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Article

Molecular Cloning and Expression Analysis of COX II from *Sitophilus zeamais*, a Potential Action Site of AITC

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Abstract: COX II containing a dual core CuA active site is one of the three core subunits of mitochondrial Cco, which plays a significant role in the physiological process. In this report, the full-length cDNA of COXⅡ gene was cloned from *Sitophilus zeamais*, which had an ORF of 684 bp encoding 227 amino acids residues. The predicted COXⅡ protein had a molecular mass of 26.2 kDa with pI value of 6.37, and multiple sequence alignment and phylogenetic analysis indicated that *Sitophilus zeamais* COXⅡ had high sequence identity 78.51% with the COXⅡ of other insect species, especially similarity to *sitophilus oryzae*. This gene was subcloned into the prokaryotic expression vector pET-32a, and induced by IPTG in *E.coli* Transetta (DE3) expression system. Finally the COXⅡ with 6-His tag was purified using affinity chromatography with Ni^{2+}-NTA agarose. WB showed the recombinant COXⅡ was about 44 kD, and the concentration of fusion protein was 50μg/mL. UV-spectrophotometer and infrared spectrometer analysis showed that recombinant COXⅡ could catalyze the oxidation of substrate Cytc, and influenced by AITC. It was found that AITC could form a hydrophobic region with COXⅡ protein via molecular docking, besides, a sulfur atom of AITC structure could form a length of 2.9 Å hydrogen bond with Leu-31. These results will provide valuable information for elucidating the role of COXⅡ in *Sitophilus zeamais* responses to AITC, meanwhile, it will helpful to carry out a point mutation in AITC binding sites for the future research.

Keywords: *Sitophilus zeamais*; COXⅡ; Soluble proteins; Enzyme activity; AITC

1. Introduction

Sitophilus zeamais is one of the major pests of stored products in the tropics and subtropics [1-2]. For a long time, management of the stored product pests has relied on synthetic insecticides and fumigants, such as metal phosphide [3-4], methyl bromide [5] and sulfuryl fluoride [6]. However, the use of fumigants has led to serious problems like 3R (resistance, resurgence, and residue) *ect* [7-9], therefore, it is urgent to find a new type of alternative fumigants to prevent and control *Sitophilus zeamais*.

In recent years, it has been proved that AITC released from glucosinolates in Brassicaceous residues have a strong fumigation activity against stored grain pests [10-12], while the mechanisms of AITC on target organisms are still relatively scarce. Our research group found that AITC had a significantly effect on mitochondrial respiratory chain Cco of *Sitophilus zeamais in vivo* and *in vitro* [13]. ROS produced by AITC could further damage the mitochondrial membrane potential, leading to the defect of mitochondrial function (Figure 1). Due to the above similar pathophysiology alterations and destructive structure of mitochondria, AITC and phosphine were proposed to act by similar mechanisms [14].

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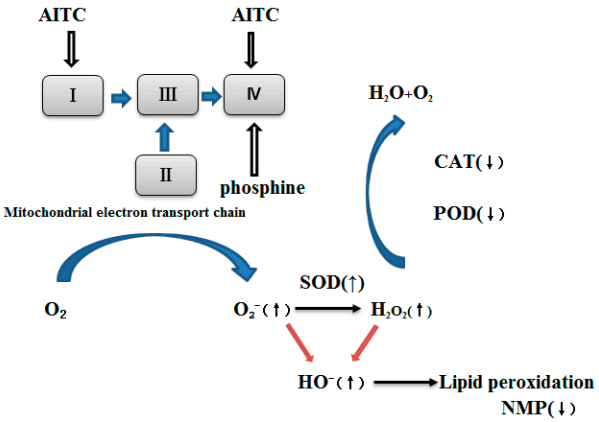

Figure 1. Proposed scheme for AITC-induced mitochondrial dysfunction with phosphine as the positive control. Arrows in parentheses represent for increase (up) or decrease (down) in activity or amount induced by AITC and phosphine fumigation

It was generally known that Cco was the terminal metal membrane protein in the electron transport chain of the eukaryotic mitochondrial inner membrane and the aerobic bacteria cell membrane electron transfer chain, and played a significant role in the physiological process [15-17]. Cco was a complex enzyme consisting of 13 different subunits in mammalian cells [18-19], and only the core subunits I–III were encoded by the mitochondrial genome [20]. These subunits were highly conserved among different organisms and were the key components of bacterial oxidases as well. Subunit I had two heme a moieties (a and a3) and a copper ion (CuB), and the latter two formed the binuclear center catalyzing oxygen reduction. Subunit II carried two copper ions in its CuA center, a hydrophilic domain oriented to the intermembrane space. During the stepwise reduction of Cox, cytochrome c docks to this domain and transfers one electron at a time to the CuA center. Much work had been done concerning Cco but a little about cloning and expression of COX Ⅱ from *Sitophilus zeamais* [21-24].

In this study, the COX Ⅱ from *Sitophilus zeamais* was cloned, and the recombinant enzyme was expressed in *E.coli* under different IPTG concentration, time and temperature. In addition, we analyzed the sequence and examined the activity of the enzyme. This work could provide the basis information for the study of mechanism of AITC against *Sitophilus zeamais*.

2. Results

2.1 *Cloning and Sequence Analysis*

A 3' cDNA fragment of 532 bp and a 5' cDNA fragment of 305 bp were amplified by 3' and 5' RACE using the special primers (Table 1). The full-length cDNA of 684 bp was then obtained by RT-PCR based on the cDNA sequences of the 3' and 5' RACE fragments. The cDNA sequence of COX Ⅱ had an ORF of 684 bp encoding 227 amino acid residues (Figure 2) with a molecular weight of 26.2 kDa, and an isoelectric point of 6.37.

Multiple alignments of COX Ⅱ deduced amino acid sequence with other insect species in NCBI database was shown below (Figure 3). COX Ⅱ exhibited a high degree of 78.51% identity conservation with other insects. To investigate the evolutionary relationships of the COX Ⅱ from *Sitophilus zeamais* with other insect COX Ⅱ, a phylogenetic tree was constructed from the deduced mino acid sequences from 14 species of COX Ⅱ (Figure 4). The analysis showed that the *Sitophilus zeamais* COX Ⅱ was located on the same branch with *Sitophilus oryzae*, however, *Sitophilus zeamais* COX Ⅱ had a distant phylogenetic relationships with *Vespa bicolor*, *Bombyx mori* and *Drosophila melanogaster*. The relationships displayed in the phylogenetic tree corresponded to their taxonomic classifications.

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1     ATATCAACATGAAAAACTCTTTTTCTTCAAGATAGAGCCTCACCTTTAATAGAACATCTT
       M  S  T  W  K  T  L  F  L  Q  D  S  A  S  P  L  M  E  H  L
61    ACATTATTTCATGACCATACTATTTTAATCTTAATTTTAATTACAATTTTAGTTAGACAA
       T  L  F  H  D  H  T  I  L  I  L  I  L  I  T  I  L  V  S  Q
121   ATACTATTAAGAATATTATTAAATAAACTCTCACACCGATTTTTACTTGAAGGTCAATTA
       M  L  L  S  M  L  L  N  K  L  S  H  R  F  L  L  E  G  Q  L
181   ATTGAAACTATTTGAACTATTCTACCTGCTATTATTCTTATTTTAATTGCCTTACCCTCA
       I  E  T  I  W  T  I  L  P  A  I  I  L  I  L  I  L  A  L  P  S
241   CTTCGATTATTATACATTCTAGACGAAATTAATAACCCTTCTATTACTATTAAAATTATC
       L  R  L  L  Y  I  L  D  E  I  N  N  P  S  I  T  I  K  I  I
301   GGACATCAATGATATTGATCATATGAATATTCTGATTATAAAAATATTGAATTTGATTCC
       G  H  Q  W  Y  W  S  Y  E  Y  S  D  Y  K  N  I  E  F  D  S
361   TATATAATTCCTACCAAAGAACTTAACTCTTTTAATTTTCGTCTCCTAGAAGTAGATAAT
       Y  M  I  P  T  K  E  L  N  S  F  N  F  R  L  L  E  V  D  N
421   CGAACTCCTTTTCCTTATAAAACTCAAATTCGACTCTTAGTTACGTCTGCAGATGTAATT
       R  T  P  F  P  Y  K  T  Q  I  R  L  L  V  T  S  A  D  V  I
      CuA binding site [ion binding site]
481   CATTCTTGGACAGTACCAAGTATAGGAATTAAAATTGATAGAACCCCAGGACGCTTAAAT
       H  S  W  T  V  P  S  M  G  I  K  I  D  S  T  P  G  R  L  N
                                  CuA binding site [ion binding site]
541   CAAGCCAATTTAATTGCTAATCGTTCAAGAATCTTTTTTGGCCAATGTTCTGAAATTTGC
       Q  A  N  L  I  A  N  R  S  S  I  F  F  G  Q  C  S  E  I  C
      CuA binding site [ion binding site]
601   GGAGCAAACCATAGATTTATACCTATCATTTTAGAAAGAATTAAACCCAATTTATTTTTA
       G  A  N  H  S  F  M  P  I  I  L  E  S  I  K  P  N  L  F  L

661   AACTGAATTATTTCCAAAACTTAA
       N  W  I  I  S  K  T  *
```

Figure 2. Nucleotide and amino acid sequence of *Sitophilus zeamais*. CuA binding site [ion binding site] containing 6 conserved feature residue pattern: H C E C H M were indicated by grey color. The start codon and the stop codon were represented with an asterisk (*)

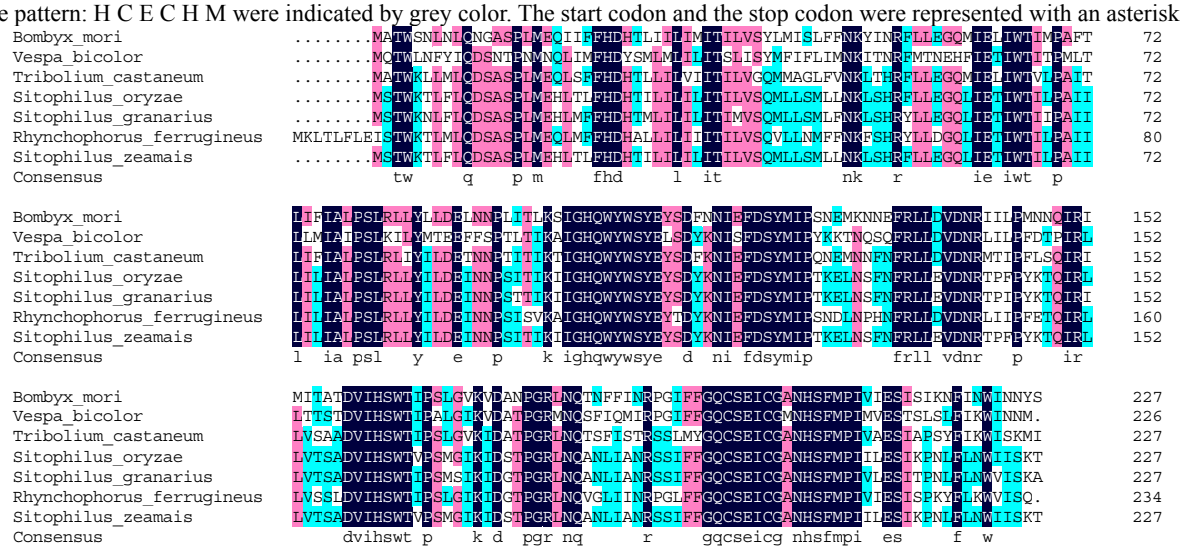

Figure 3. Multiple alignments of the amino acid sequence with other known COXⅡ. The multiple alignment was analyzed on DNAMAN 8.0. Dark blue: identity =100%; red: 75%≤identity <100%; light blue: 50%≤identity ≤75%. The absent amino acids in the alignment were indicated by dots (.), identical residues are indicated with lowercase. The GenBank accession numbers of the COXⅡ were as follows:*Bombyx mor* (AAK85720.1), *Vespa bicolor* (AIE42617.1), *Tribolium castaneum* (AIM19612.1), *Sitophilus oryzae* (ANW06522.1), *Rhynchophorus ferrugineus* (YP-009183673.1),*Sitophilus granarius* (JN163959.1),respectively.

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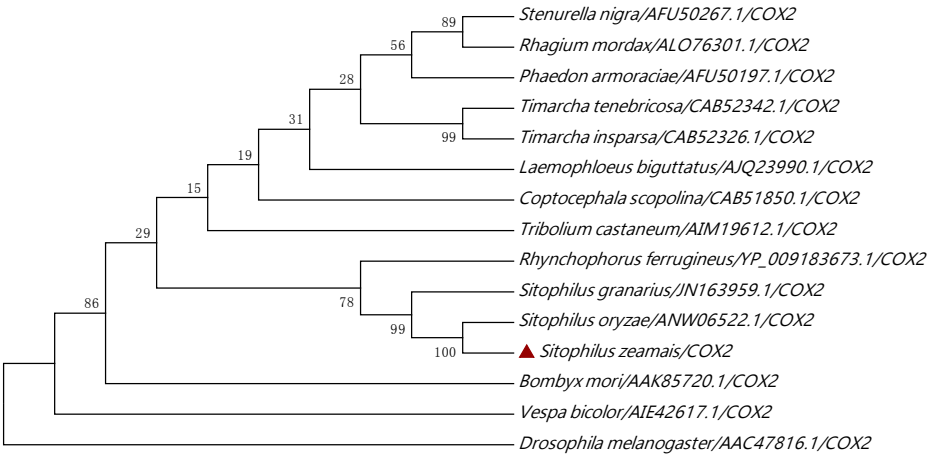

Figure 4. Phylogenetic analysis of the amino acid sequences of *Sitophilus zeamais* COXⅡ. The Neighbor-Joining phylogenetic trees were constructed using the bootstrap method on MEGA 5.1 and the number of Bootstrap replications was 1000. The protein sequencesused in these trees were as follows: *Timarcha tenebricosa*(CAB52342.1), *Timarcha insparsa*(CAB52326.1), *Rhagium* mordax(ALO76301.1), *Drosophila melanogaster*(AAC47816.1), *Bombyx mori*(AAK85720.1), *Vespa bicolor*(AIE42617.1), *Tribolium castaneum*(AIM19612.1), *Rhynchophorus ferrugineus* (YP_009183673.1), *Phaedon armoraciae*(AFU50197.1), *Coptocephala scopolina* (CAB51850.1), *Laemophloeus biguttatus* (AJQ23990.1).

2.2 *Effect of temperature, IPTG concentration and processing time on solubility of COX Ⅱ*

To investigate the effect of temperature on the solubility of recombinant COXⅡ protein, N-terminal Trx/6-His/S tagged COXⅡ protein was over-expressed in *E.coli* Transetta(DE3) at 37℃, 25℃and 16℃, respectively. It was found that the majority of the recombinant protein was insoluble at 37℃and 25℃ after sonication (Figure 5A). Compared with the protein generated at 37℃ and 25℃, when the recombinant cells overexpressed at 16℃ produced a higher percentage of soluble protein. The recombinant protein was further determined by WB analysis (Figure 5B). In general, the soluble protein yield corresponded negatively to the elevating temperature.

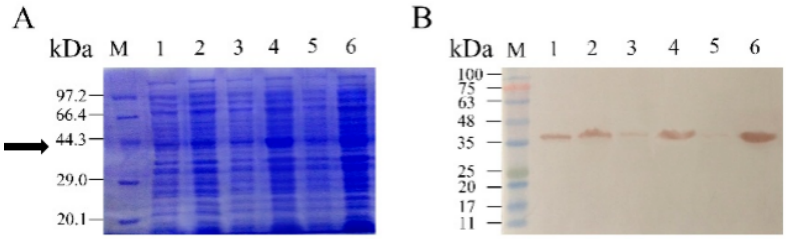

Figure 5. Analysis of the effect of temperature on the solubility of fusion COXⅡ protein. (**A)** SDS-PAGE was carried out by a 12 % polyacrylamide gel. (**B**) Western blotting analysis of fusion COXⅡ using anti-His tag antibody. The fusion COXⅡ was expressed in *E. coli* Transetta (DE3) at 16℃ (lanes 1 and 2), 25℃(lanes 3 and 4), 37℃ (lane 5 and 6). The soluble fractions are shown in lanes 1,3 and 5, respectively. Lane M, molecular mass marker proteins. The arrow indicates the expected size of fusion COXⅡ protein.

Attempts had also been conducted to increase the fraction of soluble protein in *E. coli* by adding IPTG at different levels (Figure 6). Higher IPTG was found to have negative influence on the rate of cell growth, which suppressed the *E.coli* Transetta (DE3) cell growth. The solubility of COXⅡ content reached its highest in the presence of 1mM IPTG.

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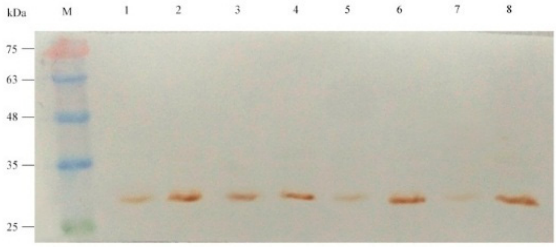

Figure 6. Western blot analysis of the effect of IPTG concentration on the solubility of recombinant fusion COXⅡ protein. The fusion COXⅡ was expressed in *E. coli* Transetta (DE3) under 0.5 mM (lanes 1 and 2), 1.0 mM (lanes 3 and 4), 1.5 mM (lanes 5 and 6), 2.0 mM (lanes 7 and 8), The soluble fractions are shown in lanes 1, 3, 5 and 7 respectively. Lane M, molecular mass marker proteins.

Different processing time was also carried out to investigate its influence on the solubility of COXⅡ. The results showed that with the extension of culture time, the protein content was increasing at the range from 6 to 24 h, (Figure 7), however, the soluble content of protein was decreased with the prolongation of the culture time after cultured 24h. In a word, it was concluded that the solubility of COXⅡ was expressed highly in *E.coli* Transetta (DE3) at condition of 1 mM IPTG, 16℃, 24 h, and 200 rpm.

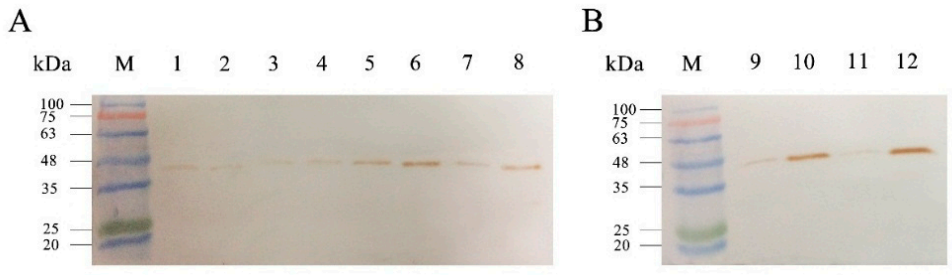

Figure 7. Western blot analysis of the effect on the solubility of recombinant COXⅡ protein in the different treatment time. The fusion COXⅡ was expressed in *E. coli* Transetta (DE3) with 6 h (lanes 1 and 2), 12 h (lanes 3 and 4), 24 h (lanes 5 and 6), 36 h (lanes 7 and 8), 48 h (lanes 9 and 10), and 60 h (lanes 11 and 12). The soluble fractions were shown in lanes 1, 3, 5, 7, 9 and 11 respectively. Lane M, molecular mass marker proteins.

2.3 *Purification of COXⅡ protein and Western Blot Assay*

The COXⅡ protein was purified as described in "materials and methods" section, and the purified recombinant protein (eluted at 200 mM concentration of imidazole) presented a single band on SDS-PAGE (Figure 8A). The Mw of purified protein band was about 44 kDa larger than the predicted 26 kDa due to the N-terminal Trx/6-His/S-tagged. The Trx-free recombinant COXⅡ protein was generated by enterokinase digestion and migrated as a single band estimated as 26 kDa (Figure 8 A) which corresponded to the size of predicted Mw. The WB signal was detected for recombinant enzyme produced from pET32a vectors (Figure.8 B). The concentration of purified COXⅡ was 50 mg•L^{-1} based on Bradford assay.

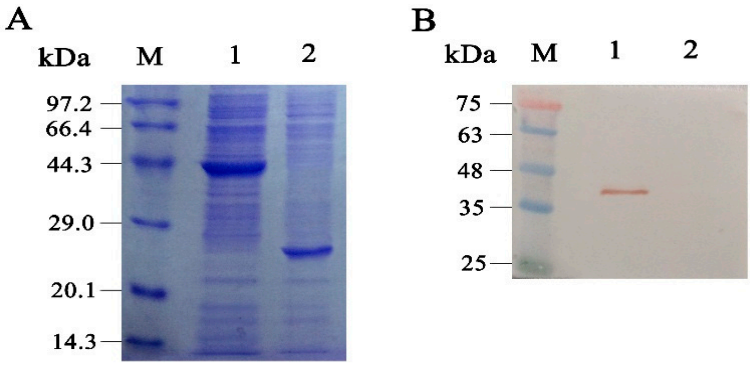

Figure 8. SDS-PAGE and Western blot analysis of purified recombinant COXⅡ. (**A**) The samples were separated on 12% gels; and (**B**) Western blot analysis of recombinant COXⅡ using anti-His tag antibody. Lane M: protein molecular standard, Lane 1: purified Trx/6×His/S-tag/COXⅡ from pET32a, Lane 2:Trx-free COXⅡ protein digested by enterokinase.

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2.4 Enzymatic activity of the COX Ⅱ

The purified tag-free protein was characterized by using the UV-visible. Under certain concentration of substrate Cytc, the absorption peak of Cytc at 520nm and 550nm decreased gradually with the increase of enzyme concentration. Besides, by adding different concentrations of AITC to the substrate Cytc, there was no change at the maximum absorption peak, while adding purified COX Ⅱ to the solution containing AITC and reduced Cytc, the absorption peak was still unchanged. So, the fusion COX Ⅱ protein could have an activity towards Cytc and affected by AITC according to the above treatments of three groups (Figure 9). In order to confirm whether AITC combination with recombinant COX Ⅱ, the reaction system of both them was detected by infrared spectrometer. while hypochromic shift was observed at 3400nm, which indicated that AITC is combined with the end of COX Ⅱ (Figure 10)

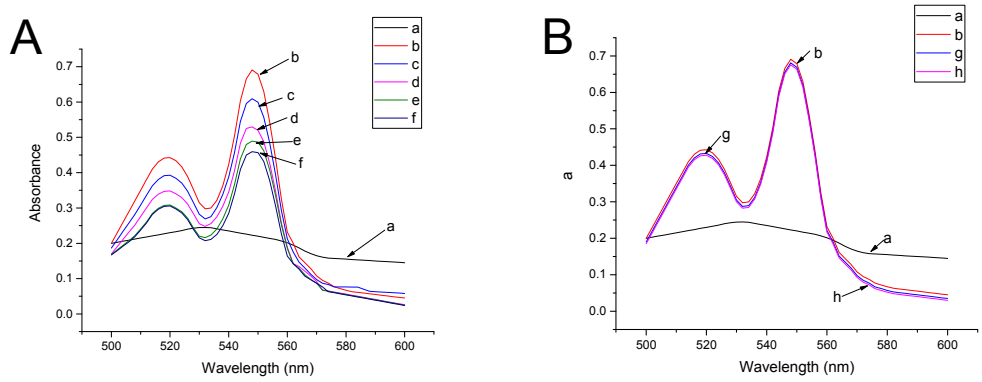

Figure 9. Measuring the activity of *Sitophilus zeamais* COX Ⅱ .**a:** Oxidized Cyt c; **b:** Reduced Cyt c **c:** Reduced Cyt c C+2μL purified COX Ⅱ; **d:** Reduced Cyt c C+4μL purified COX Ⅱ; **e:** Reduced Cyt c C+8μL purified COX Ⅱ; **f:** Reduced Cyt c C+16μL purified COX Ⅱ; **d:** Reduced Cyt c C+2μL AITC; **d:** Reduced Cyt c C+2μL AITC+4μL purified COX Ⅱ

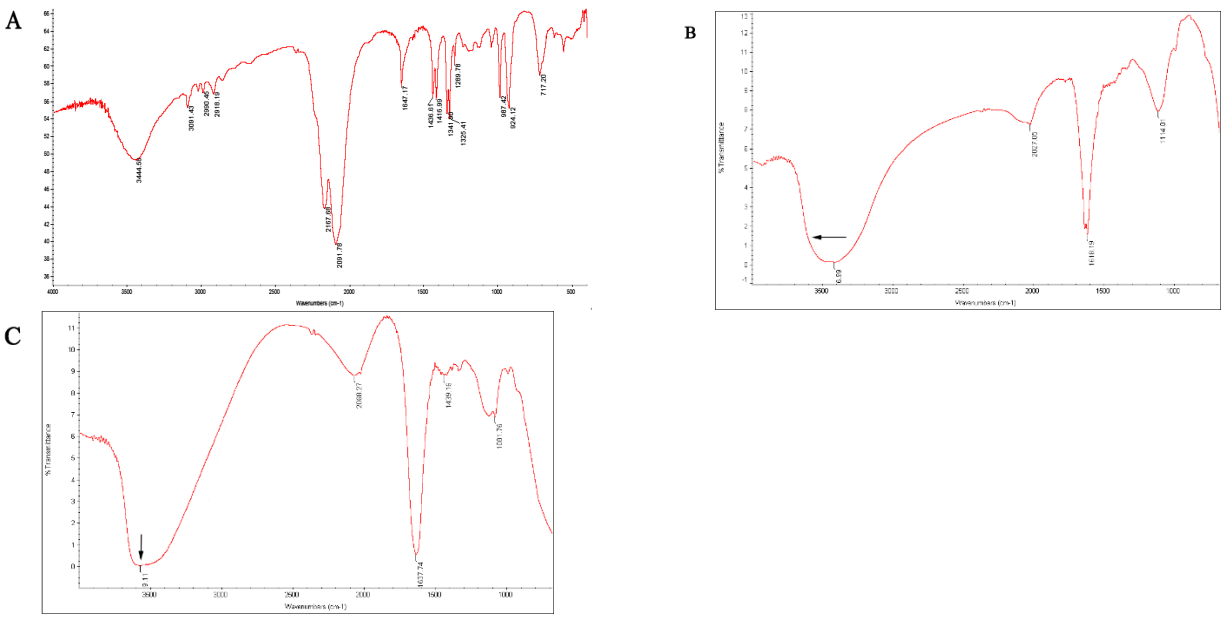

Figure 10. Infrared spectrometer scan map of samples. (A) Absorption peak of AITC. (B) absorption peak of purified COX Ⅱ.(C) absorption peak of AITC and purified COX Ⅱ. Absorption peak at 3400 wavenumber of N-terminal or C-terminal of the purified COX Ⅱ was shifted to 3600 wavenumber after adding AITC.

2.5 Homology modeling and molecular docking

To further understand the action sites of AITC effected on COX Ⅱ. The specific location of AITC interaction with Cco was simulated by molecular docking using the Vina 1.1.2 Autodock and Discovery Studio (DS) (Accerlrys, USA) software at the molecular level. Analysis showed that the propylene group of AITC was located in a

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hydrophobic cavity formed by the amino acid residues Ile-34, Pro-69, Ile-72, Leu-73 and Ile-76, forming a strong hydrophobic interaction (Figure 11).

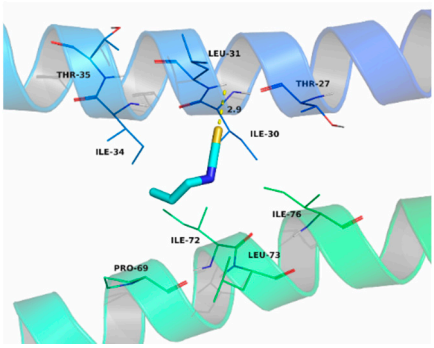

Figure 11. Molecular docking of AITC effect on the active site of *Sitophilus zeamais* COX Ⅱ by using Vina 1.1.2 Autodock and Discovery Studio software at the molecular lever.

3. Discussion

In recent years, a number of mechanisms have been proposed for the activities of AITC [25-33], such as microtubule, proteasome, nucleus, mitochondria, ABC transportes and TPR channel. However, identifying the molecular targets is a first step to make sure the molecular mechanisms of AITC. Mitochondria of insect had been proved to be the main AITC's target. [12, 34].Our research group found that AITC had a significantly effect on mitochondrial respiratory chain Cco of *Sitophilus zeamais in vivo* and *in vitro* [13]. ROS induced by AITC could further damage the mitochondrial membrane potential, leading to the defect of mitochondrial function. Basing on the similar pathophysiology alterations and destructive structure of mitochondria, AITC and phosphine are proposed to act by similar mechanisms [14]. So, Cco target of AITC was proposed.

There is a great interest in Cco due to its role in the respiratory chain of mitochondria including transfer of electrons from Cyt c to O_2 and function coupled with proton pump[35-37].To investigate the function and activity of COX Ⅱ, it is necessary to obtain the pure enzyme by separation from tissues, or expression and purification from a heterogeneous system. In this study, COX Ⅱ gene from the *Sitophilus zeamais* was cloned, sequenced and analyzed. COX Ⅱ ORF contain A+T=72.7%, G+C=27.3%,consistenting with previously reported[38]. Bio-informatic and phylogenetic analysis clearly suggested that COX Ⅱ shared high sequence similarity with COX Ⅱ genes of other insects, especially similarity to *sitophilus oryzae*. The reason for the results was that the COX Ⅱ subunit was highly conserved in the long process of biological evolution. Multiple alignment analysis and phylogenetic trees also showed that varied of COX Ⅱ identity and homology had a certain degree of difference as well as the relative conservation. This was able to exercise its unique biological function among different species.

There are two different coding systems for the mitochondrial genome and nuclear gene coding. According to the invariance of the sequence of the target protein sequence, the obtained COX Ⅱ subunit gene was modified, synthesized and then expressed. Different expression vectors (Peasy blunt E1, pET28a, pET30a, pET32a, pET42a) were used to express COX Ⅱ fusion protein. However, only pET32a-COX Ⅱ could be expressed recombinant protein in the *E.coli* Transetta(DE3) and be detected by the SDS-PAGE and WB. After removal of fusion tag with enterokinase, the monomeric form of COX Ⅱ could not be visualized by WB because of the lack of anti-COX Ⅱ antibodies.

Overexpression of protein in heterologous systems often results in the formation of inclusion bodies [39]. In the *E. coli* expression system, the formation of inclusion bodies can be formed owing to the high growth rates of cell lead to the protein unfolded to form the correct conformation [40]. Therefore, improving solubility is a major goal in studying protein production processes. Recently, the use of low growth temperatures to increase soluble protein yields has been reported [41-42]. In present study, most of the COX Ⅱ protein expressed in *E.coli* was as inclusion bodies. Therefore, various strategies have been designed to overcome this drawback. When expressed at 37℃, a considerable amount of the total protein in *E. coli* BL21 (DE3) was obtained, however only a little soluble protein was detected. Finally, considerable soluble protein was obtained when expressed at 16℃, accompanied by a reduced total protein yield. The results suggested that the conformational quality of COX Ⅱ protein is significantly

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influenced by temperature. When grown at low temperature, COX Ⅱ proteins with the slow rates growth in *E. coli* cell have enough time to fold in the correct conformation [43].

After removal of fusion tag with enterokinase, UV-visible was used to characterize purified COX Ⅱ active site. Although the *E. coli* system was a very convenient expression system to obtain purified recombinant protein *in vitro*, it was usually unsuitable to be used for the expression of the recombinant protein from most eukaryotes, such as insects, plants, and so on, because of highly divergent codon usage and protein misunfolding [43, 44]. The Cytc could not be oxidized into a fully oxidized state, the reasons are as follows:(1) the activity of COX Ⅱ needed the interaction of Cco subunits, for example, subunit III played an important role in the integrity of the structure and function of the enzymes[45-49]; (2) purified protein concentration was too low to maintain the integrity structure of biological macromolecules, resulting in changes in the spatial structure of the copper center in the enzyme; (3) as the reaction system was carried out, the pH of the buffer was changed leading to the change of the structure of the enzyme, which affected the activity of the enzyme [50].

The absorption peak of Cytc at 520 nm and 550 nm decreased gradually with the increase of enzyme concentration. While adding AITC or "AITC + purified COX Ⅱ" into Cytc system, the absorption peak did not change significantly. To explain this phenomenon, a molecular docking and analysis was conducted. The results demonstrated that the propylene group of AITC was located in a hydrophobic cavity formed by the amino acid residues Ile-34, Pro-69, Ile-72, Leu-73 and Ile-76, forming a strong hydrophobic interaction. Moreover, the sulfur atom in AITC with amino acid residues Leu-31 growth for the interaction of a hydrogen bond 2.9 Å. It might be predicted that AITC have the strong inhibitory action to the COX Ⅱ activity.

4. Materials and methods

4.1. Insects

Sitophilus zeamais cultured in the laboratory were provided by the Northwest Agriculture and Forestry University, Shannxi Province in China. Feeding wheat was placed in an oven (80℃) for 2 hours, and then adjusted the moisture content to 14%, divided and inoculating at 28℃±1℃,75%±5% relative humidity, a photoperiod of 16:12(Light: Dark), 25±1℃ for 7 days. *Sitophilus zeamais* strain was reared for generations and selected the same size of insects for the experiment.

4.2. Chemicals and Plasmids

*Eco*RI, *Xho*I, prime STAR Max Premix, *E.coli* DH5α, T4 DNA ligase and protein marker were purchased from Takara (Dalian,China). *E.coli* Transetta (DE3) was obtained from TransGen Biotech (Beijing, China). Horseradish peroxidase (HRP)-labeled Anti-His Tag Mouse Monoclonal Antibody and HiFiScript gDNA Removal cDNA Synthesis Kit were ordered from Kang bio-technology of the century (Beijing, China). The E.Z.N.A Plasmid Mini kit was provided by Omega (Guangzhou, China). Cytochrome c from bovine heart was purchased from Sigma (USA), and pET32a were used for protein expression. All other chemicals were analytical grade and obtained from Shanghai Sangon (Shanghai, China).

4.3. RNA extraction and cDNA synthesis for Molecular Cloning

The total RNA from *Sitophilus zeamais* was extracted by using the modified CTAB method [51].The quality and concentration of extracted RNA was examined by 1% agarose gel electrophoresis and SpectraMax Plus^{384}. The total RNA was digested with DNase I to remove the genomic DNA and for cDNA synthesis. 5'RACE and3'RACE-Ready cDNA were synthesized from 1 μg of total RNA using the SMART™ RACE cDNA Amplification Kit (Clontech, Dalian, China) as described by the manufacturer, respectively. The products were stored at -20℃ for future use.

4.4. *Molecular Cloning of COX Ⅱ cDNA by 3' and 5'RACE*

The primers used for 3' and 5'RACE amplification were designed (Table 1) according to the known partial sequence of *Sitophilus zeamais* in the NCBI database using the Primer Premier 5(PP5). 3'-RACE PCR was conducted using C2-1F1 and 10× Nested universal Primer A (NUP) as the primer pair for the PCR reaction. For 5'RACE, PCR was carried out using C2-1R1 and 10×Universal Primer a Mix (UPM) as the primer pair for PCR amplification. For both 3' and 5'RACE,the PCR reaction conditions were:95℃ for 3 min, followed by 32 cycles of 30 s at 95℃, 45 s at 55℃ and 1 min at 72℃, and then final extension at 72℃ for 5 min. The PCR products were separated on a 1% agarose gel electrophoresis and purified using Gel Extraction Kit (Omega, Guangzhou, China). The purified fragments were ligated into the pMD-19T vector (Takara, Dalian, China) and positive clones were

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sequenced (Sangon, Shanghai, China). Based on the obtained sequences of the 3' and 5'RACE, the splicing full-length of COXⅡ gene was amplified with specific primers C2-qF and C2-qR.

Table 1. List of primers used in this study.

Name	Sequence (5'-3')	Primer used
C2-1R1	ATTAAATTGGCTTGATTTAGACGACCT	5'RACE
5'-RACE CDS Primer	(T)25V N	5'RACE
SMART II A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACATGGG	5'RACE
C2-1F1	CACCTTACCTTATTCCATGATCACACT	3'RACE
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30V N	3'RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	5'RACE/3'RACE
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5'RACE/3'RACE
UPM-short	CTAATACGACTCACTATAGGGC	5'RACE/3'RACE
C2-qF	ATATCAACATGAAAAACTCTTTTTCTT	Full-length cloning
C2-qR	TTAAGTTTTGGAAATAATTCAGTTTAA	Full-length cloning
C2-qF-1	ATGTCAACATGGAAAACTCTTTTTCTTC	PCR
C2-qR-1	TTAAGTTTTGGAAATAATCCAGTTTAAA	PCR
C2-E-F1	G**GAATTC**ATGTCAACATGGAAAACTCTTTTTC	PCR
C2-X-R1	CCG**CTCGAG**TTAAGTTTTGGAAATAATCCAGT	PCR
T7 promoter primer	TAATACGACTCACTATAGGG	PCR
T7 Terminator Primer	GCTAGTTATTGCTCAGCGG	PCR
M13F(-47)	CGCCAGGGTTTTCCCAGTCACGAC	PCR
M13R(-48)	AGCGGATAACAATTTCACACAGGA	PCR

4.5 *Sequence analysis*

The COXⅡ amino acid sequence was deduced using the ExPASy Protparam tool. The theoretical isoelectric point (pI) and molecular weight (Mw) of the deduced proteins were also predicted with using this web Tool. A multiple alignment analysis of the amino acid sequences was carried out using DNAMAN8.0 software. The secondary structure prediction was performed with Prabi-Gerland. Conserved domains were detected using the Conserved Domains Search tool. Transmembrane regions were predicted using the TMHMM server. Phylogenetic analyses were performed using MEGA 5.1 software with the neighbor-joining method, followed by phylogeny test options of 1000 bootstrap replicates.

4.6 *Vector construction and Expression at small scale*

The COXⅡ ORF was amplified with specific primer C2-E-F1 and C2-X-R1 (Table 1) containing *EcoR*I and *Xho*I restriction enzyme sites (underlined). The amplicons were purified, ligated and sequenced under the same conditions described above. The plasmid of sequenced clones were extracted from *E.coli* DH5α and digested by restriction endonuclease *EcoR*I and *Xho*I. The digested COXⅡ ORF was cloned into *EcoRI* and *XhoI* site pre-digestion of pET-32a plasmid using T4 DNA ligase and transformed into *E. coli* Transetta (DE3). The positive recombinant clones were further verified by digestion and sequencing. The recombinant proteins containing Trx/6-His/S-tag/ at N-terminus for purification.

For fusion protein expression, *E. coli* strains containing plasmids pET-32a-COXⅡ were inoculated to 5 mL of Luria-Bertani (LB) medium including 100 μg•mL Ampicillin(AMP). Cells were cultured overnight at 37℃ with shaking at 200 rpm.50 μL cell suspension was inoculated to 5 mL of LB medium. when cell growth reached the exponential phase (OD_{600}:0.5-0.7),Then inducing with 1 mM IPTG at 16℃,25℃,37℃ with shaking at 200 rpm for 24 h, Cell pellets were harvested by centrifuged and sonicated for 5 min. After centrifugation for 20 min at 12,000×g at 4 ℃, the soluble and insoluble fractions were denatured and then submitted to a 12 % SDS-PAGE to determine the yield and solubility of expressed protein.

Different concentrations of IPTG (0.5mM, 1.0mM,1.5mM,2.0mM,) were investigated to determine the effect of IPTG on solubilization of COXⅡ protein under the same conditions described above. Meanwhile, the survey of tested time (6 h,12 h, 24 h,36 h, 48 h, 60 h) was conducted to look into its effect on the solubility of COXⅡ protein under 16℃,1mM IPTG 200rpm, and soluble protein content were analyzed on a 12 % SDS-PAGE gel.

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4.7 *Scale-up expression,Purification and Western Blot Analysis*

According to the small-scale expression cultures exhibited the optimal amount of soluble protein, recombinant *E. coli* was induced in 1.8 L LB medium in the presence of 1 mM IPTG at 18℃ under 24 h, 200 rpm, Bacterial cells were harvested by centrifugation and resuspended in 1 mL of 25 mM PBS buffer (pH 7.4) per 100 mg wet weight of cells, and then disrupted by sonication. Supernatant was separated from cell debris by centrifugation (12,000×g, 30 min, 4 ℃) and clarified by passing them through a 0.45 μm filter before being applied to a Ni^{2+}-NTA agarose gel column (TransGen, Beijing, China). The column was previously equilibrated with 10 volumes of PBS buffer to blance at a flow rate of 2mL/min according to the instruction manual. The column was washed with 6 volumes of wash buffer (10 mM PBS buffer, pH 7.4, 10 mM imidazole) after the sample was drained. The bound proteins were eluted from the affinity resin with 3 volumes of elute buffer (balance buffer containing a linear gradient of 50-400 mM imidazole), fractions were collected and analyzed on a 12 % SDS-PAGE gel.

Purified protein was verified via WB analysis with Horseradish peroxidase (HRP)-labeled Anti-His Tag Mouse Monoclonal Antibody and detected with a diaminobenzidine kit according to the instructions. The recombinant fusion COXⅡ protein was dialyzed against PBS buffer/$CaCl_2$ (10 mM/L mM, pH 7.4) and then incubated overnight at room temperature with enterokinase (Solarbo, Beijing, China) to generate tag-free protein. The protein concentration was determined using the Bradford method [52].

4.8 *Assay of Enzymatic Activity*

Cytc (Sigma, USA), was mainly the oxidized status, which can be prepared utilizing sodium ascorbate (solarbio, Beijing, China), 5μL of purified enzyme was added into 195μL of sample test buffer (16 mM Na_2HPO_4, 276 mM Na_2HPO_4, pH 5.8). The activity of protein was demonstrated by monitoring the oxidation process of the reduced cytochrome c (Cytc), the UV spectrophotometer (Hitachi-3310, Japan) was used to characterize the reaction. A total of four treatment groups as follows (Table 2).

Furthermore, absorption peaks change of the purified COXⅡ combination with AITC was detected according to the infrared spectrometer(Nicolet Avatar 330, USA). Through the observation of three different treatment of the peak pattern(Table 3), to determine whether the AITC and purified COXⅡ have a combination.

Table 2. Assay of purified COXⅡ Activity

grops	experimental treatments(200μL)
1	reduced Cyt c
2	reduced Cyt c+purified COXⅡ
3	reduced Cyt c+purified COXⅡ+AITC
4	reduced Cyt c+AITC

Table 3. Binding assay of AITC and purified COXⅡ

grops	experimental treatments(200μL)
1	1μL AITC+199μL buffer
2	2μL purified COXⅡ+198μL buffer
3	1μL AITC +2μL purified COXⅡ+197μL buffer

4.9 Homology modeling and molecular docking

With cytochrome C oxidase from bovine heart (PDB ID:1V54, PDB ID:1OCC) as a template, the three-dimensional structure of the COXⅡ was constructed by Discovery Studio (DS) (Accerlrys, USA) software homology modeling module, and then using the homologous protein method to search for the active pocket of the COXⅡ, the Vina 1.1.2 Autodock software was used to carry out molecular docking study.

5. Conclusions

In summary, we have successfully cloned the COXⅡ gene from the *Sitophilus zeamais,* and expressed the COXⅡ fused with Trx/His/S-tags in the *E.coli* cells. In addition, the fusion proteins are purified and evaluated its activities towards Cyt c and AITC. Meanwhile, combining with molecular docking experimental was conducted. The results indicated that recombinant COXⅡ was functional and might be one of the sites of action of AITC. The results

also provided that basis information for mechanism of AITC against adult *Sitophilus zeamais*.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations:

COX II	Cytochrome c oxidase subunit II
Cco	Cytochrome c oxidase
ORF	open reading frame
WB	Western Blotting
AITC	allyl isothiocyanate
ROS	Reactive oxygen species
Mw	molecular weight
IPTG	isopropyl β-D-thiogalactopyranoside

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