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# Cloning, expression, purification and characterization of an aflatoxin-converting enzyme from *Armillaria tabescens*

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**Abstract**: [**Objective**] Aflatoxin $B_1$ ($AFB_1$) is extremely mutagenic, toxic and a potent carcinogen both to humans and livestock. Aflatoxin-oxidase (AFO) was an aflatoxin-converting enzyme previously purified by us from *Armillaria tabescens*. In order to know better about the molecular characterization of this distinct enzyme, we expressed, purified and characterized the His6 tag fused aflatoxin-oxidase. [**Methods**] Based on sequences of peptides fragments of AFO previously obtained by Electrophoresis-Electrospray Ionization tandem mass spectrometry (ESI-MS/MS), we cloned the cDNA of AFO using Switching Mechanism At 5′ end of the RNA Transcript (SMART) Rapid Amplification of cDNA Ends (RACE) technology and expressed this gene as a fusion protein in *Pichia pastoris* by using pPIC9-afo as vector. We purified the fusion enzyme using nickel affinity chromatography. We identified the recombinant aflatoxin-oxidase (rAFO) by both western blot and peptide mass fingerprinting (PMF). Moreover, we characterized several enzymatic properties of the rAFO using $AFB_1$ as the substrate including Km value, optimum temperature, optimum pH, thermal stability and pH stability. [**Results**] The AFO gene is 2321 bp long with a coding region of 2088 bp encoding 695 amino acids. Peptide mass fingerprinting (PMF) identification showed a 63.2% coverage of the molecule compared to the theoretical tryptic cleavage of the rAFO. The recombinant aflatoxin oxidase was purified 5.99-folds using nickel affinity chromatography. It has a specific activity of 234 U/mg. Kinetics studies showed that the rAFO converted $AFB_1$ with the Km value of $3.93 \pm 0.20 \times 10^{-6}$ mol/L under its optimal conditions of pH6.0 and 30℃. Thermostability investigation revealed that the rAFO had a half-life of 90 min at 30℃, and pH stability results suggested that the rAFO was relatively stable when pH ranged from 5.5 to 7.5. [**Conclusion**] It appears to be the first successful production of the recombinant aflatoxin oxidase (rAFO) with $AFB_1$-converting ability from *Armillaria tabescens*. The purified rAFO with preferably $AFB_1$-converting activity confirms that this recombinant aflatoxin oxidase is now ready for further studying.

**Keywords**: *Armillaria tabescens*, Aflatoxin-oxidase (AFO), Rapid amplification of cDNA ends(RACE), *Pichia pastoris*
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Aflatoxins are highly toxic secondary metabolites predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus*[1-4]. The occurrence of aflatoxins contamination is global with severe problems especially prevalent in developing countries. Among them, aflatoxin $B_1$ ($AFB_1$) is one of the most potent naturally occurring mutagens and carcinogens known. Food contaminated with $AFB_1$ poses a serious health threat when consumed by human and animals, and when present at unacceptable levels, the contamination may also result in severe economic problems worldwide[5-6].

Elimination and reduction of aflatoxin in foods is a persistent problem of growing concern to the agricultural and food industry. But effective decontamination of foods and feeds via the traditionally used physical and chemical methods is not economically feasible. Thus biodegradation of aflatoxins, using microorganisms or enzymes, can be a highly promising choice, since it is efficient, specific, and environmentally friendly.

Some microbial isolates have been reported with different levels of degradation abilities, and experimental data had revealed that some of these active ingredients might be enzymes[7-16]. However, only a few researches reported on purification and further characterization of these active enzymes[11], and there was no report on gene cloning and recombinant expression of these enzymes.

In our previous study, intracellular extracts from the edible fungus *Armillaria tabescens* was comfirmed with $AFB_1$-converting ability[10], moreover, an $AFB_1$-converting enzyme named aflatoxin-oxidase (AFO) by the author was purified from the extracts, and characterization of this AFO showed that it could effectively concert $AFB_1$ within 30min implying its promising application prospect[17].

In present study, in order to obtain large amounts of this promising enzyme rapidly for its further researches and potential applications, the gene of AFO was cloned into *Pichia pastoris* with a His6-tag coding sequences at the C termini. The engineered *Pichia pastoris* with AFO gene was induced with methanol and expression of recombinant AFO was confirmed by western blot. Purification of the His6-tagged AFO was done by nickel affinity chromatography. Afterwards, purified enzyme was identified by peptide mass fingerprinting (PMF). Finally, biochemical characterization of the rAFO was performed including determination of pH and temperature optima, substrate kinetics and pH and temperature stability.

## 1 Materials and methods

### 1.1 Materials

*Armillaria tabescens* (5.0092) was purchased from China General Microbiological Culture Collection Center (CGMCC). The reagents of RACE and PCR were using Clontech and Qiagen protocols. Restriction endonucleases and T4 DNA ligase were from NEB. pMD18-T vector was bought from TaKaRa. Trizol and Pichia expression kit were obtained from Invitrogen. Proteins standard used as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) markers were from Bio-Rad. For the enzyme assay, $AFB_1$ standard was purchased from Sigma. The chemicals for HPLC were of HPLC grade and other chemicals used were all of analytical grade.

### 1.2 Total RNA extraction and full-length cDNA cloning of aflatoxin-oxidase

*Armillaria tabescens* was cultured as below. The culture medium used contained 0.5% (w/v) malt extract, 1% (w/v) yeast extract, 0.1% $K_2HPO_4$, 0.05% $MgSO_4$ and 0.01% vitamin $B_1$, 0.01% (w/v) $CaCl_2$. And maltose (5%, w/v), glucose (5%, w/v), fructose (2%, w/v), lactose (2%, w/v) or sucrose (2%, w/v) was used as the carbon source. The medium was brought to pH6.0 with 0.1N KOH prior to sterilization. A spore suspension of the fungus was prepared by adding sterile water (10 mL) to stock cultures on potato dextrose agar (PDA). The suspension was then added to 100 mL medium in a baffled flask. The latter was shaken at 180 r/min in an orbital incubator at 28℃ for 7 days. The biomass concentration was estimated at 18.37 g/L by a dry

weight measurement. Then the culture was prepared by adding about 25 g wet mycelia (obtained by sterile operation filtrated) to 250 mL medium in a baffled flask. The flask was shaken at 200 r/min for 7 days at 27℃. Then the mycelia were harvested and frozen under −80℃. The frozen tissue was then ground to a powder and the total RNA extracted using Trizol.

Since ten peptide fragments of AFO were identified by Electrophoresis-Electrospray Ionization tandem mass spectrometry (ESI-MS/MS)[17], degenerated primers: sense P1: 5'-TGGGARGGNTTYACNGC-3', antisense G2: 5'-GCNGTRAANCCYTCCCA-3', sense G1: 5'-CARGAYGCNAAYGGNGA-3' and antisense P2: 5'-TCNCCRTTNGCRTCYTG-3' were designed based on two of them, whose amino acid sequences were WEGFTA and QDANGE, respectively. Then an internal fragment of AFO gene termed as E1 was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with total RNA as a template using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions. The amplication product E1 extracted from 1% agarose gel was cloned by T/A ligation in the pMD18-T vector (Takara) and transformation of *Escherichia coli* DH5α for DNA sequence confirmation.

Two specific primers: the primer S1 (5'-TAGGCGAAGTGTCGTCGTCAATGGAA-3') and the primer S3 (5'-GAAGTTATCGGCTTTCCAGTCAGAGGGT-3') were designed based on the sequence of E1 for amplification of the 5'- and the 3'- ends of AFO gene. Then the 5' and 3' ends of AFO gene termed as E2 and E3 respectively were obtained by using a SMART™ RACE cDNA Amplification Kit (Clontech, Austria)[18]. Both 5'- and 3'- amplification products were ligated into pMD18-T vector (Takara) and transformed into *Escherichia coli* DH5α for sequencing. The full-length cDNA sequence of AFO was obtained by jointing E1, E2 and E3 via the software DNAMAN. The AFO full-length cDNA was composed of the 5'- noncoding region, the open reading frame(ORF) and the 3'- noncoding region.

### 1.3 Construction of yeast expression vectors containing the ORF of AFO

The open reading frame (ORF) of AFO fused with an extra His6-tag at the C-terminus was amplified by PCR using the forward primer P3 (5'-GTCGAATTC**ATG**GCCACCACAACTGTC-3', EcoRI site underlined, and initiation codon in bold) and the reverse primer P4 (5'-AAGGAAAAAAGCGGCCGC**T**
**CA***ATGGTGGTGGTGATGGTG*CAATCGTCTCTCAATG
AAACTTTC-3', *Not*I site underlined, a His6 tag in italic and termination codon in bold). The PCR reactions were carried out using Pfu DNA polymerase with AFO 5'-ready cDNA as template under the following conditions: initial denaturation at 94℃ for 5 min followed by 5 cycles at 94℃ for 30 s, 72℃ for 4 min and 35 cycles at 94℃ for 30 s, 68℃ for 4 min, with a final extension at 72℃ for 10 min. Then the PCR product designated as afo was cloned into yeast vector pPIC9 downstream of α-factor signal peptide sequence by *EcoR* Ⅰ and *Not* Ⅰ restriction sites. And the recombinant vector after sequence confirmation was designated pPIC9-afo.

### 1.4 Recombinant AFO expression and Western Blotting identification

For expression of recombinant AFO, histidine-deficient *Pichia pastoris* strain GS115 was electrotransformed with pPIC9-afo linearized by *Sal* Ⅰ according to the manual of Pichia expression kit (Invitrogen). $His^+$-transformed methylotropic ($His^+$, $Mut^+$) yeast was selected using histidine-absent medium containing dextrose (MD) or methanol (MM) as the only carbon source.

Three randomly chosen transformants as well as an empty vector control were precultivated in buffered complex glycerol medium (BMGY) at 30℃ with shaking at 200 r/min to early logarithmic growth phase. The cells were harvested by centrifugation at 5000 × g for 15 min at 4℃. Then the cell pellets were resuspended in 10 mL of buffered complex methanol medium (BMMY) and added to 100 mL with BMMY in a baffled flask to an initial $OD_{600}$ of 2 − 6. The production of recombinant AFO was conducted at 30℃ for 4 days with shaking at 200 r/min. Methanol was added to the culture medium daily to a final concentration of 0.5%.

After 4-day methanol induction, the culture supernatant was obtained by centrifugation at 5000 × g for 15 min at 4℃ for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting identification. Supernatant samples were separated on 12% SDS-PAGE according to the method of Laemmli[19]. The gel was stained with Coomassie brilliant blue R-250 and a low molecular marker (Bio-Rad) was used for the estimation of protein size.

For western-blotting, supernatant samples were subjected to a SDS-PAGE as described above, then proteins in the gel without stain were transferred onto polyvinylidene difluoride (PVDF) membrane and blocked by 5% dried skim milk. Subsequently, the membrane was probed with horse-radish peroxidase (HRP)-conjugated anti-penta-His Antibody (Qiagen) and following washing steps, protein bands were revealed by exposure to diaminobenzidine (DAB) substrate (Qiagen).

### 1.5 Enzyme assay and protein determination

The activity of the rAFO was expressed by the decrease of $AFB_1$ between the reaction group and the control group. For the reaction group, $AFB_1$ (final concentration, 100 μg/L) was incubated at 30℃ for 30 min in a 200 μL assay mixture containing 0.04 mol/L $Na_2HPO_4$-0.02 mol/L citric acid buffer (pH6.0) and 10 μl of suitably diluted enzyme. The reaction was terminated by mixing with 300 μL methanol. For the control group, equal enzyme was added after the reaction was pre-terminated by methanol. For both reaction and control groups, the residual $AFB_1$ in the terminated mixture were extracted using 500 μL chloroform for three times, then all the extract were transformed to a new eppendorf tube and evaporated under nitrogen gas. The evaporated residue was dissolved in 200 μL mobile phase for the quantitative analysis with HPLC. Meanwhile, 0.125 μg – 2.5 μg $AFB_1$ standard were also dissolved in 200 μL mobile phase and analyzed by HPLC for quantitation of $AFB_1$. HPLC analysis was performed on a Shimadzu HPLC System (LC-20AT Prominence Liquid Chromatograph, Japan) using a Shim-pack VP-ODS C18 (150 mm × 4.6 mm, 4.6 μm) column. The mobile phase was methanol-acetonitrile-aquafer (60/150/490, V/V/V) at a flow rate of 1ml/min and the sample temperature was set at 40℃. $AFB_1$ was detected using a RF-10AXL fluorescence detector (Shimadzu, Japan) with excitation wave length of 365 nm and emission wave length of 425 nm. Raw data were evaluated by the HPLC LCsolution software system (Shimauzu, Japan).

One unit of the rAFO activity was defined as the amount of enzyme that transforming 1 pmol of $AFB_1$ equivalent per minute. Specific activities are expressed as units per milligram of protein.

Protein concentrations were measured by the method of Bradford with bovine serum albumin (BSA) as the standard.

### 1.6 Purification of recombinant AFO

For purification of the recombinant AFO, transformant whose supernant with higher special activity was subjected to a high cell density fermentation using a 5L jar fermenter (BTF-5L, Biotop, China). The whole process of fermentation was consist of three stages; (i) a glycerol batch phase generating biomass; (ii) a glycerol feed phase for continued biomass generation and derepression of the alcohol oxidase promoter AOX1; and (iii) a methanol feed phase for induction of expression[20]. The transformant was cultivated at 30℃ in 2 L of basal salt medium (BSM) containing 0.01% antifoam. Methanol induction fermentation was maintained for 77 h after glycerol batch and fed-batch phases.

Purification procedures were carried out at 4℃ unless otherwise stated. Broth were harvested after nearly 4 days of fermentation (centrifugation, 10000 × g, 4℃, 30 min), and the supernatant was filtrated through membrane filters with pore size of 0.45 μm (CFP-4-E-4X2MA, GE USA). The filtrate with the volume of 1800 mL was concentrated about 20-fold and buffer-exchanged into equilibrium buffer (0.05 mol/L Tris-Cl pH8.0) using a Kvick Lab SCU (molecular weight cutoff of 10 kDa, GE, USA). The concentrated

enzyme was applied to AKTA explore100 (GE Healthcare) equipped with a HisTrap HP 1 mL column pre-equilibrated with equilibrium buffer (0.05 mol/L Tris-Cl pH8.0). After sample was loaded on, the column was washed with equilibrium buffer (10 mL) followed by the same buffer containing 10 mmol/L imidazole (10 mL) at a fiow rate of 1 mL/min. Then His6-tag fused rAFO were eluted with equilibrium buffer containing 500 mmol/L imidazole. Active fractions were combined and concentrated using an Amicon Ultra-15 (molecular weight cutoff of 30 kDa, Millipore).

### 1.7 Peptide mass fingerprinting (PMF) identification of purified rAFO

The purified rAFO was subjected to peptide mass fingerprinting (PMF) analysis for further identification. The electrophoresis strip of the rAFO was excised from the Coomassie stained gel and the gel pieces were subjected to DTT reduction and alkylation. Digestion was performed with trypsin, and aliquots of the supernatants were then incorporated into a fast evaporation nitrocellulose matrix with α-cyano-4-hydroxy cinnamic acid as the matrix substance. The mass spectrometric measurements were performed on a Reflex MALDI-TOF mass spectrometer (Bruker, Germany) equipped with an ion gate and pulsed ion extraction. The acceleration voltage was set to 20 kV and the reflector voltage 23 kV. This work was performed by the National Center of Biomedical Analysis, Academy of Military Medical Sciences, China.

### 1.8 Biochemical characterization of purified rAFO

Investigation to find out the optimum pH for the rAFO activity was carried out in $Na_2HPO_4$-citric acid buffers with various pH values (pH4.0 – 8.0). While the optimum temperature for the rAFO activity was determined by incubating the enzyme in 0.04 mol/L $Na_2HPO_4$ – 0.02 mol/L citric acid buffer (pH 6.0) at different temperatures (20℃ – 50℃). Other procedures involved in activity determination were all the same as the standard assay.

Then activities of the rAFO were measured under the optimal conditions determined above at different $AFB_1$ concentrations range from 100 μg/L – 300 μg/L. The Vmax and Km value were calculated using a Lineweaver-Burk plot.

On stability studies, the rAFO was incubated in $Na_2HPO_4$-citric acid buffers with various pH values (pH4.0 – 8.0) at 30℃ for 90 min to determine its pH stability, and then remaining activities of these treated enzymes were measured by the standard assay procedure. For thermal stability determination, the purified rAFO in 0.04 mol/L $Na_2HPO_4$ – 0.02 mol/L citric acid buffer (pH 6.0) was incubated at different temperatures (20℃ – 50℃) for 90 min. These treated enzymes were subject to the standard enzyme assay after being cooled on ice for 30 min.

## 2 Results

### 2.1 Full-length cDNA and ORF cloning of the AFO gene

An 803 bp fragment of the AFO cDNA named E1 was isolated by RT-PCR using the degenerated primers and total RNA as the template. Based on the sequence information of E1, specific primers for the enzyme were designed and used for RACE-PCR to obtain the 5'- and 3'-cDNA ends. And PCR products of 3'- and 5' RACE termed as E2 and E3 were 750 bp and 1530 bp respectively. By joining E1, E2 and E3, the full-length cDNA of AFO was 2321 bp long with a coding region of 2088 bp, corresponding to a protein of 695 amino acid residues. This cDNA sequence of AFO was deposited in GenBank (Accession No. AY941095).

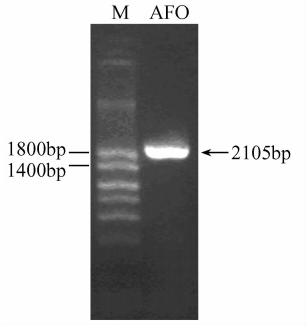


Fig. 1 PCR amplification of the open reading frame (ORF) of AFO. M: DNA ladder; AFO: product of the open reading frame (ORF) amplification by PCR.

ORF cloning of AFO gene fused with an extra His6-tag at the C-terminus revealed a PCR product of 2105 bp (Fig. 1) whose sequence was completely in accordance with that of joined full-length cDNA.

### 2.2 Expression, and Western Blotting identification of the rAFO

Since the recombinant secretion vector pPIC9-afo was introduced into *Picha. pastoris* by homologous recombination at the chromosomal AOX1 promoter locus. The secretive expression of the rAFO was performed under the induction of methanol in shaking flask culture. The culture supernatants of three randomly selected transformants were sampled for the western blotting identification. As illustrated in Fig. 2, a single band was detected in two of the three transformants, while it was absent in both the empty vector control and transformant 3. The special activities of the supernatant of the transformant 1 and 2 were determined as described in section 2.5. Quantitation analysis showed that $AFB_1$ has a good linear relation with peak area score when its concentration was within the range of 25 μg/L – 500 μg/L ($Y = 0.0003X - 4.0801$, $R^2 = 0.999$). Activity determination revealed that the specific activities of the supernatant of the transformant 1 and 2 were 6.1 U/mg and 4.3 U/mg respectively. Thus transformant 1 with higher specific activity was chosen for further experiments.

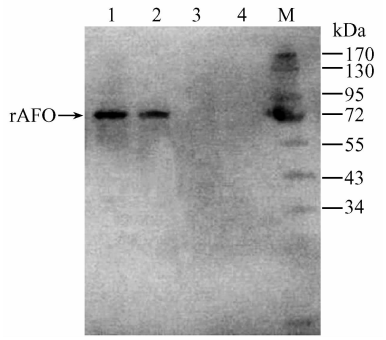


Fig. 2　Identification of rAFO by western blotting. Cell-free supernatant of transformant 1 (lane 1); transformant 2 (lane 2); transformant 3 (lane 3); empty vector control (lane 4); M: protein marker. The rAFO-sepecific bands are indicated by an arrow (approximately 76 kDa). For each sample 1 μg. supernatant was loaded in the tracks of the gel.

### 2.3 Purificaion and PMF identification of the rAFO

The transformant 1 was cultivated in a jar fermenter for the rAFO production. Supernatant of 1800mL was harvested after 77-hour methanol induction fermentation and then concentrated about 20-fold for subsequent affinity chromatography purification. Table 1 summarizes the results of a typical purification procedure. The rAFO was purified 5.99-folds from culture supernatant with a yield of 61.3% to a specific activity of 234 U/mg. The apparent molecular mass of the purified rAFO was estimated to be 76 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3).

The purified rAFO was analyzed by peptide mass fingerprinting (PMF) after trypsin digestion. The peptide mass fingerprint was submitted to protein database and searching results revealed a 63.2% coverage of the molecule when compared to the theoretical tryptic cleavage of the rAFO, indicating the correct expression of the rAFO.

Table 1　Summary of the purification of recombinant AFO

| Step | Total activity (U) | Total protein (g) | Specific activity (U/mg) | Recovery (%) | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 46566 | 1.19 | 39 | 100 | 1 |
| Concentrated crude extract | 38044 | 0.87 | 44 | 81.7 | 1.13 |
| Histrap FF | 28545 | 0.12 | 234 | 61.3 | 5.99 |

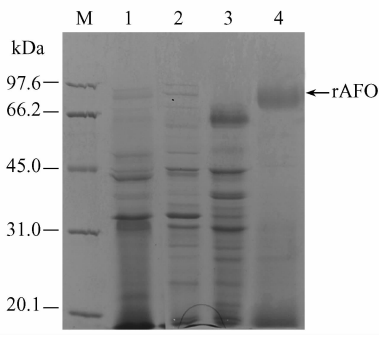


Fig. 3　Purification of rAFO. M: protein marker; Lane 1: cell-free supernatant induced by methanol for 77 h; Lane 2: Eluant from HisTrap FF column with equilibrium buffer; Lane 3: Eluant from HisTrap FF column with 10 mmol/L imidazole; Lane 4: 10-fold concentrated eluant from HisTrap FF column with 500 mmol/L imidazole. For each sample, 1 μg was loaded in the tracks of the gel.

### 2.4 Biochemical characterization of purified rAFO

Enzyme kinetics of purified rAFO was investigated since it was correctly expressed with activity. Firstly, activity of the rAFO was determined under various pH values (pH4.0 – 8.0) and temperatures (20℃ – 50℃) with $AFB_1$ as the substrate. Results suggested that the optimal pH and temperature of the rAFO were pH6.0 (Fig. 4A) and 30℃ (Fig. 4B) respectively. Then enzymatic kinetics of the rAFO was investigated under the optimal conditions. Enzyme activity was measured under different $AFB_1$ concentrations range from 100 μg/L – 300 μg/L. Vmax was determined as 1.096 ± 0.06 pmol/min and the Km value was 3.93 ± 0.20 × $10^{-6}$ mol/L calculated by the Lineweaver and Burk plotting method.

On stability of the enzyme, the assays of enzyme resistance to various pH indicated that rAFO could preserve half or more of its activity in weak acid solution (pH5.5 – 6.5) at 30℃ for 90 min (Fig. 4C). And the thermostability investigation revealed that this recombinant enzyme had a half-life of 90 min at 30℃ but was deactivated when treated at 45℃ for 90min (Fig. 4D).

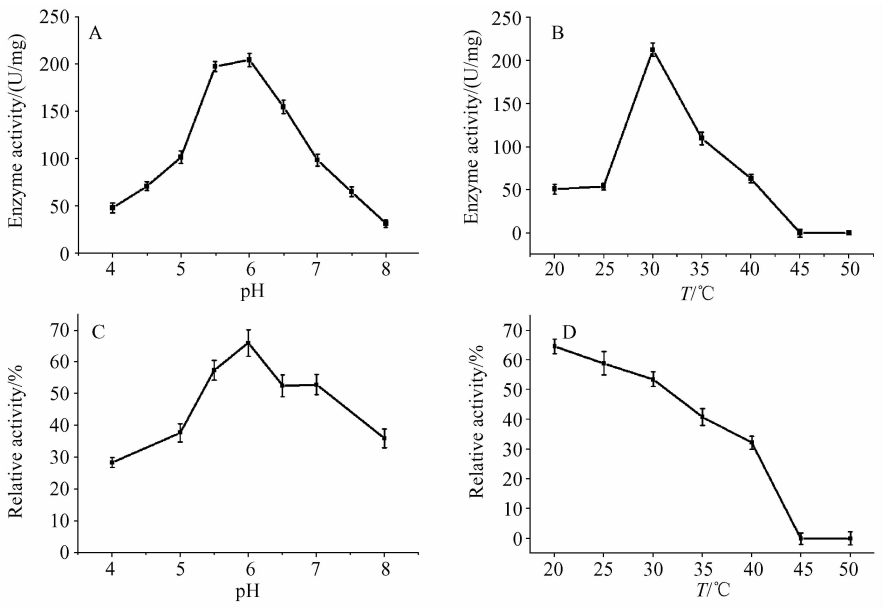


Fig. 4 Biochemical characterization of purified rAFO. (A) Optimum pH, 10 μL purified enzyme was applied for activity determination under buffers with different pH value (pH4.0 – 8.0) at 30℃; (B) Optimum temperature, 10 μL purified enzyme was applied for activity determination under different temperatures (20℃ – 50℃) at pH6.0; the special activity was calculated; (C) pH stability; (D) thermal stability. For each stability experimental, purified enzyme was was treated under different conditions for 90 min, then the treated enzyme was subjected to enzyme assay under standard conditions. Residual activity was expressed as percentage of activity of untreated enzyme. All experiments were performed in duplicate.

## 3 Discussion

Recently, interests in biotransformation of aflatoxin $B_1$ ($AFB_1$) have greatly increased. Some active extracts or enzymes had been reported with $AFB_1$ degradation function. However, many of them were limited to practical applications by either cofactor requirement[21] or long incubation time of 24 h or longer[7-8]. Aflatoxin-oxidase (AFO) isolated from *Armillaria tabescens* was firstly named aflatoxin-detoxifizyme (ADTZ) for its $AFB_1$-converting ability by our group[17]. Unlike other reported $AFB_1$-convergting

enzymes, AFO could effectively convert $AFB_1$ without any cofactors within 30min revealing a greatly potential application of this enzyme. Thus gene cloning and heterogenous expression of AFO were done for further research on its structure and function.

After the gene of AFO was cloned, a pairwise alignment was done between the amino acid sequences of the ten native AFO peptides and of the recombinant AFO, results indicated that six of these ten peptides were well matched. However, activity determination of the recombinant aflatoxin-oxidase (rAFO) provided the evidence that the rAFO could convert $AFB_1$, and the peptide mass fingerprinting (PMF) identification of the rAFO was in accordance with the pairwise alignment result. It could be considered that the rAFO had been successfully cloned and expressed. Since sample of native AFO for mass spectrometry (MS) identification was obtained from the native polyacrylamide gel electrophoresis (PAGE)[17], this meant that the purity of the purified native AFO used for MS identification was approximate 95%. Therefore, the four peptides of AFO poorly matched in the pairwise sequence alignment with the rAFO might be due to the impurity proteins of the native AFO sample.

On the other hand, the amino acid sequence of the rAFO was submitted to protein databases of NCBI for a protein blast. And result showed that the enzyme was similar to predicted basidiomycete dipeptidyl peptidases III with identities above 70%. However, electrochemical experiments using the rAFO-modified electrode as detecting element showed that rAFO could oxidize $AFB_1$ accompanying with hydrogen peroxide formation[22]. This leds us to believe that this enzyme was an oxidase, thus it was renamed aflatoxin-oxidase (AFO).

Unfortunately, we encountered difficulties in isolating and identifying the oxidized product of $AFB_1$ by the rAFO. For clearly interpretation of the catalytic mechanism of the rAFO, attempts are still being made to obtain and identify this oxidized product notwithstanding.

In addition, the rAFO could convert $AFB_1$ under mild conditions within relatively short time, indicating its promising application. But the pH and temperature stability of this enzyme was inadequate to meet practical application conditions. Thus we are also making efforts on enzymatic feature improvement by molecular directed evolution.

In conclusion, A gene encoding an enzyme afiatoxin-oxidase (AFO) with aflatoxin conversion ability was successfully cloned from *Armillaria tabescens*, and expressed in *P. pastoris*. This has made it much easily to produce this enzyme in an industrial scale. And characterization of the rAFO suggested its potential application. Further researches on elucidating the enzymatic reaction mechanism and on improving enzymatic features by directed evolution might be greatly helpful in its industrial application.

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# 假蜜环菌黄曲霉毒素氧化酶的基因克隆、表达、纯化及酶学性质分析

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**摘要：**【目的】黄曲霉毒素氧化酶(aflatoxin-oxidase，AFO)来源于假蜜环菌(Armillariella tabescens)的细胞内提取物，具有转化黄曲霉毒素 $B_1$(Aflatoxin $B_1$，$AFB_1$)的特性。为更进一步了解该酶的性质，我们克隆了 AFO 的基因，并进行了重组 AFO 蛋白的表达、纯化和酶学性质分析。【方法】本研究利用基质辅助激光解吸飞行时间质谱(MALDI-TOF-MS)获得的 AFO 短肽序列设计简并引物进行逆转录，再通过 cDNA 末端快速扩增(rapid-amplification of cDNA ends，RACE)技术获得了 AFO 基因的全长 cDNA 序列。构建重组表达载体 pPIC9-afo，在毕赤酵母中进行重组 AFO(rAFO)的融合分泌表达，用 Ni 离子螯合层析进行 rAFO 的纯化，获得有活性的 rAFO 后，对其进行肽质量指纹(peptide mass fingerprinting，PMF)鉴定和酶学性质分析。【结果】黄曲霉毒素氧化酶(AFO)基因的开放阅读框为2088 bp，编码 695 个氨基酸；肽质量指纹鉴定结果显示重组 AFO 的肽片段序列覆盖率为 63.2%。活性测定表明纯化后的重组 AFO(rAFO)比活力为234 U/mg；对 rAFO 进行酶学性质分析表明，对于底物黄曲霉毒素 $B_1$，rAFO 的 Km 值为 $3.93 \pm 0.20 \times 10^{-6}$ mol/L；反应最适温度为 30℃，最适 pH 为 6.0；30℃放置90 min后酶活力下降 50%；rAFO 在 pH5.5－7.0 之间酶活力较稳定，相对活力维持在 51%－65%之间。【结论】本文第一次成功克隆并重组表达了一种具有黄曲霉毒素 $B_1$ 转化功能的酶——黄曲霉毒素氧化酶(aflatoxin-oxidase，AFO)，纯化后的重组 AFO(rAFO)具有较好的黄曲霉毒素 $B_1$ 转化活性，为进一步研究和应用奠定了基础。

**关键词：**假蜜环菌，黄曲霉毒素氧化酶，cDNA 末端快速扩增，毕赤酵母

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