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# Isolation and Characterization of a Silkworm cDNA Encoding a Protein Homologous to the 14kDa Protein of Bovine Ubiquinol-cytochrome C Reductase

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**Abstract**

In this study, we characterized the small subunit of ubiquinol-cytochrome C reductase (*Bmuccr*) of the silkworm *Bombyx mori*, a model insect of Lepidopteron species. The *Bmuccr* gene covers a 1.4 kb genome region and contains 3 exons. The ORF contained 354bp and encoded 117 amino acid residues, which shares 69% overall amino acid sequence identities with the subunit VII of ubiquinol-cytochrome C reductase from bovine. Phylogenetic tree showed *Bmuccr* had high homology with *T. castaneum* homologous. The multiple sequence alignment of 16 subunit VII homologues shows that *Bmuccr* is very hydrophilic, has a characteristic charge distribution, and has a high helical content. Expression analysis indicated that *Bmuccr* was highly expressed in larva stage and was down-regulated in embryos stage and adult stage of silkworm. The tissue-specific expression indicated *Bmuccr* had high-expression level in tissues that consume oxygen. The analysis of domain structure of this protein suggested that it might be involved in correct assembly of the cytochrome bcl complex. Definition of the homologous of bovine subunit VII of ubiquinol-cytochrome C reductase should facilitate further analysis of structure/function relationships of silkworm cytochrome bcl complex.

**Keywords:** *Bombyx mori*, Ubiquinol-cytochrome c reductase, Multiple sequence alignment, Subunit VII, Mitochondria

## 1. Introduction

The cytochrome bcl complex (commonly known as ubiquinol-cytochrome c reductase or Complex III) is a segment of the mitochondrial respiratory chain that catalyzes reduction of Cyt c by the oxidation of ubiquinol (Hatefi et al., 1967, p. 235; Bechmann et al., 1992, pp.199). Coupled to this reaction is the translocation of protons across the inner membrane of mitochondria in eukaryotic organisms and across the cytoplasmic membrane in many bacteria. The bc1 complex of these prokaryotes consists of 3 or 4 subunits (Trumpower, 1990, p.101), while those from eukaryotes may consist of up to 11 subunits (Schägger, et al., 1986, p.224). Two large "core" proteins, three respiratory proteins that directly participate in electron transport (Cyt b, Cyt cl, and the "Rieske" iron sulfur protein), and four to six small proteins with molecular masses of less than 20 kDa.

Since bacterial bc1complexes that contain only the respiratory subunits have the same activity as the eukaryotic enzymes (Trumpower, 1990, p.101), the role of the small subunits is not quite understood. In the 11 different

polypeptide subunits of bovine ubiquinol-cytochrome C reductase complex, 7.2kDa protein (subunit X) maintains contact with cytochrome c1 and iron sulfur protein (Schagger et al., 1983, p. 307). Both subunits VII and VIII (14 kDa and 11 kDa, respectively) of *S. cerevisiae* are thought to be in close association with cyt b (Berden et al., 1988. P.195). Disruption of these subunits showed that both are essential for correct assembly of the complex (Braun et al., 1995. p.1217; Boumans et al., 1995. p.105). So these small subunits may play an essential role in the proper assembly of the bc1 complex.

Here, we report the isolation, tissue expression, and *in vitro* expression of a novel silkworm cDNA which encodes 117 amino acids that share 69% sequence identity with subunit VII of the bovine bc1 complex.

## 2. Materials and methods

### *2.1 Silkworm strain*

The silkworm *B. mori* (Strain DaZao P50) were used. All larva were fed with mulberry leaves three times a day at 25 ± 2 °C under a 12 h light/12 h dark cycle.

### *2.2 Isolation and DNA sequencing of a cDNA clone*

We have been determinate the nucleotide sequences of cDNA clones randomly selected from a silkworm cDNA library. By comparing the DNA sequences of these cDNA clones with known DNA sequences in the database, we identified a clone that encoded a protein highly homologous to the bovine ubiquinol-cytochrome C reductase, 14kDa protein (GenBank accession: NP_001029969).

Midgut tissues were collected from the larva of 3rd day old 5th instar. Total RNA was isolated with the Trizol method. The synthesis of first-strand cDNA was catalyzed by MMLV. The specific primers: ‘5 ATGGCTTTTAGAGCAACTGC 3’ and ‘5 ATATTCCTTCTCCCACTGCTC 3’, were designed for the *Bmuccr* based on the predicted coding sequences and were used for the amplification of total length cDNAs. The amplicons were then cloned into the vector pMD18-T (Takara) for sequencing.

### *2.3 Bioinformatics*

The *Bmuccr* cDNA sequence was compared with the silkworm genomic sequences using SIM4 (http://pbil.univ-lyon1.fr/sim4.php). Translation into amino acid sequence was done with SwissProt database ExPASy Translate tool (http://au.expasy.org/tools/dna.html). Homology searches were performed using Clustal w Network Service (www.ebi.ac.uk/clustalw). The secondary structure prediction was carried out using the HNN secondary structure prediction method in PBIL (http://pbil.univ-lyon1.fr/). We submitted the *Bmuccr* sequence to the Swiss-Model homology modeling server (http://swissmodel.expasy.org/SWISS-MODEL.html) to predict its three-dimensional model using the automatic modeling mode. The sequencing results were processed using Molecular Evolutionary Genetic Analysis (MEGA) version 3.1. The neighbor joining (NJ) method was used to construct phylogenic trees.

### *2.4 Expression analysis of target gene*

Total RNA were isolated from embryos 3 day before hatching, larva of 1st instar, 3rd instar, 5th instar, 5 day old pupae, and 1 day old adults using the Trizol reagent kit (invitrogen,USA). Total RNA samples from the following tissues were extracted and used to synthesize first-strand cDNA: midgut, haemolymph, fat body, silk gland, ganglion, epidermis, testis, ovary, tuba Malpighii. First-strand cDNA was synthesized using oligod(T)18, followed by PCR using the specific primers. *BmActin A3* was amplifying as housekeeping gene.

### *2.5 In Vitro Expression and Western blot*

The *Bmuccr* cDNAs without any mismatch nucleotides were cloned into the expression vector PET30a (Novagen) and were transformed into the *E. coli* expression strain BL21 (DE3). The induced *E. coli* cells were collected and lysed by lysozyme on ice. The fusion proteins present in the supernatant were subjected to electrophoresis on 12% SDS-polyacrylamide gels. Then western blot was operated. Blotted proteins were incubated with corresponding 6×HIS tag antibodies at room temperature for 2 h, followed by washing with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with horseradish peroxidase conjugated secondary antibody (IgG-HRP), the nitrocellulose membrane was rinsed extensively with TBST. Immunodetection was carried out using a substrate of DAB.

## 3. Results

### *3.1 Isolation and sequencing of cDNA, chromosomal localization, and genomic structure*

The nucleotide and deduced amino acid sequences of the novel silkworm gene, termed Bombyx mori ubiquinol-cytochrome C reductase 14kDa subunit (*Bmuccr*), are shown (Figure 1). The cDNA sequence consists of 553 nucleotides with an open reading frame of 354 nucleotides encoding a 117-amino-acid peptide of approximately 14kDa (GenBank accession No. NP_001038957). An in-frame termination codon (TGA) is located 12 nucleotides upstream of the first methionine (ATG), and the polyadenylation signal, AATAAA, begins 16 bases upstream of the polyadenylation site.

A homology search, using the BLASTN programs, revealed that the nucleotide sequences of the cDNA were identical to parts of the genomics DNA sequence Dazao Contig002343 (AADK01002343), which had been assigned to silkworm chromosome 5. A comparison of cDNA and genomic DNA sequences defined the genomic structure, which appears to span a genomics region of about 1.4kb and consists of three exons.

*3.2 Protein sequence alignments and homology modeling*

A FASTA search for homologies between the predicted amino acid sequence and archived proteins revealed 50~70% identity with several other the equivalent proteins from other species, the closest of them being the 14kDa protein of *T. castaneum* (XP_975188) (Figure 2A). The crystal structure of subunit VII of bovine cytochrome bcl complex is available in PDB (PDB ID: 1bccF) and it was used as the template to build the model of *Bmuccr* protein (Figure 2B). The apparent structural similarity indicated two proteins also share functional similarities.

*3.3 Phylogenetic analyses*

The multi-sequence alignment was performed using Clustal W to identify *Bmuccr* protein sequence distances between 16 species. The phylogenetic tree showed that the *B.mori* branched with *T. castaneum* at a level of 71% identity and with 57% bootstrap support, which indicated they were homology protein and belonged to a protein family. The similarity of *Bmuccr* with *D. melanogaster*'s and *A. aegypti*'s subunit VII of cytochrome bcl complex reached 57% and 61%, respectively, but low genetic relationship to *S. pombe*. *D. melanogaster* was closely related to *A. aegypti*, *H. sapiens* showed high genetic relationship to *B. Taurus* (Figure. 3). The results indicated that *Bmuccr* of *B. mori* was close in genetic relationship to Coleoptera species.

*3.4 Bmuccr expression pattern*

The tissue distribution of the *Bmuccr* mRNA was assessed by RT-PCR using the primer specific. As illustrated (Figure 4A), the mRNA were detected in all silkworm tissues. There was no sex difference in expression levels of the mRNA (data not shown). But expression was significantly abundant in tuba Malpighii, intestinal, epidermis, silk gland, testis and ovary, tissues that consume oxygen at high level.

The expression profile of *Bmuccr* throughout development was further analyzed from embryos to adult by RT-PCR (Figure 4B). The results showed that expression of *Bmucc*r was much lower in embryos stage. *Bmuccr* expression increased slowly in larva stage, and peaked at the 3rd stage larvae. Subsequently, *Bmuccr* transcription decreased to lower levels in pupa stage and adult stage. These observations strongly suggest that *Bmuccr* plays a specific role during larval growth and development.

*3.5 Prokaryotic Expression, SDS-PAGE, and Western blotting of the Bmuccr proteins*

The Pet30a expression vector was used to transform to competent *E. coli* BL21. The results of SDS-PAGE and Western blotting analysis of the *Bmuccr* protein is shown in Fig. 5. The expressed fusion protein Bmuccr-HIS had a molecular weight of 20.6 kDa and it was soluble in the supernatant, as detected by SDS-PAGE. Western blotting confirmed that its molecular weight was about 20.6 kDa (Figure 5)

**4. Discusion**

In this study, we have isolated a gene from *B. mori* and identified it as the *Bmuccr* gene encoding subunit VII of the bc1 complex based on sequence identity with the bovine subunit VII. It seems to be essential for correct assembly of the complex.

Because the function of the 14-kD subunit in electron transport and/or proton translocation is rather unclear, it is difficult to discuss domain-like structures of the protein. However, Molecular features of the 14-kD subunit of Cyt c reductase were very characteristic. The conserved amino acids, indicated by a black box in Fig.2, are not distributed over the entire protein, are predominantly found in the central domain and C-terminus. The N-terminus is highly variable, both in amino acid composition and in length. But The Nterminal part (corresponding to amino acids 1-36 from) has a surplus of positive charges (human and bovine 6+/1—; fruit fly 5+/0—). Most of these proteins are rather small. Import of proteins with cleavable presequences was shown to depend on the positive charge of the N-terminal extensions and on the membrane potential across the inner mitochondrial membrane with the negative side facing the matrix (Schleyer et al., 1982. p.109). This postulated N-terminal part (amino acids 1-36) of the 14-kD protein from silkworm has typical features of a mitochondrial presequence: it comprises 6 positively charged amino acids, only 1 negative residue, and 4 Ser residues. Alternatively, one of the internal amphiphilic helices may be involved in import of the 14-kD subunit into mitochondria.

The C-terminal half of this 14-kD protein contains a large number of both acidic and basic residues, and comprises five conserved Arg residues (at positions 54, 64, 67, 70, and 110 in the protein from silkworm). This 14-kD protein is predicted to have a high helical content, a feature which is confirmed by the sequence alignment. A previous study on this C-terminus in *S. cerevisiae* showed the importance of this part of the protein ((hydrophilicity and charges) for correct assembly of the bc1 complex, but not for electron transport (Hemrika et al., 1994. p. 569).

Of all cell organelles, the mitochondria play a primary role in the pathogenesis of peroxidative damage, because mitochondria consume about 90% of inhaled oxygen, and reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical are formed in the mitochondria as physiologic metabolites of the respiratory chain (Richter et al., 1995. p. 67). If malfunction, generation of reactive oxygen species results in disturb homeostasis and cellular death (Marcelo et al., 2002. p.537). Silkworm is model insect of Lepidoptera species. More detailed analysis of other subunits of cytochrome bcl complex from silkworm is needed for production of silk fibers and control of pest.

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Figure 1. The complete nucleotide and deduced amino acid sequence of *Bombyx mori* ubiquinol-cytochrome C reductase, 14kDa subunit. The start, stop codons and polyadenylation signal of *Bmuccr* are indicated by bold boxes. The primers used for the amplification of total length cDNAs and RT-PCR are underlined

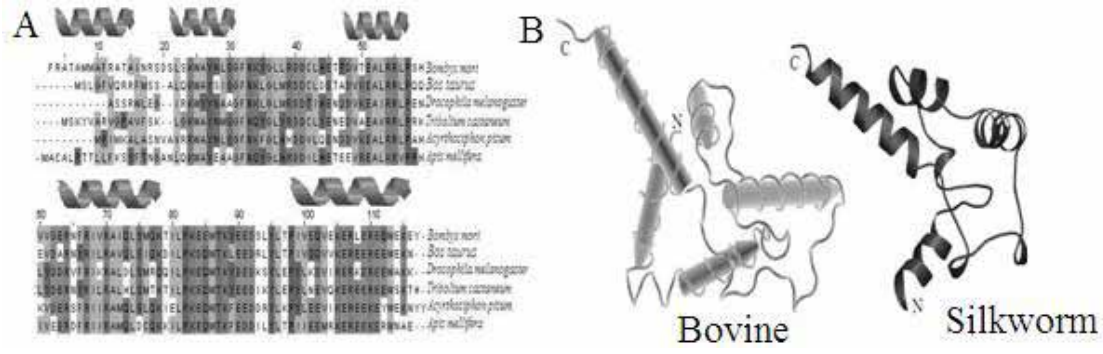


Figure 2. (A) Alignment of sequences of several ubiquinol-cytochrome C reductase 14kDa subunits. Residues that are identical in all six proteins are shaded. Five corresponding helices are indicated. (B) 3D model of ubiquinol-cytochrome C reductase 14kDa subunit VII. Bovine subunit VII protein and silkworm *Bmuccr* protein share the same fold. N and C denote the N-terminus and C-terminus, respectively.

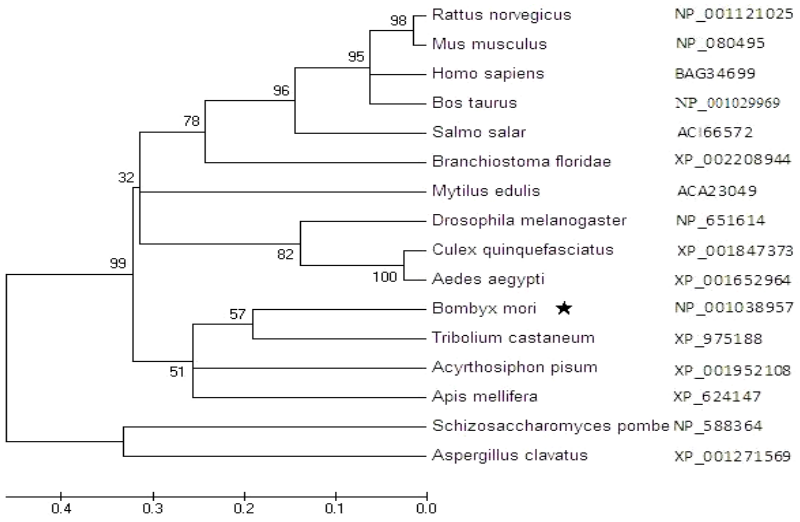


Figure 3. Phylgenetic tree showing the relationship of *Bmuccr* to other species homologous protein. The phylogenetic tree was generated based on the entire amino acid sequences and the tree-drawing software mega 4.0.

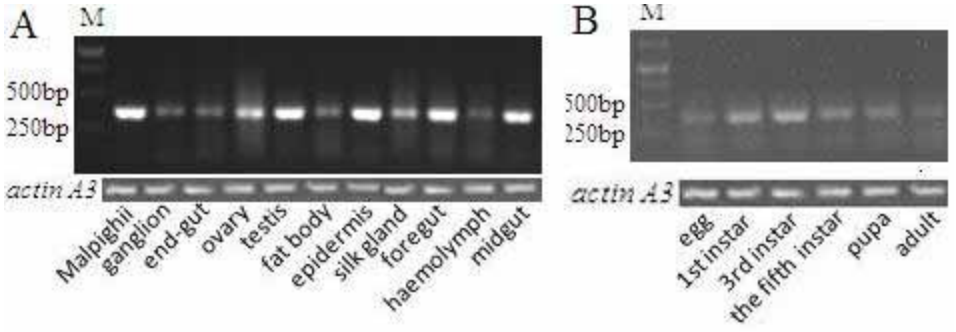


Figure 4. (A) Distribution of *Bmuccr* in different tissues of the strain Dazao. (B) RT-PCR analysis for expression of mRNAs for *Bmuccr* and *actin A3* in different development stage, whose cDNA sizes amplified are 354 and 289 base pairs.

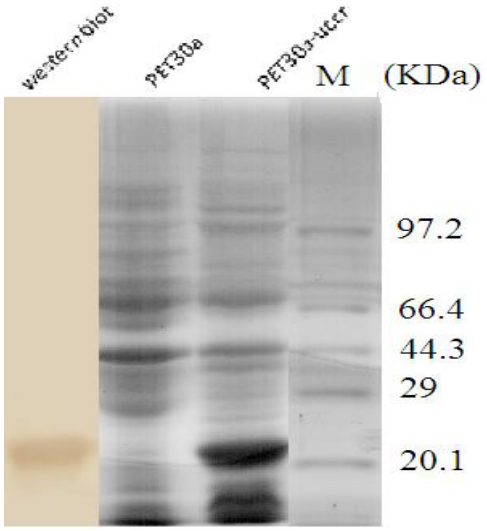


Figure 5. Expression and western blotting analysis of the pET 30a-*Bmuccr* protein.