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Cloning and Molecular Study of *Aspergillus niger* Uric Acid Oxidase in *Escherichia coli*

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Urate oxidase enzyme or uricase (UOX) is a uric acid metabolizing enzyme that exists in most terrestrial animals, except primates (including human). An elevation in uric acid levels in the blood leads to many health problems, such as gout, electrolyte disturbances, and renal failure. Recently, recombinant UOX has been approved by the FDA to be used as a therapeutic agent for the treatment of hyperuricemia and Tumor Lysis Syndrome (TLS). Moreover, it is used to manufacture diagnostic kits for enzymatic uric acid determination. In the present study, we cloned the *UOX* gene from *Aspergillus niger*. The open reading frame of an UOX of 906 bp, encoding a protein of 302 amino acids, was synthesized using previous data from Genbank and cloned in pET-15b. The predicted amino acid sequence was used as a template to screen orthologues from evolutionarily related organisms and to explore their phylogenetic relationships. The monomeric subunit secondary structure was constructed; the molecular weight from the predicted amino acid sequences was 34.0 kDa and the isoelectric point value (pI) was7.19. The recombinant enzyme was expressed in soluble form as a 6-Histidine N-terminal fusion protein in *E. coli* BL21 (DE3) and purified using Ni-NTA-agarose affinity column. The UOX enzyme showed good activity toward uric acid.

Keywords: *Aspergillus niger*, urate oxidase, gene expression, Molecular modelling, Cloning, Phylogenetic analysis

Introduction

Uric acid is formed by xanthine oxidase which converts hypoxanthine produced from purine metabolism to uric acid and xanthin. In some organisms, uric acid is oxidized to 5–10 times more soluble derivative; allantoin[1-3], by urate oxidase or uricase (UOX). High plasma uric acid concentration (>6-7mg/dL) is considered as hyperuricemia. It could be produced as a result of excessive dietary protein, fat, and alcohol as well as inherited errors of purine-pyrimidine metabolism, or protein degradation[4]. Previous studies have reported microbial non-recombinant, as well as recombinant UOX from various organisms to reduce the uric acid levels[5-7]. Recently, recombinant UOX has been approved by the FDA to be used as a therapeutic agent for the treatment for hyperuricemia[8-10], and Tumor Lysis Syndrome[11]. Moreover, it is used to manufacture diagnostic kits for the enzymatic uric acid determination[12,13]. To the best of our knowledge, the recombinant UOX from *A. niger* has not been reported previously. Nowadays, *Escherichia coli* have been successfully used as biofactory for the production of many recombinant proteins[14-17]. Therefore, we aimed to investigate the production of recombinant UOX from *A. niger*, expressed in its soluble form in *E. coli*, and to investigate its activity. The an UOX gene was cloned into a pET15a vector, expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography on a Ni-NTA-agarose matrix, to evaluate its activity. Molecular study including multiple sequence alignment, secondary structure of the protein structure and phylogenetic analysis was carried out, to shed light towards the structural and catalytic features of the *A. niger* UOX.

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Materials and Methods

Materials and strains

In this work, we cloned the coding region of UOX from *Aspergillus niger* in a pET15 vector in *E. coli*

strain *BL21 (DE3)*, and the expression of recombinant UOX was assessed in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin. Ni-agarose was purchased from GenScript, USA. All chemicals and kits were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA, Bio Basic Inc., Toronto, ON, Canada, New England Biolabs, MA, USA and Qiagen, CA, USA.

Cloning

The open reading frame corresponding to UOX gene, from *A. niger* (Genbank Accession # DQ993354), was synthesized by Genscript, USA and subcloned in pET15b(+) expression vector (Novagen, Inc. Madison, USA) between *Nde*I and *Bam*HI restriction sites under the control of T7 promotor and ampicillin resistance gene for selection. The proper frame insertion and orientation of the gene were confirmed by sequencing before expression, and the recombinant plasmid was used to transform *E. coli* BL-21 *(DE3)* for overexpression.

An UOX expression

The cells were cultured in 25 mL LB medium containing 100 µg/ml ampicillin at 37°C under shaking at 160 rpm for 12-14h. Cells were adjusted to an optical density of 1 unit at 600 nm and used to inoculate 500 mL LB medium under the same conditions. The expression was induced by the addition of 1mM IPTG with continuous shaking. The cells were harvested by centrifugation for 5 minutes at 5000 rpm and re-suspended in extraction buffer (2ml/g biomass of 0.05 M PBS pH 8+ 0.001 M PMSF + 0.001 M DTT + 5% glycerol). The cells were lysed by ultrasonication (5 pulses, 5 sec each), and the bacterial lysate was centrifuged at 13,000 x g for 5 min, 4°C to remove the cell debris.

An UOX purification

Extract from a single positive colony of 500 ml LB liquid culture was applied onto a Ni-NTA-agarose column (0.5 x 1 cm) previously equilibrated with extraction buffer containing 0.025M imidazole. Non adsorbed protein was washed off with equilibration buffer until the absorbance at 280 nm decreased to zero. Bound UOX was eluted with equilibration buffer containing 0.25 M imidazole. Collected fractions (1 ml each) were assayed for UOX activity, and the protein content was determined by Coomassie G-250, according to Bradford (1976). Imidazole was eliminated using desalting column chromatography on Sephadex G25 (30 cm x 1.5 ids) previously equilibrated by PBS and the 6His tag was excised by thrombin using RECOMT-1KT Thrombin Clean Cleave™ kit (Sigma-Aldrich, Cat# T9685) using five units thrombin/mg protein at 25°C for 16 h followed by re-chromatography on Ni-NTA-agarose affinity matrix. The non-adsorbed protein contained in the enzyme without the 6His Tag was analyzed for its protein purity SDS-PAGE[18].

Assay of enzyme activity, and kinetic analysis

The UOX activity was assessed spectrophotometrically by following the disappearance of uric acid in the form of sodium urate as detected by a decrease in absorbance at 292 nm. The reaction mixture of 1 ml contained 0.1 M borate buffer pH 9.0 and 0.12 mM of uric acid. The enzyme activity was measured by the addition of enzyme to the reaction mixture and incubating at 30°C for 10 minutes, and the reaction was stopped by the addition of 0.2 ml of 1M KOH. One unit of an UOX is defined as the amount of enzyme necessary to transform 1 µmole of uric acid into allantoin in 1 minute at 30°C and pH 9.0[19].

Alignment of amino acid sequences and phylogenetic study

The deduced amino acids from the coding regions were compared with the existing sequences in the NCBI Protein Database (https://blast.ncbi.nlm.nih. gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to identify homologous sequences through PSI-BLAST. The secondary protein structure was predicted using the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/), sequence from different strains were aligned with the Clustal W program using the MAFFT Multiple Sequence Alignment (2011), color-coded to identify conserved amino acids and the phylogenetic tree was constructed using Jalview features (2011).

Results and Discussion

Cloning of the UOX from *Aspergillus niger*

The UOX gene from *A. niger* was chemically synthesized by Genscript company based on the Genbank accession number DQ993354. The coding region consists of 906 nucleotides which represent a putative UOX, and to the best of our knowledge, this gene has not been studied at the level of recombinant protein production, before. So, we used this sequence for further bioinformatics analysis to clone the gene and to overexpress it as a recombinant fusion protein.

The amino acid composition and primary structure of UOX

The ORF of the 906 nucleotides encodes a putative protein of 302 amino acids as predicted using BioEdit Program. The length of the protein coincides with the data obtained from many fungal UOX. On the contrary, the number of residues differs from bacterial UOX like in case of *Bacillus fastidious* which has 322 amino acid residues[20]. The calculated molecular weight of the predicted monomer using the program PROTEAN was 34 kDa, and the isoelectric points were 7.19.

The secondary structure of UOX enzyme

The secondary structure of *A. niger* was carried out (Figure 1). The monomer of UOX *A. niger* gave secondary structure similar to rasburicase of *A. flavus* regarding the number and arrangement of the alpha helices and Beta sheets[21]. The results were not surprising, considering a high ratio of identity and similarity between their sequences.

Amino acid sequence alignment and phylogenetic relationships

The sequence of rasburicase from *Aspergillus flavus* was used as a template to search form other fungal UOX that are not cloned or expressed elsewhere as recombinant proteins. A comparison between all these sequences was listed in table 1. Multiple Sequence Alignment of *A. niger* uricase with other species is shown in Figure 2. The *A. niger* UOX gene sequence was taken as a template to search for other fungal UOX. A comparison between all these

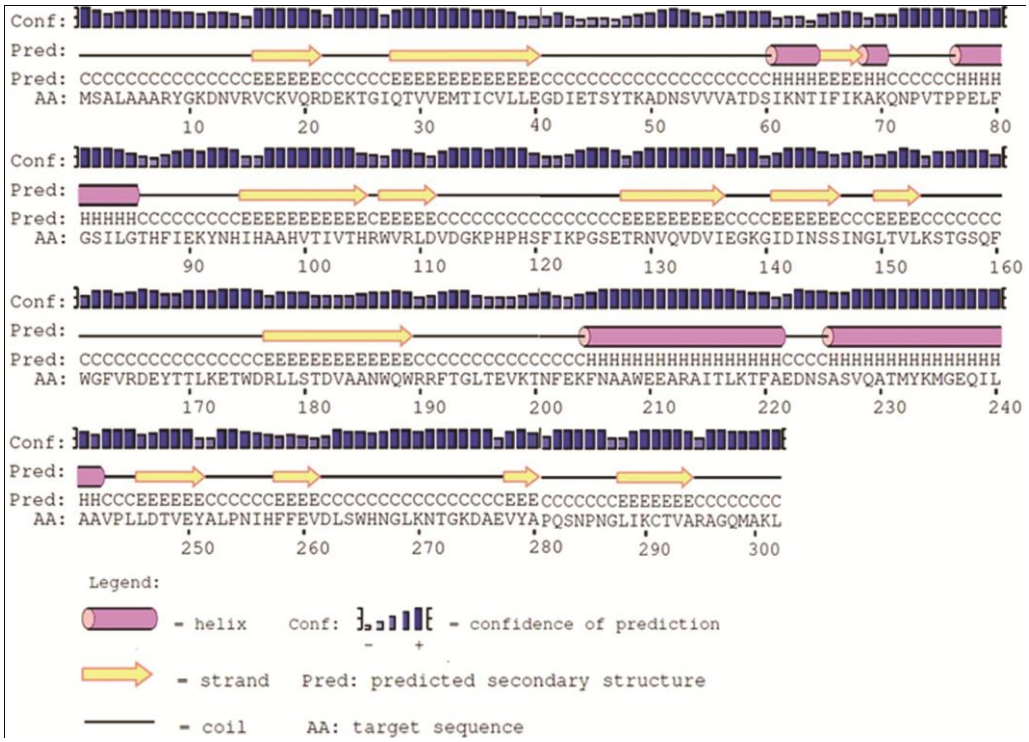

Fig.1 — Sequence annotations for UOX from *A. niger* showing the location of α-helices, and β-sheathsusing PSIPRED program.

Table 1 — Comparison of funcal UOX with rasburicase (*Aspergillus flavus*)

Accession #	Description	Max score	Total score	Query cover	E value	Identity%
XM_002377830.1	*Aspergillus flavus*	2050	2050	100%	0.0	100%
AB225284.1	*Aspergillus oryzae*	1862	1862	94%	0.0	99%
DQ993354.1	*Aspergillus niger*	1679	1679	81%	0.0	100%
XM_014678134.1	*Penicillium digitatum*	477	477	78%	3e-130	77%
XM_002151919.1	*Penicillium marneffei*	420	420	77%	6e-113	76%
XM_958021.3	*Neurospora crassa*	361	361	76%	3e-95	75%
XM_018296964.1	*Colletotrichum higginsianum*	305	305	76%	2e-78	74%
XM_003712795.1	*Magnaporthe oryzae*	270	270	77%	6e-68	73%
XM_011323208.1	*Fusarium graminearum*	237	237	77%	6e-58	72%

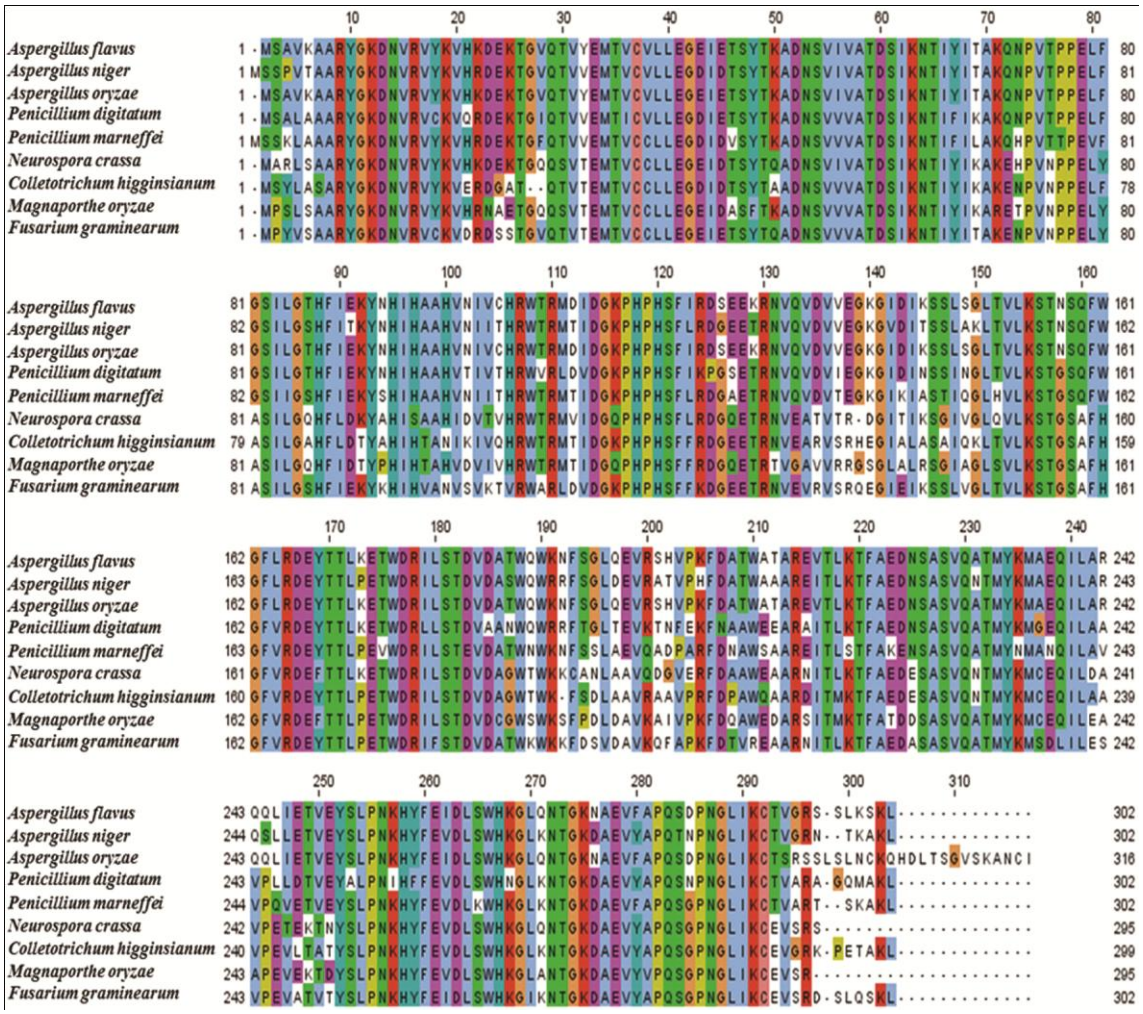

Fig.2 — Amino acid sequence alignment of *A. niger* UOX and potentially related proteins. The deduced amino acid sequence was compared with other sequences of the GenBank database (Table 1). The alignment was generated with the JalView program.

sequences is listed in Table 1. The percentage of identities was 100% with *Aspergillus flavus*, 77% with *P. digitatum*, 75% and 72% with *N. crassa* and *F. graminearum*, respectively. The phylogenetic relationship showed that *Aspergillus* sp. groups together and takes a different evolutionary line from *Penicillium* species and *Neurospora* species (Figure 3).

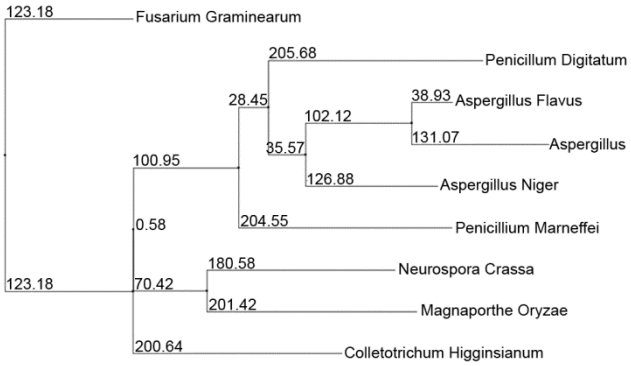

Fig.3 — Phylogenetic analysis of *A. niger* and potentially related fungal proteins from the GenBank database. The phylogenetic tree was generated with the Jal View program.

Expression and purification of recombinant UOX in *E. coli*

The coding sequence of the UOX gene from *A. niger* was cloned and expressed as 6HisTag fusion protein in *E. coli* BL21(DE3) under T7 promotor of pET-15b(+) vector. Cells from single colony harboring recombinant pET15b vector were used to inoculate LB medium for UOX expression. The cells were permitted to grow to mid-log (0.8 OD at 600 nm), to analyze the kinetics of UOX expression. Optimization of the expression conditions was done at either 0.1 or 1 mM IPTG concentration at different expression temperatures and duration (37 °C for 4 h, 30 °C for 8 h and 25 °C for 14 h). Samples of induced cells were analyzed by SDS-PAGE which revealed a

36kDa induced band which is consistent with the predicted molecular mass of the monomer of the recombinant enzyme (33.68 kDa) plus 2.18 KDa of the 20 amino acids of the His-Tag. The best induction was obtained at 37 °C in either 0.1 or 1 mM IPTG. The induction profile detected induced gene product starting from 30 minutes after addition with either 0.1 or 1 mM of IPTG, and the maximum quantity was reached after 4 h in case of using 0.1 mM IPTG and after 3 h with 1 mM IPTG. The soluble cytoplasmic extract was obtained by ultrasonication, and the clear homogenate was separated by centrifugation. The enzyme was purified by applying the cleared cell lysate on a Ni-NTA resin column in single-step affinity purification. The active purified enzyme was eluted from the column with 250 mM imidazole. The enzyme was purified with 5.6 folds over the crude extract.

UOX enzyme activity

The UOX activity was measured according to the method of Mahler (1970). The UOX from the crude extract of 1 gram biomass gave 20 units which is considered a good quantity. After affinity purification chromatography on Ni-NTA-agarose matrix, 95% of the activity was recovered. The purified extract was rapidly subjected to desalting on a Sephadex G25 column (1.4 id. × 30 cm) previously equilibrated by tris HCL buffer pH 8.0, to eliminate the imidazole, and to prepare the enzyme for the next step for excision of the 20 amino acids of the tag using thrombin enzyme. After desalting, we added glycerol to 20% concentration and the eluate was used for thrombin digestion for 16 h at room temperature (25 °C). A noticeable loss of enzyme activity was observed (Table 2). The results coincide with that of previous researches, were lactose and raffinose were used to improve the thermal stability of UOX from *A. flavus*. Lactose showed protective effects on UOX stability, while for raffinose, it is relatively compromised[22]. In the present study, urate oxidase from *A. niger* was successfully expressed in *E. coli* as a soluble active enzyme. Producing such active enzyme is of particular commercial importance since the soluble active form avoids many laboratory works that should be done in case of recombinant proteins produced in the form of inclusion bodies which requires subsequent trials for solubilizing and refolding of such proteins. The process of production and purification is powered by the addition of the 6 × His tag in the construct which facilitated the affinity purification of the recombinant protein in a simple one-step purification using Ni- NTA column. The yield after Ni-NTA-agarose column is about 75% which is close to the yield reported previously[22-24] for commercial rasburicase and native *B. subtilis* urate oxidase. The presence of the tag did not affect enzyme activity; in the contrary the elimination of the tag makes the enzyme less stable.

Table 2 — Summary of the purification stpes of anUOX

Step	Total activity (U/g biomass)	Yield%
Crude	20	100
Ni-NTA-agarose column	15	75.0
Sephadex G25 column	13	65.0
Thrombin treatment	2	10.0
Activity in lactose	1.95	10.0
Activity in raffinose	1	5.0

UOX stability at -20 °C and 25 °C and the effect of metal of UOX activity

We studied the stability of the recombinant UOX from *A. niger* during storage. The enzyme retained 50% of its activity after freezing and thawing three times at -20°C for 48h each time. However, the enzyme lost 90% of its activity after incubation for 16 h at 25°C. The addition of 3 µl of 50 mM of either cysteine, NaCl, or $CaCl_2$ enhanced the enzyme activity (doubles the enzyme activity) while $CuCl_2$ inhibited the UOX activity by 40%. The low stability of UOX is an obstacle to many researchers, and several methods have been postulated to stabilize UOX including the addition of lactose and raffinose[22], and making directed mutation in the nucleotide sequence replacing one amino acid with other[25]. Mutants of *Bacillus subtilis* 6E9 and 8E279 exhibited 2.99 and 3.43 times higher catalytic efficiency, respectively.

Abbreviations

Urate oxidase (UOX); Uric acid (UA); isopropyl 1-thio-β-galactopyranoside (IPTG); *Aspergillus niger (A. niger);* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); phosphate buffer saline (PBS); phenyl methyl sulfonyl fluoride (PMSF); dithiothretol (DTT).

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