Recombinant production of *Aspergillus Flavus* uricase and investigation of its thermal stability in the presence of raffinose and lactose

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Abstract *Aspergillus flavus* uricase (Rasburicase) with a molecular mass of 135 kDa is currently used for the treatment of gout and hyperuricemia occurring in tumor lysis syndrome. To characterize the effects of raffinose and lactose osmolytes on the uricase structure, its coding sequence was cloned, expressed in *E. coli* BL21, and purified by Ni-NTA agarose affinity chromatography. Thermal inactivation studies at 40 °C showed that nearly 15% of UOX activity was preserved, while the presence of raffinose and lactose reduced its activity to 35 and 45% of its original activity, respectively. Investigation of UOX thermal stability at 40 °C in the course of time showed that the enzyme relatively lost almost 60% of its original activity after 40 min, whereas more than 50% of UOX activity is preserved in the presence of lactose. Estimation of thermal inactivation rate constant, k_{in} , showed that the UOX k_{in} and UOX k_{in} in the presence of raffinose was unchanged (0.018 min^{-1}), whereas for the presence of lactose, it was 0.015 min^{-1} . Half-life and T_m analysis showed that UOX half-life is almost 38 min and addition of raffinose did not change the half-life, whereas the presence of lactose had remarkable impact on UOX half-life (46 min). The presence of raffinose increased UOX T_m to a lesser extent, whereas lactose notably enhanced the T_m from 27 to 37 °C. Overall, our findings show that lactose has protective effects on UOX stability, while for raffinose, it is relatively compromised.

Keywords Uricase · Osmolyte · Thermal stability · Recombinant · *Aspergillus flavus*

Introduction

Uricase or urate oxidase (UOX) (EC 1.7.3.3) is an enzyme, belonging to the family of oxidoreductases, which catalyzes the oxidation of uric acid to 5-hydroxyisourate and H_2O_2 and eventually allantoin and CO_2 (Shaaban et al. 2015; Wu et al. 1989). Functional uricase is present in most organisms and microorganisms but primates, birds, and some reptiles' species have lost it during evolution of their genome (Dabbagh et al. 2016; Keilin 1959; Kratzer et al. 2014). As a result, any imbalance in production and excretion rate of uric acid leads to hyperuricemia manifested as gout (Schumacher and Chen 2006; Sherman et al. 2008b). The concentration of uric acid in human serum is 1.5–6.0 mg/dl for women and 2.5–7.0 mg/dl for men (Maiuolo et al. 2016). Any increase in the catabolism of purines or decrease in uric acid excretion leads to build up of uric acid in the form of monosodium urate crystals in joints or soft tissues (Pascual and Pedraz 2004). Moreover, hyperuricemia is also observed tumor lysis syndrome (TLS) and is caused by massive tumor cell lysis followed by catabolism of the nucleic acids to uric acid after chemotherapeutic treatment (Pession et al. 2008; Pui et al. 2001a, b).

Allopurinol, a competitive inhibitor of xanthine oxidase is a common agent for blocking the conversion of hypoxanthine and xanthine to uric acid (Pession et al. 2008), and thereby, allopurinol reduces uric acid production not its conversion to uric acid and hence excretion. In addition, UOX from *Aspergillus flavus* (Rasburicase) is mostly used for treatment of gout disease and hyperuricemia occurring in TLS (Alakel et al. 2017). The advantage of urate oxidase treatment over allopurinol is that it reduces preexisting

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hyperuricemia and does not lead to accumulation of xanthine or hypoxanthine which occurs after allopurinol uptake.

Structurally, *Aspergillus flavus* UOX is an enzyme with 302 amino acids and molecular weight of 34.2 kDa. The functional form of enzyme is a globular homotetramer with mass of 135 kDa (Gabison et al. 2010; Retaillieu et al. 2004, 2005). Cloning of the gene encoding *Aspergillus flavus* UOX was performed in 1992 (Legoux et al. 1992). To date, several groups have cloned and over-expressed UOX in various hosts either as a fusion protein with tags or non-tagged protein (Fazel et al. 2014; Legoux et al. 1992; Li et al. 2006).

Osmolytes are small organic compounds naturally found in living cells and protect cells against osmotic stresses (Bolen 2001; Khan et al. 2010). Basically, they are classified into non-protecting and protecting osmolytes. A typical example for non-protecting osmolytes is urea which accumulates in mammalian renal medulla cells. The protecting osmolytes fall into three classes: amino acids, certain methylamines, and polyols which include glycerol, sucrose, trehalose and other sugars (Bolen 2001). It is evident that osmolytes stabilize protein structures and protect them from unfolding. The mechanism by which osmolytes mediate protein stability is called preferential exclusion or preferential hydration of water (Street et al. 2006). On the other hand, preferential exclusion of an osmolyte from immediate vicinity of a protein implies that its concentration around the protein is lower than its concentration in the bulk phase. In other words, concentration of water molecules surrounding a protein must be higher than that in the bulk phase (Bolen 2001). This phenomenon drives the equilibrium of protein folding toward the native form.

Several studies evaluated the effects of osmolytes on the stability of various proteins and enzymes such as catalase (Sepasi Tehrani et al. 2013), monoclonal antibody (Barnett et al. 2016), and recombinant interferon beta-1b (Mahjoubi et al. 2015). However, the impact of osmolytes on uricase enzyme stability is not fully delineated (Caves et al. 2013). Despite the importance of *Aspergillus flavus*, UOX is a therapeutic enzyme and widely used for hyperuricemia therapy, there is lack of enough data on the effect of osmolytes on UOX thermal stability, activity, and function. Therefore, here, we first produced a recombinant UOX and second investigated its thermal stability in the presence of two osmolytes raffinose and lactose.

Materials and methods

NcoI, *XhoI*, T4 DNA ligase, isopropyl- β -D-thiogalactopyranoside (*IPTG*), and molecular mass marker (*SM0431*) were purchased from Fermentas. DNA ladder (*SL5051*)

and agarose were obtained from Sinaclon Co. Plasmid extraction kit and PCR purification kit were obtained from Bioneer Corp. Ni-NTA agarose (30210) was from QIAGEN. Tryptone, and yeast extract was obtained from Scharlau company. Lactose, raffinose, and all SDS-PAGE chemicals were purchased from Merck. Kanamycin and Uric acids sodium salt (U2875) were from Sigma-Aldrich.

Microorganisms and plasmids

pET-28a(+), *E. coli* DH5 α , *E. coli* BL21 (DE3) *pLysS* cells were obtained from Pasteur institute of Iran. *Aspergillus flavus* UOX gene was retrieved from Genbank under the accession number X61766.1 and its sequence synthesized and cloned into *pGEM-B1* vector by Bioneer corp. Then, it is sub-cloned into *pET-28a(+)* under the control of a strong bacteriophage T7 promoter between *NcoI* and *XhoI* which its expression is induced by IPTG or lactose.

Construction of expression vector

To subclone UOX gene from cloning vector *pGEM-B1* into *pET-28a(+)*, *pGEM-B1* harboring UOX was digested with *NcoI* and *XhoI*, was loaded on the 1% agarose gel and the expected band (929 nucleotide) was cut out and purified using DNA gel extraction kit. Next, ligation reaction started by mixing digested UOX gene with digested *pET-28a(+)* in the ratio of 5:1 and T4 DNA ligase at 24 °C for 2 h. The resulting construct named *pET-28a(+)-AFUOX*. Once the plasmid constructed, it was transformed into *E. coli* DH5 α competent cells using the heat-shock method and eventually bacterial cells plated on LB agar plates containing 50 μ g/ml of kanamycin and incubated at 37 °C overnight (O/N).

Confirmation of cloning

Following heat-shock transformation of ligation product into *E. coli* DH5 α competent cells and growing them on kanamycin agar plates at 37 °C O/N, two positive bacterial colonies were randomly picked up from agar plates and grown in LB medium, and their plasmid was extracted by plasmid miniprep kit. The purified plasmid went under digestion check with two *NcoI* and *XhoI*. Finally, *pET-28a(+)-AFUOX* construct was sequenced on the ABI 3730XL DNA Analyzer by Bioneer Corp using T7 promoter and T7 terminator universal primers.

UOX induction and purification

A fresh bacterial colony harboring the constructed *pET-28a(+)-AFUOX* was used to inoculate into 10 ml of LB medium containing 50 μ g/ml of kanamycin followed by

incubation at 37 °C under vigorous shaking O/N. Then, 200 ml of LB media was inoculated with 2 ml of O/N culture and grown at 37 °C with vigorous shaking until the culture OD reached at 0.6–0.7. IPTG was then added to the final concentration of 1 mM and the culture was grown at 22 °C for 12 h. Following incubation, bacteria pellet was collected and resuspended in lysis buffer (Tris buffer, pH 7.4, containing 300-mM NaCl and 5-mM imidazole) and sonicated (10 cycles of 15-s pulses at 50% amplitude, 70 W) on ice. Purification of His6-tagged fusion UOX was carried out using Ni-NTA column. Briefly, the column was first washed with 9 ml of distilled water and then equilibrated with 9 ml of lysis buffer (3 column volumes). Following centrifugation at 10,000g, supernatant was loaded onto a Ni²⁺-NTA column. Then, the column was washed with 20 ml of wash buffer (Tris buffer, pH 7.4 containing 300-mM NaCl and 25-mM imidazole). Thereafter, recombinant UOX was eluted using elution buffer (Tris buffer containing 300 mM imidazole). Finally, the purity of the UOX protein was analyzed by SDS-PAGE 12.5% stained by Coomassie Brilliant Blue (Imani et al. 2017). Protein concentration was then determined by Bradford assay using BSA as the protein standard (Bradford 1976).

HPLC analysis of uricase

In addition to SDS-PAGE, one can use analytical RP-HPLC for analysis of protein purity. Typically, reversed-phase chromatography was performed on a ZORBAX 300SB-C18 column (4.6 mm × 150 mm) (Agilent Technologies 1200 series, USA). 20 µl of purified uricase was injected onto the C18 column which was preequilibrated with 0.08% TFA and eluted with mobile phase of acetonitrile/borate buffer (60/40 v/v ratio) under the flow rate of 1 ml/min. The column eluent was monitored at 220 nm and the area of peak was analyzed by HPLC software and considered as a percentage of purity.

Uricase assay

Since UOX converts uric acid to allantoin and hydrogen peroxide and only uric acid absorbs light at 293 nm, the rate of oxidation of uric acid to allantoin is directly followed spectrophotometrically at 293 nm. The enzymatic reaction was performed at room temperature and the reaction volume contained 960 µl of 20-mM boric acid buffer pH 8.5, 20 µl of 48-µM uric acid solution and 20 µl of UOX enzyme. The reaction was stopped after 6 min and the decrease in absorbance was measured at 293 nm. One unit of UOX activity was defined as the amount of enzyme that catalyzes the conversion of 1 µM uric acid to allantoin per minute at pH 8.5 (<http://www.worthington-biochem.com/up/assay.html>; Li et al. 2006; Mahler et al. 1955). The

enzyme activity was calculated using the following equation:

$$U/ml = \frac{(\Delta A_{293}/\text{min test} - \Delta A_{293}/\text{min blank})(df)}{12.6 \times 0.02}$$

whereas df is the dilution factor, 12.6 is the millimolar extinction coefficient of uric acid at 293 nm, and 0.02 is the volume (in ml) of enzyme used.

Uricase characterization

Optimum temperature and osmolytes

The optimum temperature was evaluated by measuring urate oxidase activity after preincubation at defined temperature from 5 to 60 °C with 5 °C interval for 6 min. To find out the optimum concentration of osmolytes on thermal stability of UOX, various concentrations of raffinose and lactose were examined on the enzyme activity at 40 °C. Practically, equal amounts of UOX and 2X concentrations of osmolytes were mixed and incubated at 40 °C for 30 min, and after cooling on ice for 30 min, the enzyme activity was measured by monitoring of the absorbance at 293 nm.

Thermal inactivation and stability

To examine the thermal inactivation, purified UOX is incubated at temperature range (from 0 to 70 °C) for 10 min and then transferred onto ice for 30 min to restore its native folding. Thereafter, the enzymatic reaction was performed at room temperature and the activity was measured by reading absorbance at 293 nm. For thermal stability studies, enzyme was incubated at 40 °C for 60 min for a defined period of time (5 min). Then, 20 µl of UOX was withdrawn and incubated on ice for 30 min and assayed. The residual activity was expressed as a percentage of the original activity. It must be mentioned that to test the effect of osmolytes on UOX activity, above-mentioned enzymatic assays were performed in the presence of raffinose and lactose.

Results

Construction of pET-28a(+)-AFUOX construct

The UOX gene was cloned into *pET-28a(+)* expression vector between *NcoI* and *XhoI* restriction sites. To verify the constructed recombinant vector, both restriction digestion and sequencing were performed. The exact cloning site of UOX within vector is shown in Fig. 1a. The undigested and digested *pET-28a(+)-AFUOX* are shown in the Fig. 1b. As shown in Fig. 1, the inserted fragment cuts out from the

construct is 929 bps. Finally, to ensure the correct orientation of the inserted fragment of UOX gene, the construct was sequenced with an ABI 3730XL DNA Analyzer.

Uricase production

The expression of UOX was induced with 1 mM IPTG. SDS-PAGE analysis showed a higher purity level following purification by Ni^{+2} -NTA affinity chromatography. The purified UOX is apparent in Fig. 2a lane 2. Analytical RP-HPLC has been reported as a powerful technique for the determination of protein purity (Khaksa et al. 1998). To further confirm the UOX purity, RP-HPLC analysis was performed. As evident in Fig. 2b, there is only a major peak with a smaller shoulder on the right. Main peak corresponds to the purified UOX with a retention time of 1.37 min. Areas of major and minor peaks were calculated 98 and 2%, respectively.

Optimum temperature of uricase

To determine the optimal temperature for the function of the recombinant UOX, its activity was measured under various temperatures. As shown in Fig. 3, uricase is highly active in the range of 15–25 °C with the peak point at 25 °C. Remarkable reduction in the activity was observed over 35 °C, so that the enzyme lost approximately 35% of its original activity.

Thermal inactivation studies

The thermal inactivation of uricase was investigated in the presence of optimum concentrations (20%) of raffinose and lactose by following enzyme activity at different temperatures. As shown in Fig. 4, by increasing the temperature, uricase loses its original activity. Enzyme activity is diminished 60% when it is incubated at 30 °C for 10 min. Incubation of uricase

with 20% of raffinose and lactose preserved enzyme activity over 40 and 60% of its original activity, respectively. By calculating the temperature at which the enzyme activity was diminished by 50% of original activity, we reach T_m for uricase in the absence and presence of raffinose and lactose as 27, 30, and 37 °C, respectively (Table 1).

Thermal stability studies

In addition to thermal inactivation studies, the thermal stability of uricase was investigated at 40 °C for 60 min. As indicated in Fig. 5, after 5 min, there is a 25% decline in enzyme activity. However, at the same time, the uricase incubated in osmolytes only loses 15% of its activity relative to its initial activity. Following longer times, a decrease in uricase activity was observed for in the absence and presence of osmolytes.

Considering enzyme inactivation as an irreversible one-step process and following enzyme activity versus time, one can determine enzyme inactivation rate constant (k_{in}) and half-life using a first-order rate equation. By plotting $\ln$ of enzyme activity versus time which is linear, the slope is $-k_{in}$ (Fig. 6). The half-life ($t_{1/2}$) of the activity (the time needed to lose half of the activity) can be calculated using $t_{1/2} = 0.693/k$. As apparent in Table 1, uricase k_{in} in the absence and presence of raffinose is 0.018 min^{-1} whereas it is reduced when incubated with lactose (0.015 min^{-1}). Surveying enzyme half-life in the absence and presence of osmolytes, the data indicated that raffinose did not affect uricase half-life but lactose elongated its half-life for 8 min.

Discussion

An enzyme with diverse applications in medicine and industry ranging from therapeutics and diagnosis to biosensors technology, namely urate oxidase, has been the

Fig. 1 Molecular map of *pET-28a(+)-AFUOX* construct and verification of cloning. **a** Place of UOX on *pET-28a(+)* plasmid and total size which is 6160 bp after cloning. **b** Miniprep construct (lane 2) and double digested construct (lane 3). The lower band is 929 bp cloned UOX gene. The map drawn by *ApE 2.0.47 A* Plasmid Editor software

Fig. 2 Verification of uricase purity by SDS-PAGE (a) and RP-HPLC (b). **a** Lane 1 protein marker; lane 2 fraction of purified uricase; **b** HPLC chromatogram of purified UOX. The column was C18 column. Mobile phase of acetonitril/borate buffer (60/40 v/v ratio) with the flow rate of 1 ml/min. The wavelength used for recording of column eluent was 220 nm

Fig. 3 Effect of various temperatures on enzyme activity. Purified uricase was incubated under different temperatures for 6 min and assayed as described in the “Materials and methods” section. The error bars are based on the standard of the mean ($n = 3$), where n is the number of independent experiments

subject of most research studies (Arora et al. 2014; Camalleri and Malaguarnera 2007; Kant et al. 2016; Pasut et al. 2008; Sherman et al. 2008; Zhou et al. 2016). The major drawback in protein utilization is mainly its conformational and kinetic stability weakness (De Laet et al. 2016). This setback prompts researchers to improve proteins stabilization through diverse strategies. In this study, we cloned, expressed, purified, and, for the first time, investigated the impact of two osmolytes, raffinose, and lactose on uricase thermal stability.

To produce and obtain a sufficient amount of UOX, we synthesized and cloned UOX gene into *pET-28a(+)* between two restriction sites *NcoI* and *XhoI* under control of T7 promoter (Fig. 1a). For confirmation of cloning, both double digestion test (Fig. 1b) and sequencing were performed (data not shown). Since purification of a protein demands a sophisticated purification step which, in turn, diminishes its yield of purification, we inserted 6xHis tag

coding sequence at the C-terminus of recombinant UOX before stop codon, so that one-step purification method using affinity chromatography is successfully applied for purification. RP-HPLC is a powerful technique for purification and analysis of small molecules, peptides, and proteins (Golabi et al. 2016; Moussa et al. 2010; Ranga et al. 2009). In order to analyze UOX purification, both SDS-PAGE and RP-HPLC techniques were employed. As Fig. 2 states, UOX was successfully purified, so that the HPLC results indicated over 98% purity.

Once UOX purified, optimum temperature and pH were characterized. As indicated in Fig. 3, the optimal temperature for enzyme activity is 25 °C, while at other applied temperatures, the activity is decreased. Estimation of UOX optimum pH under different pH range (pH 2–13) showed that UOX is highly active at pH 8.5 (data not shown).

To elucidate UOX structural and functional stability characteristics, thermal inactivation and thermal stability

Fig. 4 Thermal inactivation of uricase in the presence of osmolytes. 20 μ l of purified enzyme incubated in temperature range of 0–70 °C for 10 min and then transferred on ice for 30 min and assayed. The remaining activity was expressed as a percentage of original activity. (open square) Uricase, (filled triangle) uricase in 20% raffinose, and (open circle) uricase in 20% lactose. The error bars are based on the standard of the mean ($n = 3$), where n is the number of independent experiments

Table 1 Inactivation parameters of uricase in the absence and presence of osmolytes

	k_{in} (min^{-1})	$t_{1/2}$ (min)	T_m (°C)
E	0.018	38.5	27
E + Raf	0.018	38.5	30
E + Lac	0.015	46.2	37

The errors are associated with the parameters falls within $\pm 10\%$ of the values

experiments were conducted. As shown in Fig. 4, measurement of thermal inactivation of UOX indicates approximately 70% loss in activity compared to the original activity after incubation at 30 °C for 10 min. However, in the presence of raffinose and lactose, uricase almost lost 55 and 40% of its initial activity, respectively. Estimation of enzyme activity at 40 °C showed that nearly 15% of UOX activity was preserved after 10 min of incubation. However, incubation of enzyme in the presence of raffinose and lactose improves the activity by 35 and 45%, respectively, of its original activity. The results suggest that in the presence of raffinose and lactose, UOX displays high resistance to heat inactivation in comparison to UOX without osmolytes. Furthermore, investigation of UOX thermal stability at 40 °C in the course of time showed that the enzyme relatively lost more than 60% of

its original activity after 40 min, while in the presence of lactose, UOX preserved more than 50% of its activity. The effect of raffinose on the thermal stability of UOX is only significant at the early 30 min of incubation. However, in the presence of lactose, UOX preserves its activity for approximately 55 min.

To further elucidate the effects of osmolytes on thermal stability of UOX, we investigated thermal inactivation rate constant, k_{in} , in the absence and presence of osmolytes. Our results showed that the UOX k_{in} and UOX k_{in} in the presence of raffinose was the same (0.018 min^{-1}) while the presence of lactose had considerable effect of k_{in} . The slope of thermal inactivation of UOX in the presence of lactose is milder rather than for that of for UOX alone and UOX in the presence of raffinose, so that its value is 0.015 min^{-1} . These values indicate resistance of UOX structure in the presence of lactose and hence driving a delay in thermal inactivation.

In addition, considering enzyme half-life and T_m as criteria of enzyme stability, we calculated UOX half-life and T_m in the presence of osmolytes. As shown in Table 1, the UOX half-life is almost 38.5 min and the presence of raffinose did not change the half-life, while the presence of lactose had remarkable impact on UOX half-life, so that it shifted the enzyme half-life to 46 min. Determination of T_m indicated that the presence of raffinose increased UOX

Fig. 5 Thermal stability of uricase in the presence of osmolytes. Uricase was incubated at 40 °C for 70 min and at a given time period (5 min), 20 μ l of UOX was removed after incubation on ice for 30 min assayed. (open square) Uricase, (filled triangle) uricase in 20% raffinose, and (open circle) uricase in 20% lactose. The error bars are based on the standard of the mean ($n = 3$), where n is the number of independent experiments

Fig. 6 Logarithmic representation of UOX thermal stability in the absence and presence of raffinose and lactose. (open square) Uricase, (filled triangle) uricase in 20% raffinose, and (open circle) uricase in 20% lactose. The error bars are based on the standard of the mean ($n = 3$), where n is the number of independent experiments

T_m to some extent, whereas lactose notably enhanced the T_m from 27 to 37 °C.

Although the exact mechanism(s) governing UOX thermal inactivation remains unclear, it is mostly suggested that its tertiary and quaternary structure play a potential role in its susceptibility to environmental conditions. Moreover, several studies have emphasized the importance of osmolyte application for stabilization of proteins structures (Barnett et al. 2016; Mahjoubi et al. 2015). However, there is no special molecular mechanism by which

osmolytes affect protein stability, but it is accordingly called preferential exclusion of an osmolyte from immediate vicinity of a protein or preferential hydration of water (Street et al. 2006). It is agreed that osmolytes push the equilibrium of protein folding reaction [unfolded (U) $\leftrightarrow$ native (N)] toward N state (Sharma et al. 2012). This is due to the surface area of U state relative to N state which in turns makes osmolytes to interact with it more likely than N state and hence destabilize it. Destabilizing U state favors N state. Thermal stability effect of raffinose and lactose most likely be explained by the above-mentioned mechanism: Raffinose and lactose preferentially are excluded from uricase backbone but when uricase undergoes unfolding by increasing the temperature, lactose interacts with unfolded state, destabilizes it, and drives the U state towards N state. Under given condition, UOX shows remarkable resistance against unfolding. According to the values of k_{in} and half-life of UOX in the absence and presence of osmolytes, it is evident that lactose is more protective than raffinose as an osmolyte.

Because protein stabilization using osmolytes has been one of the most effective strategies in protein preservation and enhancement of pharmaceutical protein shelf life, the results of our study showed lactose, and to some extent, raffinose could enhance the thermal stability and thermal inactivation of uricase enzyme. In addition, lactose endows uricase environment a condition in which it could tolerate and stand longer times against heat. Protective effect of lactose is also reflected in half-life and T_m of UOX comparing that of raffinose. Taking all together, lactose is recommended as a potent agent for preservation and

increasing half-life of uricase. Examination of UOX in the presence of other protective osmolytes and structural analysis by spectroscopic techniques are suggested, as well.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

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