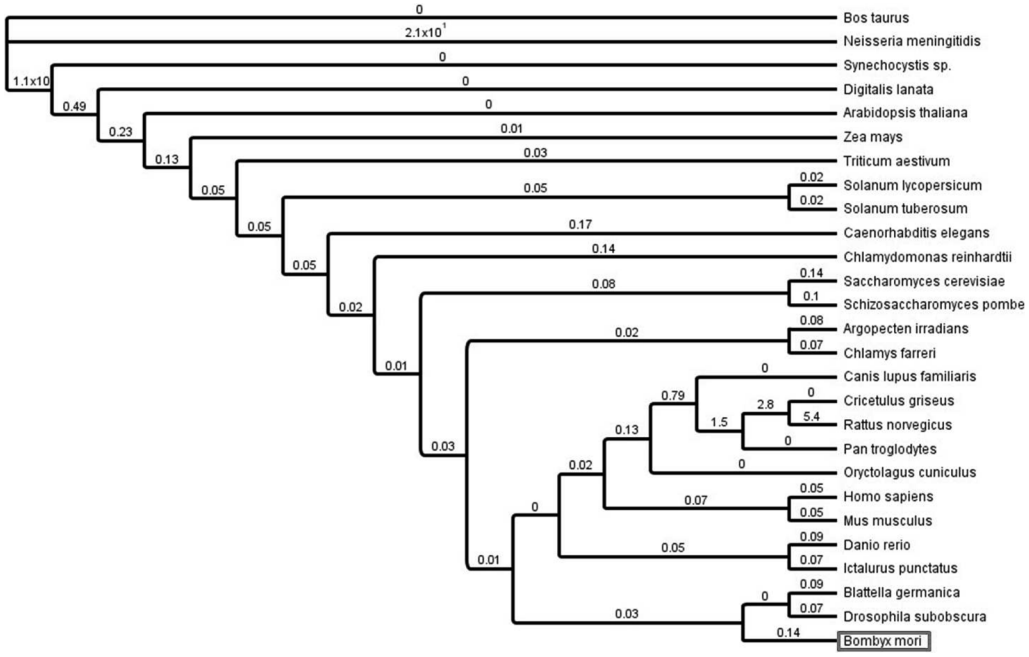

Fig. 4. Phylogenetic tree of amino acid sequence of cyclophilin A from *B. mori* and other organisms. Phylogenetic relationship of CypA constructed by the neighbor-joining method. Bootstrap support values for the NJ tree are shown at the nodes (out of 1,000 replicates). The sequences were obtained from *A. thaliana* (P34790), *A. irradians* (ABM92916), *B. germanica* (CAA60869), *B. mori* (AAX76988), *S. scrofa* (P04374), *C. elegans* (P52009), *C. lupus familiaris* (AAF78600), *C. reinhardtii* (T07950), *C. farreri* (AAR11779), *C. griseus* (P14851), *D. rerio* (AAQ91263), *D. lanata* (T50768), *D. subobscura* (AAB87889), *H. sapiens* (P30405), *I. punctatus* (AAY86951), *M. musculus*(Q99KR7), *N. meningitidis* (AAZ91295), *O. cuniculus* (AAF22215), *P. troglodytes* (ABB77876), *R. norvegicus* (P10111), *S. cerevisiae* (P14832), *S. pombe* (P18253), *S. lycopersicum* (P21568), *S. tuberosum*(T50771), *Synechocystis sp.* (S74880), *T. aestivum* (AAK49426), *Z. mays* (P21569).

weight of 18.2 kDa (Fig. 1). This gene locates in the 9th silkworm chromosome, and contained two exons and one intron. Two exons were found in contig 10795 (Fig. 2). Fig. 3 was shown 14 residues for binding the immunosuppressive agent CsA and two Cyp-type peptidyl-prolyl *cis-trans* isomerase signatures. Also, the deduced amino acid sequence of *b*CypA was 79.2% identical with *B. germanica,* 78.4% with *M. meningitides,* 74.5% with *M. musculus,* 73.9% with *H. sapiens,* 66.6% *H. maize*, but it showed a relatively low identity of 65.4% with *S.cerevisiae*.

Phylogenetic analysis

The evolutionary position of *b*CypA was determined by comparing it with other cyclophilins through bootstrap analysis. Bootstrap analysis was used with 1,000 replicates to test the relative support for the branches produced by the NJ analysis. A phylogenetic tree was constructed based on the amino acid sequence of 27 Cyps by the NJ method (Fig. 4). A phylogenetic tree showed that *b*CypA was closest to CypA from *Blattella germanica* and *Drosophila subobscura.*

Relative quantification real-time PCR

To confirm whether *b*CypA mRNA is ubiquitously expressed, the present of its transcript in several larva tissues was examined by Real-time PCR. Real-time PCR analysis was done using mRNA extracted from *B.mori* tissue of 5th instar 3 days, including the Midgut, Fat body, Malpighian tubule, Skin, Middle silk gland, Posterial silk gland, Ovary and Testis. *B.mori* Actin3 mRNA was used

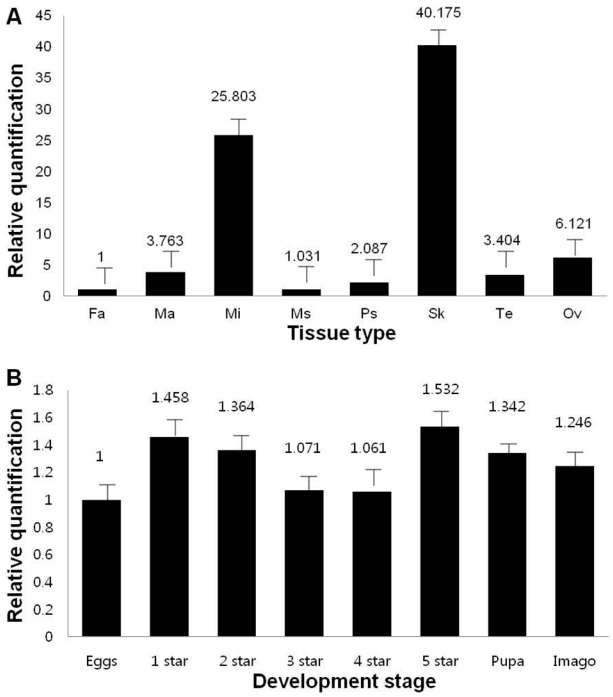

Fig. 5. Transcriptional activity of *b*CypA in tissues and development stages of *B. mori* using Real-Time PCR. Total RNA was isolated from the following organs (Fa, fat body; Ma, Malpighian tubule; Mi, Midgut; Ms, Middle silk gland; Ps, Posterial silk gland; Sk, Skin; Te, Testis; Ov, Ovary) and development stages of *B. mori*. An elevated level of expression occurred in the Skin and Midgut compared to other tissues (A). *b*CypA was found universally distributed in all development stage (B).

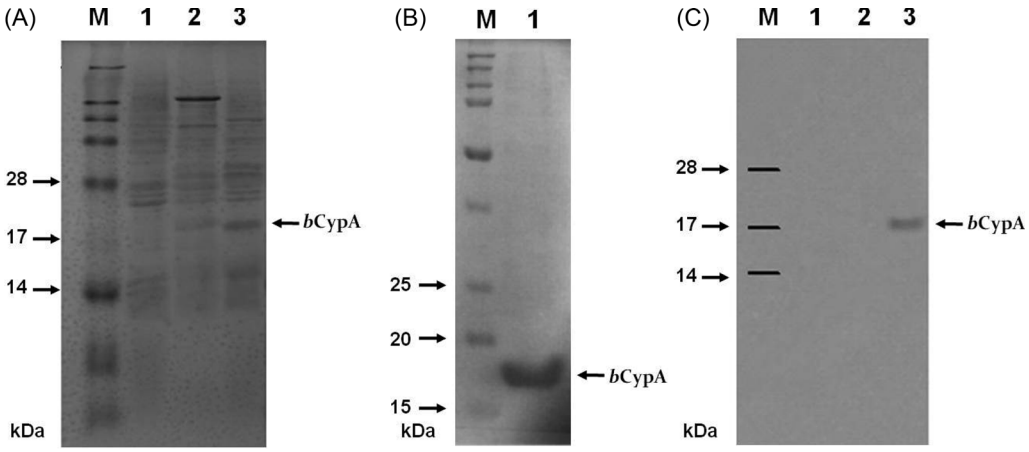

Fig. 6. Expression of *b*CypA recombinant protein. Sf-9 cells (3.0×10^6) were infected with recombinant virus encoding *b*CypA/His6 at a total m.o.i. of 5. Cells were harvested 72 hr after infection. SDS-PAGE analysis of *b*Cyp A recombinant protein (A). Affinity purified *b*CypA recombinant protein using a Magnetic Nickel agarose beads (B). Western blot analysis of *b*Cyp A recombinant protein was performed using a human CypA antibody (C). M, protein size marker; Lane 1(1), protein extracted from normal cells; lane 2(2), protein extracted from cells infected with wild type virus; lane 3(3), protein extracted from Sf9 cells infected with *vAc-b*CypA.

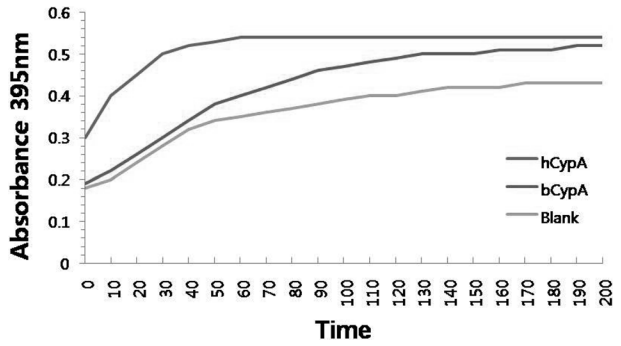

Fig. 7. Cis-trans peptidyl isomerization activity of *b*CypA. Spectrophotometric proline isomerization assay performed as described by Heitman *et al*. (1993) using 300ng six-histidine-tagged *b*CypA and 300ng human CypA. Time course of the cis -> trans isomerizatio of the Ala-Pro peptide bond int the assay peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, as measured by the A_{395} after the coupled hydrolysis by chymotrypsin. The graph is representative of several independent expreiments.

as a control. *b*CypA mRNA transcript was expressed universally distributed in all investigated tissues. Specialy, the expression of the Skin and Midgut was highest (Fig. 5A). Also, to examine the distribution of *b*CypA mRNA expression throughout development, the present of its transcript in development stage was examined by Real-time PCR. Real-time PCR analysis was done using mRNA extracted from eggs (1-day-old), larva (from 1 instar 1-day-old to 5 instar 1-day-old), pupa (5-day-old) and imago (0-day-old female). *b*CypA mRNA transcript was found universally distributed in all development stage (Fig. 5B).

Expression and Characterization of *B.mori* cyclophilin A

*b*CypA fused at the C-terminus with six-histidine was over-expressed in Sf9 cells using BEVS. The protein was affinity-purified with MagExtractor His-taq kit and a molecular mass of about 18.2 kDa, as expected. The recombinant protein gave a strong positive signal when incubated with an anti-human CypA monoclonal antibody (Fig. 6). The isomerase activity assay was followed using the standard spectrophotometric assay based upon the chymotrypsin mediated cleavage of the trans isomer of the peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide. *b*CypA is able to catalyze the cis to trans isomerization of the Ala-Pro bond as shown in Fig. 7.

Cyclophilins are first identified as the binding proteins for cyclosporine A and have been found in a wide range of species from prokaryotes and eukaryotes. Their rotamase activity was shown to be essential in the quality control of newly synthesised proteins as well as in the refolding and repair of partially denatured proteins inside the cells. The *b*CypA was isolated from Bombyx mori and had peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. Also, *b*CypA mRNA transcript was found universally distributed in all development stage and in all investigated tissues. Accordingly, we suggest that *b*CypA is a family of cyclophilin A and will be useful for mass productions of bioactivity recombinant proteins.

Acknowledgement

This study was supported by year Post-doctor Fellowship Program (Project No.PJ006650012011) of national Academy of Agricultural Science, Rural Development Administration, Republic of Korea.

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