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Full Length Research Paper

cDNA, genomic sequence cloning and overexpression of cytochrome c oxidase gene (*COX6b1*) from the *Ailuropoda melanoleuca*

Jun Li[1,2], Yiling Hou[1,2], Wanru Hou[1,2]*, Bing Sun[1,2] and Xiulan Su[1,2]

[1]College of Life Science, China West Normal University, 637009, Nanchong, Sichuan, People's Republic of China.
[2]Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), People's Republic of China.

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Cytochrome c oxidase (COX) is a component of the mitochondria respiratory chain. COX6b1 is one of the COX small subunits encoded by nuclear genes. In currently study, the cDNA and the genomic sequence of *COX6b1* were successfully cloned from the *Ailuropoda melanoleuca* with the RT-PCR technology and Touchdown-PCR, respectively. The length of cDNA fragment cloned is 341 bp in size, containing an open reading frame (ORF) of 261 bp encoding 86 amino acids. The length of the genomic sequence is 4657 bp, which was found to possess 3 exons and 2 introns. Alignment analysis indicated that the nucleotide sequence and the deduced amino acid sequence are highly conserved with other six species studied. Topology prediction showed that there are one N-glycosylation site, one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites, two N-myristoylation sites and one microbodies C-terminal targeting signal site in the COX6b1 protein of the *A. melanoleuca*. The *COX6b1* gene was also overexpressed in *Escherichia coli* and the result indicated that *COX6b1* fusion with the HIS-tagged gave rise to the accumulation of an expected 13.46 kDa polypeptide. The expression product obtained could be used to purify the protein and study its function further.

Key words: Cytochrome c oxidase subunit Vib polypeptide 1 (*COX6b1*), clone, overexpression, *Ailuropoda melanoleuca* (Giant Panda), sequences analysis.

INTRODUCTION

Cytochrome c oxidase (COX; EC 1.9.3.1), the terminal enzyme complex of the mitochondria respiratory chain, is a multisubunit enzyme located in the mitochondrial inner membrane. It oxidizes cytochrome c and transfers electrons to molecular oxygen. Meanwhile, COX is an important energy-generating enzyme critical for the proper functioning of most cells, especially those of highly oxidative organs, such as the brain (Capaldia, 1990). In mammals, it is comprised of 13 protein subunits of which three large subunits (subunit I to III) are encoded by the mitochondrial genome (mtDNA), constituting the catalytic core of the enzyme, and the remaining 10 subunits (IV, Va, b, VIa, b, c, VIIa, b, c and VIII) are nuclear genes (nDNA) products, which are synthesized on cytoplasmic ribosomes and are imported into the mitochondria by leading sequences (Cao et al., 1988; Barrientos et al., 2002).

Study had confirmed that all 13 subunits are essential for a functional holoenzyme (Liang et al., 2006). The mtDNA-encoded subunits provide the catalytic core of cytochrome-c oxidase, and the nuclear-encoded subunits may be involved in the regulation and assembly of the complex (Yanamura et al., 1988). As for *COX6b1* gene, Massa et al. (2008) found that it is associated with severe infantile encephalomyopathy. Cui et al. (2010) also pointed out that the loss of *COX6b1* genes leads to mitochondria dysfunction that could cause apoptosis of the blastocyst-stage embryos.

Ailuropoda melanoleuca, a rare and endangered species currently found only in China, has a very high

*Corresponding author. E-mail: hwr168@yahoo.com.cn. Tel/Fax: +86-0817-2568653.

ecological, scientific, economic, cultural and aesthetic value. At present, the *COX* genes have been extensively characterized in mammals (Bachman, 1995; Sacconi et al., 2005; Wu et al., 2007; Du et al., 2007), whereas there is a handful of report about the *COX6b1* genes of *A. melanoleuca*.

In this study, RT-PCR technique was used for amplifying the cDNA of *COX6b1* gene from the total RNA. Touchdown-PCR technique was employed to amplify the genomic sequence of the *COX6b1* from extraction DNA with the skeleton muscle of the *A. melanoleuca*. The characteristics of this gene was identified by homologous analysis according to the related sequences in GenBank, and the overexpression of the gene was conducted in *Escherichia coli* using pET28a plasmids. The research can provide a scientific data to enrich the species database.

MATERIALS AND METHODS

Skeletal muscle was collected from a dead *A. melanoleuca* at the Wolong Conservation Center of the *A. melanoleuca*, Sichuan, China. The collected skeletal muscle was frozen in liquid nitrogen for DNA and RNA isolation.

DNA and RNA extraction

Approximately 500 mg muscle tissue of *A. melanoleuca* was ground to fine powder in liquid nitrogen, and the powder was then immediately transferred into a 2 ml centrifuge tube and carefully added to 1 ml cold lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA and 0.5% SDS). The mixture was incubated at 65℃ at least 1 h with gently shaking occasionally. After incubation, the mixture was centrifuged with 10000 rpm for 10 min at 4℃. Subsequently, the supernatant was carefully transferred to a new 2 ml centrifuge tube and an equal volume of chloroform : isoamylalcohol (24:1) was added and mixed gently by inversion for 1 min, followed by keeping for 10 min at room temperature and centrifugation at 10000 rpm for 10 min at 4℃. The aqueous phase was carefully transferred to a fresh tube, and two third volumes of ice-cold isopropanol were added. Finally, the DNA obtained was then dissolved in TE buffer and kept at -20℃.

Total RNAs were isolated from the muscle tissue of *A. melanoleuca* using the Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to the manufacturer's instructions. RNA sample was dissolved in RNase-free water, and kept at -70℃.

DNA and RNA sample quality was determined by electrophoresis on 1.0% agarose gel and quantification was performed spectrophotometrically.

Primers design, RT-PCR and cloning of cDNA sequence

The PCR primers were designed by Primer Premier 5.0, according to the mRNA sequence of *COX6b1* from *Homo sapiens* (NM_001863), *Bos taurus* (NM_176675), *Pongo abelii* (NM_001131741), *Sus scrof*a (NM-001097497), *Mus musculus* (NM_025628) and *Rattus norvegicus* (NM_001145273). The specific primers of cDNA sequence are as follows: *COX6b1*-F: CTTTGCTGAGGGTCACATTG; *COX6b1*-R: GAGAGGTG [G/A] GTGGAGCCAG.

Total RNAs were utilized to synthesis the first-stranded cDNAs using a reverse transcription kit with Oligo dT as the primers according to the manufacturer's instructions (Promega). The 20 µl of first-strand cDNA synthesis reaction system included 1 µg of total RNAs, 5mM of $MgCl_2$, 1mM of dNTPs, 0.5µg of Oligo dT_{15}, 10U/µL of RNase inhibitor, and 15 U of AMV reverse transcriptase, and was incubated at 42℃ for 60 min.

The first-strand cDNA synthesized was used as a template for cds sequence amplification. The total reaction volume for DNA amplification was 25 µl. The reaction mixtures contained 1.5 mM of $MgCl_2$, 200 µM of each of dATP, dGTP, dCTP and dTTP (Omega), 0.3 µM of each primer, 5.0 units of Taq plus DNA polymerase (Sangon Co, Shanghai, China). DNA amplification was performed using a MJ Research thermocycler, Model PTC-200 (Watertown, MA) with a program of 4 min at 94.0℃, followed by 30 cycles at 94.0℃ for 30 s, 45℃ for 30 s, and 72.0℃ for 1 min, then ended with the final extension for 10 min at 72.0℃. After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1× TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide and visualized under UV light. The expected fragments of PCR products were harvested and purified from gel using a DNA estraction kit (Omega, China), and then ligated into a pET28a vector at 16℃ for 12 h. The recombinant molecules were transformed into *E. coli* complete cells (JM109), and then spread on the LB-plate containing 50 µg/ml ampicillin, 200 mg/ml IPTG (isopropyl-beta- D-thiogalactopyranoside) and 20 mg/ml X-gal. Plasmid DNA was isolated and digested by *Pst*I and *Sca*I to verify the insert size. Plasmid DNA was sequenced by Huada Zhongsheng Scientific Corporation (Beijing, China).

Cloning the genomic sequence of *COX6b1*

The PCR primers were designed based on the cDNA sequence of the *COX6b1* from the *A. melanoleuca* obtained earlier and complete genomic sequences of the *COX6b1* from four mammals studied including *H. sapiens*, *M. musculus*, *R. norvegicus* and *B. taurus*. The specific primers of genomic sequence are as follows: *COX6b1*-F: AGAGTCAGCACCATGGCAG; *COX6b1*-R: GAGAGGTGGGTGGAGCCAG.

The genomic sequence of the *COX6b1* gene was amplified using Touchdown-PCR with the following conditions: 94℃ for 30 s, 62℃ for 45 s, 72℃ for 2 min in the first cycle and the annealing temperature deceased 1℃ per cycle; after 10 cycles conditions changed to 94℃ for 30 s, 52℃ for 45 s, 72℃ for 2 min for another 25 cycles. The fragment amplified was also purified, ligated into the clone vector and tansformed into the *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Sangon (Shanghai, China).

Construction of the expression vector and overexpression of recombinant *COX6b1*

PCR fragment corresponding to the COX6b1 polypeptide was amplified from the *COX6b1* cDNA clone with the forward primer, 5'-CAGAATTCATGGCAGAAGACA (*EcoR I*)-3' and reverse primer, 5'-CGAAGCTTCAGATCTTCCCA (*Hind III*)-3', respectively. The PCR was performed at 94℃ for 3 min; 35 cycles of 30 s at 94℃, 45 s at 55℃ and 1 min at 72℃; 8 min at 72℃. The amplified PCR product was cut and ligated into corresponding site of pET28a vector (Stratagen). The resulting construct was transformed into *E. coli* BL21 (DE3) strain (Novagen) and used for the induction by adding IPTG at an OD600 of 0.6 and culturing further for 4 h at 37℃, using the empty vector transformed BL21 (DE3) as a control. The recombinant protein samples were induced after 0, 0.5, 1.5, 2, 2.5, 3, 3.5 and 4 h and then separated by SDS-PAGE and stained with Commassie blue R250.

Data analysis

The sequence data were analyzed by GenScan (http://genes.mit. edu/GENSCAN.html). Homology research of the A. melanoleuca COX6b1 gene as compared with the gene sequences of other species was performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). Open reading frame (ORF) of the DNA sequence was searched by ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein structure of the COX6b1 sequence cloned was deduced using predict protein software (http:// cubic. Bioc. columbia.edu/predictprotein/). Multiple sequence alignment was performed by DNAMAN 6.0. The prediction of protein functional sites and biochemical characteristics depend on the software ExPASy Proteomics Server. Protein tertiary structure was simulated by SWISS MODEL (http://swissmodel.expasy.org/). The construction of evolutionary tree relies on MEGA4.0RESULTS

RESULTS

Analysis of the cDNA of *COX6b1* from the *A. melanoleuca*

The electrophoresis showed that the amplified cDNA fragment was about 500 bp in size (Figure not shown). The sequencing result indicated that the length of the cDNA cloned is 436 bp, corresponding to the eletrophoretic result. Blast research analysis revealed that the *COX6b1* cDNA sequence cloned share high similarities with other mammals reported, including *H. sapiens*, *B. taurus*, *S. scrofa*, *P. abelii*, *M. musculus* and *R. norvegicus,* especially the similarity is up to 95.79% with *B. Taurus* (Taber 1). On the basis of the high identity, we concluded that we had cloned the cDNA encoding the *COX6b1* protein of *A. melanoleuca*. The *COX6b1* cDNA sequence has been submitted to Genbank (accession number: HQ326486), which contained a 261-bp-length ORF encoding 86 amino acids (Figure 1).

Analysis of the genomic sequence of *COX6b1*

The electrophoresis showed that about 5000-bp-length DNA fragment was amplified with primers *COX6b1* -F and *COX6b1* –R (Figure not shown). The sequencing indicated that the length of the DNA fragment cloned is 4657 bp. The genomic sequence of the *COX6b1* has been submitted to Genbank (accession number: HQ326485). The comparison of nucleotides sequence between the genomic and cDNA sequences indicated that genomic sequence cloned contains three exons and two introns with software Lasergene. The distribution of three exons is as follows: 13 to 118 bp; 1487 to 1587 bp; 4584 to 4637 bp (Table 2).

Comparison of the genomic sequences with some vertebrates (including *H. sapiens*, *B. taurus*, *M. mulatta, P. abelii, M. musculus* and *X. tropicalis*) was performed by DNAMAN 6.0. The result demonstrated that their sequences have high similarity. Nevertheless, the number and length of exons are different among the species when they are compared (Table 2).

Prediction and analysis of protein functional sites in *COX6b1* protein of the *A. melanoleuca*

The results indicated that the *COX6b1* protein sequence of *A. melanoleuca* share over 85% similarities with six species when they are compared, especially high similarities with *B. Taurus* (96.51%) and *S. scrofa* (97.67%). Primary structure analysis revealed that the molecular weight of the putative COX6b1 protein is 10.16 kDa with a theoretical pI of 8.83. Topology prediction showed that there are one N-glycosylation site, one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites, two N-myristoylation sites and one microbodies C-terminal targeting signalsite in *COX6b1* protein of *A. melanoleuca* (Figure 2). There was complete consistent in the comparison of functional sites, except that *B. taurus* has one more protein kinase C phosphorylation site than other species related.

Overexpression of the *COX6b1* gene in *E. coli*

The *COX6b1* gene was overexpressed in *E. coli*, using pET28a plasmids carrying strong promoter and terminator sequences derived from phage T7. Therefore, the *COX6b1* gene was amplified individually by PCR and cloned in a pET28a plasmid, resulting in a gene fusion coding for a protein bearing a HIS-tag extension at the N terminus. The product of expression was tested by SDS-PAGE analysis of protein extracted from recombinant *E. coli* BL21 strains (Figure 4). The results indicated that the protein *COX6b1* fusion with the N-terminally HIs-tagged gave rise to the accumulation of an expected 13.46 kDa polypeptide that formed inclusion bodies. Apparently, the recombinant protein was expressed after an hour of induction, and it reached the highest level at 3 h after induction. These results suggested that the protein is active and it is just the protein encoded by the *COX6b1* from the *A. melanoleuca*. The expression product obtained could be used to purify the protein and study its function further.

DISCUSSION

COX is a key enzyme in the process of oxidative phosphorylation of mitochondria respiratory chain, and its damage can directly affect mitochondria function. Studies has also shown that mutation in a nulear-encoded COX subunit, *COX6b1*, will cause drial encephalomyopathy due to complex IV deficiency (Massa et al., 2008). In this study, we cloned successfully, *COX6b1* gene from the *A. melanoleuca,* which share high identity on the nucleotide sequence and the deduced amino acid sequence with *H.*

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1   ctttgctgaccctcacattgagctgcgagttgctgctgtggtgtgtttagagtca
56  gcacc   atg gca gaa gac atc aag acc aaa atc aag aac tac
1           M   A   E   D   I   K   T   K   I   K   N   Y
97  cag acc gcc cct ttt gac agc cgc ttc ccc aac cag aac cag
13   Q   T   A   P   F   D   S   R   F   P   N   Q   N   Q
139 act agg aac tgc tgg cag aac tac ctg gac ttc cac cgc tgt
27   T   R   N   C   W   Q   N   Y   L   D   F   H   R   C
181 gag aag gca atg act gct aaa ggg ggt gat gtc tcc gtg tgt
41   E   K   A   M   T   A   K   G   G   D   V   S   V   C
223 gaa tgg tac cgg cgt gtg tac aag tcc ctc tgc ccc ata tcc
55   E   W   Y   R   R   V   Y   K   S   L   C   P   I   S
265 tgg gtg tca gcc tgg gac gac cgc cgg gca gaa ggc aca ttt
69   W   V   S   A   W   D   D   R   R   A   E   G   T   F
307 cct ggg aag atc tga actggctccacccacctctc
83   P   G   K   I   *
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Figure 1. Nucleotide sequence and putative amino acid sequence of *COX6b1* cDNA from the *A. melanoleuca*. The asterisk (*) represents termination codon.

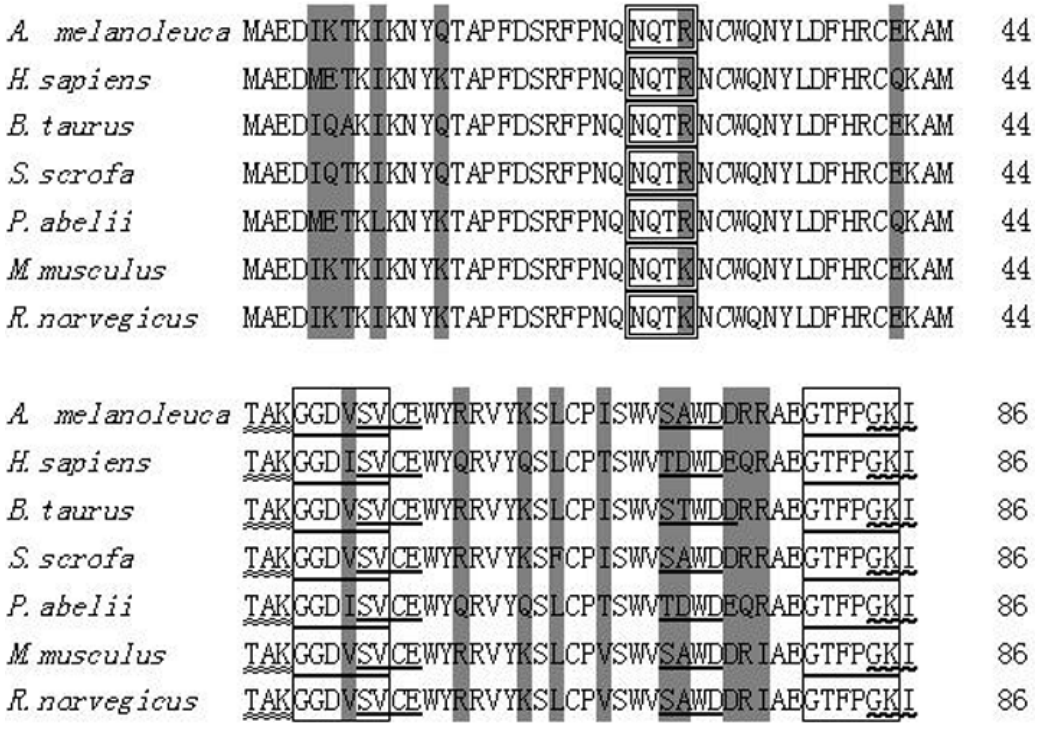

Figure 2. Comparison of functional sites based on the *COX6b1* amino acid sequences among the different species. ▣: N-glycosylation site; ⎵: protein kinase C phosphorylation site; ▁: casein kinase II phosphorylation site; □ : N-myristoylation site; ⎵: microbodies C-terminal targeting signal; ▮ polymorphic site.

Table 1. The comparison of gene sequence and encoding sequence with *Cox6b1* among the *A. melanoleuca*, human and other four species (%).

Parameter	*H. sapiens*	*B. taurus*	*P. abelii*	*S. scrofa*	*M. musculus*	*R. norvegicu*
cds similarity (100%)	90.42	95.79	89.27	95.02	91.57	91.19
aa similarity (100%)	86.05	96.51	84.88	97.67	95.35	95.35
Molecular weight (kD)	10.19	10.16	10.19	10.19	10.07	10.07
Isoelectric point (PI)	6.85	8.51	6.85	8.51	8.81	8.81

Table 2. The length comparision of the *Cox6b1* genomic sequence between A. *melanoleuca* and other six.

Species	Size (bp)	Number of exons	Join site in the CDS	Accession number
A. melanoleuca	4657	3	13-118; 1487-1587; 4584-4637	HQ326485
H. sapiens	10562	3	3022..3127, 6349..6449, 10372..10425	NG_012193
B. taurus	8879	2	1644..1749, 8690..8744	NC_007316
M. mulatta	11268	3	2866..2974, 7119..7219, 11081..11134	NC_007876
P. abelii	9889	2	5274..5375,9697..9750	NC_012610
M. musculus	9178	3	1547..1652, 2637..2737, 8990..9043	NC_000073
X. tropicalis	10357	2	8856..>8957, 10160..10213	NW_003164399

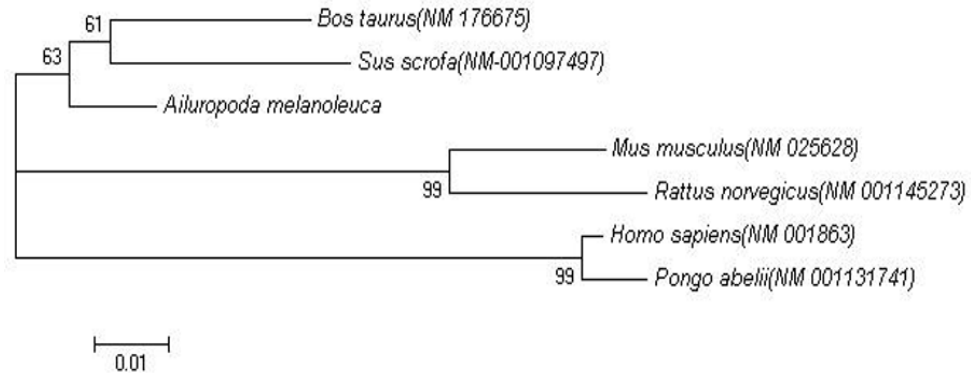

Figure 3. ME evolution tree constructed based on cds sequence of variety species.

sapiens, *B. taurus*, *P. abelii*, *S. scrofa*, *R. norvegicus* and *M. musculus* as well as on the deduced amino acid sequence. The pI and molecular weight with *COX*6b1 of *A. melanoleuca* are close to the compared six species (Table 1). These results suggested that the gene we cloned is *COX6b1* gene of *A. melanoleuca*.

Schmidt et al. (2002) examined the amino acid replacements for 16 genes that encode the proteins of the COX holoenzyme in eight vertebrate species, and found that *COX6b1* have undergone acceleration in amino acid replacement rates. They thought the mature portions of these genes are likely to have undergone a functionally significant change to adapt in nature. In this study, a variety of polymorphic sites were found on the nucleotide sequence, but they belong mostly to synonymous mutation. Furthermore, a lot of amino acid variation was also found by the comparison of amino acid sequence (Figure 2). The result shows that the change of *A. melanoleuca and B. Taurus, S. scrofa* in amino acid appeared largely consistency. We also analyzed the function sites of *COX6b1* and found that the function sites are greatly consistent in seven mammal species when they are compared, except that the *B. Taurus* has one more casein kinase II phosphorylation site (Figure 2). These results are in agreement with the results obtained by the ME (minimum evolution) evolutionary tree of *COX6b1* (Figure 3). In addition, Lu et al. (2010) found that the untranslated region (UTR) polymorphisms of *COX6b1* subunit gene is pervasive and considered COX subunit gene heterogeneity may mediate COX functional variation. In this paper, the genomic sequence of *COX6b1* was compared by DNAMAN 6.0, and UTR

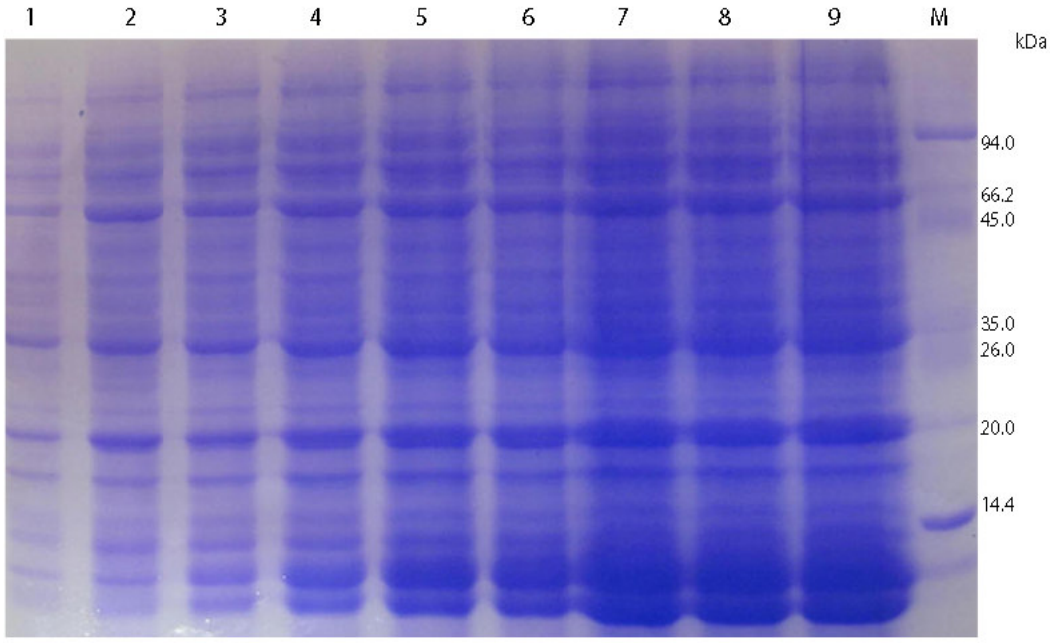

Figure 4. Protein extracted from recombinant *E. coli* BL21 strains were analyzed by SDS-PAGE gel stained with Commassie blue R250. Numbers on the right shows the molecular weight and the arrow indicates the recombinant protein bands induced by IPTG with 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h (lanes 2 to 9), respectively. Lane 1 represents the products of the *E. coli* strains with the empty vectors.

polymorphisms of *COX6b1* subunit gene were also found.

As is well known, the biogenesis of cytochrome c oxidase is a complex process controlled by two distinct genomes. Meanwhile, NRF (nuclear respiratory factor) also act as an important mediator of coordinated regulation of all ten nuclear-encoded COX subunit genes (Scarpulla, 2002; Shilpa et al., 2009). *COX6b1* is one of COX small subunits encoded by nuclear genes. Similarly, NRF will also have an effect on *COX6b1*. Furthermore, Taanman's experiment in 1990 have demonstrated that the steady-state levels of the COX6b transcript were different in the tissues examined in human liver, skeletal muscle, MOLT-4 cells and fibroblasts. In this paper, we achieved only efficient express in a prokaryotic organism. The gene's transcription level needs further research.

The cDNA and the genomic sequence of *COX6b1* gene were cloned successfully from the skeleton muscle of the *A. melanoleuca*, respectively, which were sequenced and analyzed preliminarily. The cDNA of the *COX6b1* gene was also overexpressed in *E. coli*. The data will enrich and supplement the information about *COX6b1*. In addition, it will contribute to the protection for gene resources and the discussion of the genetic polymorphism.

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