Efficient purification of a recombinant tag-free thermostable *Kluyveromyces marxianus* uricase by pH-induced self-cleavage of intein and expression in *Escherichia coli*

Bangchun Wang¹ · Laipeng Luo¹ · Dongmei Wang² · Rui Ding¹ · Jiong Hong²

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Abstract

Uricase as an important healthcare-related protein is extensively used in the treatment of tumor lysis syndrome and in the manufacture of serum uric-acid diagnostic kits. In this study, a gene of a new thermostable uricase (KmUOX) was cloned from thermotolerant yeast *Kluyveromyces marxianus*. The uricase was fused with a self-cleaving intein and cellulose-binding affinity tag and expressed in *Escherichia coli* BL21 (DE3). Through the binding to inexpensive cellulose and in situ intein cleavage induced by a pH change, tag-free uricase (KmUOX) was efficiently purified with a 77.11% yield via a single-step column purification strategy. This tag-free uricase showed K_m , V_{max} , and K_{cat} values of 67.60 μ M, 56.35 μ M/(min mg), and 32.74 S^{-1} , respectively. Furthermore, this pure uricase was relatively thermostable and retained 79.75% of activity when incubated at 40 °C for 90 h. Thus, this pH-induced self-cleavable intein system combined with a cellulose matrix for affinity chromatography is proven here to be an effective and low-cost method for recombinant-uricase purification. Moreover, the stability of KmUOX makes it useful for clinical applications.

Keywords Intein · *Kluyveromyces marxianus* · Tag free · Thermostable · Uricase

Introduction

Uric acid, a normal component of urine, is a product of the metabolic breakdown of purine nucleotides. Abnormal levels of uric acid are strongly associated with hyperuricemia, which may lead to pathological consequences in several organs, including the brain, kidneys, subcutaneous tissues, and joints, and is a causative agent of gout, urolithiasis, and gouty nephropathy (Cammalleri and Malaguarnera 2007).

Uricase (urate oxidase, UOX) is an enzyme important for purine degradation. Most vertebrates possess uricase, except humans and higher apes. It catalyzes uric-acid degradation, and produces allantoin, H_2O_2 , and CO_2 in the presence of oxygen (Fazel et al. 2014; Li et al. 2017). Because allantoin is tenfold more soluble than uric acid and is easily excreted in urine, uricase is used to convert uric acid into allantoin and reduces the risk of (and may cure) the above diseases (Patel et al. 2017). Rasburicase, a recombinant version of urate oxidase, was first approved by the U.S. Food and Drug Administration for the treatment of tumor lysis syndrome in 2001 (Pui et al. 2001). Furthermore, uricase with a 4-aminopyridine peroxidase system is extensively employed as a reagent in the clinical analysis of uric acid in serum (Liu et al. 1994). So far, many uricases from various microbes, including *Candida utilis* (Koyama et al. 1996), *Bacillus subtilis* (Pfrimer et al. 2010), *Aspergillus flavus* (Legoux et al. 1992), and *Pseudomonas aeruginosa* (Shaaban et al. 2015), have been recombinantly expressed. Rasburicase mentioned above is the recombinant uricase cloned from *A. flavus* and expressed in *Saccharomyces cerevisiae* (Pui et al. 2001). Nowadays, a large number of excellent uricases with thermal

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✉ Rui Ding
dingrui@ahmu.edu.cn

✉ Jiong Hong
hjiong@ustc.edu.cn

¹ Institutes of Life Science, Anhui Medical University, No. 81 Mei Shan Road, Hefei 230032, Anhui, China

² School of Life Science, University of Science and Technology of China, No. 96 Jinzhai Road, Hefei 230036, Anhui, China

stability, chemical stability, and long-term storage stability are still needed for clinical applications.

Compared with the conventional multistep protein purification protocols, the affinity chromatography system that takes advantage of the intrinsic affinity of fusion proteins for different carbohydrates not only facilitates downstream processing of the recombinant proteins, but also strongly decreases the purification cost (Wan et al. 2011; Wang and Hong 2014). For such carbohydrate-based affinity columns, a cellulose-binding domain is an attractive affinity tag, because it is safe and relatively inexpensive and binds nonspecifically to other proteins. In most cases, however, the affinity tag of a recombinant protein should be removed by protease digestion. The general drawbacks of protease-mediated tag cleavage are the need for a high enzyme-to-protein ratio, long incubation time for complete tag cleavage, an additional step for tag removal, and high protease cost (Arnau et al. 2006; Nishiya et al. 2002). Self-cleaving intein, which enables on-site tag cleavage, can be introduced to further simplify the purification process by varying pH or a thiol reagent concentration (Hong et al. 2008a). One research group reported purification of the uricase cloned from *A. flavus* by intein-mediated self-cleavage and by means of a chitin-binding affinity tag (Alishah et al. 2016). Nonetheless, only 0.2-mg l⁻¹ tag-free recombinant uricase was obtained after 40-h incubation with dithiothreitol (DTT), and more optimization research is needed for large-scale purification. In addition, the cost is strongly increased by the use of DTT and chitin during the purification process.

In our study, we found that a thermostable yeast strain, *Kluyveromyces marxianus* NBRC 1777, produces uricase that appears as a clear halo on a uric-acid plate around the clones (Supplementary Fig. S1). In the present study, the structure of a hypothetical uricase gene, *KmUOX*, was deduced from the genomic sequence of *K. marxianus* (GenBank Accession No. BAP70065) and was cloned from genomic DNA. This uricase gene was fused with a cellulose-binding module (CBM3) and a gene of self-cleaving intein. Finally, the CBM3-intein-uricase fusion protein was expressed in *Escherichia coli* BL21 (DE3). Through the binding to inexpensive cellulose and *in situ* cleavage of intein via a pH change, a tag-free uricase was efficiently purified. Moreover, the tag-free recombinant enzyme was characterized and found to be a new thermostable uricase.

Materials and methods

Reagents and microorganisms

All the chemicals used in this study were of analytical or higher grade. Restriction enzymes and modifying enzymes were purchased from Thermo Fisher Scientific.

K. marxianus NBRC 1777 was acquired from the NITE Biological Resource Center (Tokyo, Japan). *E. coli* XL10-Gold was used to construct and amplify the expression plasmid, and *E. coli* BL21 (DE3) was used for the expression of the recombinant uricase obtained from *K. marxianus* (KmUOX). The yeast extract–peptone–dextrose (YPD) medium was employed to aerobically culture *K. marxianus*. The lysogeny broth (LB) medium was used to cultivate *E. coli*. Regenerated amorphous cellulose (RAC) was prepared from microcrystalline cellulose (FMC PH-105) by a method described previously (Wang and Hong 2014).

Plasmids and strains

Plasmid pCIU was constructed for expression of the CBM3-intein-KmUOX fusion protein. The gene of KmUOX was amplified with primer pair *KmUOX*–*XhoI*-F and *KmUOX*–*PstI*-R (Supplementary Table S1) from *K. marxianus* NBRC 1777 genomic DNA as a template. After digestion with *XhoI* and *PstI*, the *KmUOX* DNA was inserted at the same restriction sites into pCIG, which was constructed from plasmid pTWIN1 (New England Biolabs, Ipswich, MA USA) in our previous study (Hong et al. 2008a). The resultant plasmid was named as pCIU (Supplementary Fig. S2), within which the uricase, KmUOX, was expressed with CBM3 (GenBank Accession No. AF283517.1) and *Ssp DnaB* intein (GenBank Accession No. CDK12648.1) fused to the N terminus. pCIU was then transfected into *E. coli* BL21 (DE3) and the resultant strain was named as *E. coli* KmUOX.

Recombinant expression of KmUOX in *E. coli*

E. coli KmUOX was inoculated into 300 ml of the LB medium supplemented with 100-μg ml⁻¹ ampicillin in a 1-L Erlenmeyer flask, and incubated at a rotary shaking rate of 250 rpm and 37 °C. The expression was induced with 0.2-mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h when optical density at 600 nm (OD₆₀₀) reached 0.8–1.0. The cells were harvested by centrifugation (4000×g, 10 min, 4 °C) and washed once with 50-mM Tris–HCl buffer (pH 8.5). Each cell pellet was then resuspended in 30 ml of lysis buffer (30 ml of 50-mM Tris–HCl buffer pH 8.5) and lysed in an ice bath by ultrasonication under the following conditions: 3-s pulse, total 480 s, at 50% power using Sonics Vibra cell Model VCX130 (SONICS & MATERIALS INC. Newtown, CT, USA). The cell lysate was centrifuged at 15,000×g for 40 min at 4 °C to remove the cell debris completely.

Purification of tag-free KmUOX

After we mixed 30 ml of the cell lysate supernatant with 10 ml of RAC (100 mg) at room temperature for 30 min, CBM3-intein-KmUOX was adsorbed on the matrix. After centrifugation at $4000\times g$ for 10 min and washing with 40 ml of wash buffer (50-mM Tris-HCl, 0.5-M NaCl, and 1-mM EDTA, pH 8.5) two times, the RAC with adsorbed CBM3-intein-KmUOX was resuspended in 40 ml of cleavage buffer (50-mM Tris-HCl, 0.5-M NaCl, and 1-mM EDTA, pH 6.5). The optimal self-cleavage temperature was then determined. The mixture was dispensed at 0.5-ml/tube into 1.5-ml microcentrifuge tubes, and was incubated at 4, 25, 30, 40, or 50 °C. Next, samples were taken at different time points (1, 3, 5, 7, 9, and 13 h), and the activity of uricase in the supernatant was determined.

After the optimal cleavage temperature was determined, large-scale purification of tag-free uricase was performed. As mentioned above, 10 ml of RAC (100 mg) was mixed with 30 ml of the cell lysate at room temperature for 30 min. After a wash and resuspension of the RAC carrying adsorbed uricase in 40 ml of cleavage buffer, the suspension was incubated at 40 °C for 9 h and then loaded onto an empty column. The tag-free KmUOX flowing through the buffer was recovered (Fig. 1).

Optimal pH and pH stability

To determine the optimal pH, enzymatic activity of the purified tag-free KmUOX was determined using 50-mM buffer with a pH range of 4–12. The buffer systems with pH

Fig. 1 Procedure for purification of tag-free KmUOX

ranges of 4–5, 6–10, and 11–12 consisted of sodium acetate buffer, Tris-HCl buffer, and disodium hydrogen phosphate-sodium hydroxide buffer, respectively. The relative activity at optimal pH was designated as 100%. The pH stability of KmUOX was examined by incubating the enzyme samples in the above-mentioned buffers for 48 h at various temperatures (4, 25, or 37 °C), and the residual enzymatic activity was measured under standard assay conditions; the initial enzymatic activity was designated as 100%.

Optimal temperature and thermostability

The optimal temperature of the purified tag-free KmUOX was determined by measuring the enzymatic activity in a temperature range of 25–55 °C (25, 30, 35, 37, 40, 42, 45, 50, and 55 °C). The relative activity at the optimal temperature was designated as 100%. Thermostability was evaluated by incubating the enzyme at various temperatures (0, 25, 30, 35, 40, 45, 50, or 55 °C) for 15 min. The residual activity was then determined under the standard assay conditions; the enzymatic activity at 0 °C was designated as 100%. Furthermore, tag-free KmUOX was incubated at 40, 45, or 50 °C for a longer period (from 15 min to 90 h). The residual activity was determined, and the activity at 0 h was designated as 100%.

Effects of metal ions, organic chemicals, and surfactants

To evaluate the effect of metal ions on the tag-free KmUOX, the purified enzyme was incubated for 1 h at 4 °C with the following 1-mM metal salts: KCl, MnCl_2 , CaCl_2 , NaCl, CuCl_2 , MgSO_4 , FeSO_4 , NiSO_4 , $\text{Fe}_2(\text{SO}_4)_3$, or EDTA. To evaluate the influence of organic chemicals, the enzyme was incubated with 20% (v/v) each of ethanol, methanol, acetaldehyde, acetone, DMSO, DTT, N-hexane, or β -mercaptoethanol at 4 °C for 1 h. To assess the effect of surfactants, the enzyme was incubated with 20% (v/v) PEG4000 or 20% (v/v) Tween 80 at 4 °C for 1 h. Finally, the enzyme was incubated with 0.02-mM H_2O_2 at 4 °C for 1 h to determine the influence of H_2O_2 . The activity was then measured under the standard conditions as described below. The activity without the above chemicals was designated as 100%.

The enzymatic activity assay and protein quantification

The enzymatic activity of urate oxidase was assayed in a 1.5-ml reaction system. Fifty microliters of the enzyme was added to the reaction mixture containing 400 μl of 2-mM uric acid and 1 ml of 50-mM Tris-HCl buffer (pH 9). After 5 min of incubation at 42 °C, 50 μl of 3-M H_2SO_4 was added

to stop the reaction. The decrease in uric-acid concentration was determined by measuring absorbance at 293 nm. One unit of enzymatic activity was defined as the amount of the enzyme required to transform 1 μmol of uric acid into allantoin in 1 min at 42 °C and pH 9 (Li et al. 2017). The protein concentration was determined by the Bradford method, based on a standard protein, bovine serum albumin (Bradford 1976). The purified protein was then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel and Tris–glycine buffer (Laemmli 1970).

Determination of kinetic constants

These constants were studied at various concentrations of uric acid (0.055–0.53 mM) as a substrate and were then calculated by means of the Lineweaver–Burk plot (Li et al. 2017).

Statistical analysis

All the experiments were conducted in duplicate. The reported data represent the average of at least three independent experiments. Error bars represent standard error of the mean.

Results

Cloning of the original uricase gene and amino acid sequence analysis of *KmUOX*

The production of uricase in *K. marxianus* NBRC 1777 was confirmed with the halo assay (Supplementary Fig. S1). Next, a putative uricase gene, named *KmUOX*, was found in genomic DNA by genomic mining. The amino acid sequence of *KmUOX* was aligned with that of several widely studied uricases. *KmUOX* was found to be highly homologous to the eukaryotic uricase, and showed a 63.31% similarity with the uricase of *C. utilis* (CuUOX; GenBank Accession No. P78609) and a 43.91% similarity with the uricase of *A. flavus* (GenBank Accession No. CAA43895), whereas *KmUOX* is relatively less homologous to the bacterial uricase and was found to share a 21.49% similarity with the uricase from *B. subtilis* (Fig. 2).

Although some eukaryotic uricases, including CuUOX, are known to be copper ion-binding proteins (Chu et al. 1996; Suzuki et al. 2004), the putative copper ion-binding motif, H-X-H-X-F, was not found in *KmUOX* (Fig. 2). This result is consistent with the observation that EDTA did not inhibit *KmUOX* activity. The two regions and two motifs that are commonly present in the uricases analyzed in other studies (Suzuki et al. 2004; Koyama et al. 1996) were

designated as region A (Y/H-G-K-X-X-V), region B (N-S-X-V/I-V/ I-A/P-TD-S/T-X-K-N), motif 1 (V-L-K-T-T-Q-S), and motif 2 (S-P-S-V-Q-K/H/N-T-L-Y), respectively. Figure 2 indicates that *KmUOX* possesses region A ($^9\text{Y-G-K-D-N-}^{14}\text{V}$). Nevertheless, the amino acid sequence of region B in *KmUOX*, $^{55}\text{N-T-P-I-V-P-T-D-T-V-K-}^{66}\text{N}$, proved that the second amino acid residue could be T (^{56}T in Fig. 2). Motifs 1 and 2 in Fig. 2 were identified as consensus patterns of the eukaryotic uricase, and motif 1 ($^{148}\text{V-L-K-S-T-G-}^{154}\text{S}$) and motif 2 ($^{235}\text{S-P-S-V-Q-A-T-M-}^{243}\text{Y}$) in *KmUOX* are similar to those of CuUOX. The active site of the uricase, which is located at the interface of the dimers, was found to be highly conserved and is marked with an asterisk in Fig. 2. Uric acid interacts with the enzyme through hydrogen bonds with residues R183 and Q239 and through aromatic π -stacking with F166 (Li et al. 2017). Moreover, a catalytic water molecule is located above the uric acid, which interacts with the enzyme through hydrogen bonds with N265 and T61 from another subunit.

Expression of CBM3-intein-*KmUOX* in *E. coli*

The expression plasmid, pCIU, was constructed (Supplementary Fig. S2) and then introduced into *E. coli* BL21 (DE3) for fusion protein expression. CBM3-intein-*KmUOX* expression was induced by IPTG, and the *E. coli* cells were lysed by sonication. The resultant fusion protein was detected by SDS-PAGE (Fig. 3, lanes 1 and 2), and its activity in the lysate supernatant was 11.36 U ml^{-1} (Table 1). According to the results of SDS-PAGE, the cleavage was also detected in the sample of RAC carrying adsorbed CBM3-intein-*KmUOX* (Fig. 3, lane 3). In addition, the amount of cleaved CBM3-intein-*KmUOX* in solution after RAC adsorption was negligible (Fig. 3, lane 4). These results indicated that the cleaved protein in lane 1 of Fig. 3 was produced during the SDS-PAGE sample preparation, but most of the fusion protein remained uncleaved during the expression.

Purification of the tag-free *KmUOX* uricase

Over 90% of CBM3-intein-*KmUOX* was adsorbed on RAC when the lysate was mixed with RAC (Fig. 3, lanes 3 and 4; Table 1). After a wash with wash buffer, the RAC with adsorbed CBM3-intein-*KmUOX* was resuspended in cleavage buffer. The optimal temperature for self-cleavage of CBM3-intein-*KmUOX* was determined by incubating the fusion protein at various temperatures in cleavage buffer (Supplementary Fig. S3). The cleavage rate increased with the increasing temperature. The highest cleavage rate was observed at 40 °C, with 89.67% of the uricase being released into the supernatant in 9 h. Therefore, 40 °C was selected as the cleavage temperature for the subsequent experiments.

Fig. 2 Alignment of the amino acid sequences of uricases from *A. flavus* (AfUOX), *B. subtilis* (BsUOX), *C. utilis* (CuUOX), and *K. marxianus* (KmUOX). The bars indicate region A, region B, motif 1, motif 2, and a putative Cu (II)-binding site. *Key amino acid residues in the active site

Large-scale purification was conducted next. CBM3-intein-KmUOX was adsorbed on RAC and rinsed with wash buffer. After that, the RAC with the adsorbed target protein was resuspended in cleavage buffer and incubated at 40 °C for the on-site cleavage of the CBM3-intein tag. After 9 h of incubation, the suspension was loaded onto an empty column, and tag-free KmUOX was recovered in the buffer flowing through the column (Fig. 3, lane 6); CBM3-intein was still adsorbed on RAC (Fig. 3 lane 5). From 300 ml of the culture for protein expression, 5.2 mg of pure tag-free KmUOX was obtained with a specific

activity of 50.54 U mg⁻¹, and purity was improved 9.43-fold (Fig. 3; Table 1). Specific activity of KmUOX was high in comparison with the uricases obtained from other organisms and analyzed in other studies (Table 2); the specific activity was higher than that of the commercial enzyme rasburicase (18.2 U mg⁻¹) (Fazel et al. 2014). It was also higher than the activity of an *E. coli*-expressed recombinant uricase (38.4 U mg⁻¹) obtained from *C. utilis* and was almost the highest as compared with specific activities reported in the literature (Table 2).

Fig. 3 SDS-PAGE analysis of the KmUOX expression and purification. (1) The lysate supernatant (crude enzyme). (2) Cell debris. (3) RAC carrying adsorbed CBM3-intein-KmUOX. (4) The lysate after RAC adsorption. (5) Cellulose-bound protein after cleavage. (6) Tag-free KmUOX cleaved from RAC. (7) Protein molecular weight markers. The three markers—**a**, **b**, and **c**—indicate different bands, CBM3-intein-KmUOX, CBM3-intein, and KmUOX, respectively. Self-cleavage also occurred during the SDS-PAGE sample preparation (boiling with loading buffer); this problem led to the detection of tag-free KmUOX in lanes 1 and 3

The optimal pH and pH stability of tag-free KmUOX

After the tag-free pure KmUOX was obtained, its optimal pH and pH stability were determined. KmUOX still retained more than 90% of its initial activity in a pH range of 6–11 after 48-h incubation at 4 °C. Nonetheless, the stability of recombinant KmUOX decreased sharply when this range was exceeded. A similar trend was observed when pH stability was determined at room temperature (25 °C) or 37 °C (Fig. 4). On the other hand, the retained activity at 25 or 37 °C was slightly lower than that at 4 °C in the pH range below 5. The optimal pH of this recombinant uricase was 9 (at 42 °C), which is similar to those of most uricases (Fig. 4).

The optimal temperature and temperature stability of the tag-free KmUOX

The optimal temperature and thermostability were also identified (Fig. 5). The optimal temperature was found to be 42 °C, and the retained enzymatic activity at 40 and 45 °C was 91.63% and 93.32%, respectively. After incubation of the enzyme at various temperatures for 15 min, the retained activity was determined. The retained activities at 40 and 45 °C were 95.55% and 74.55%, respectively (Fig. 5a). The retained activity decreased significantly when the incubation temperature was above 55 °C (Fig. 5a). The enzyme was

Table 1 Purification of the tag-free KmUOX

Fraction	Volume (ml)	Activity (U ml ⁻¹)	Total activity (U)	Protein (mg ml ⁻¹)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%) [*]	Purified fold
Soluble cell lysate	30	11.36	340.8	2.12	63.6	5.36	100	1
RAC adsorbed [*]	–	–	314.7	–	13.66	23.04	92.34	4.30
Lysate after RAC adsorption	30	0.87	26.1	1.66	49.94	0.52	7.66	–
Tag-free enzyme	40	6.57	262.8	0.13	5.2	50.54	77.11	9.43

^{*}The yield of RAC adsorbed was calculated through total activity subtracted the activity in lysate after RAC adsorption

Table 2 Uricase from various microorganisms

Original microbes	Expression hosts	Specific activity (U mg ⁻¹)	Optimal temperature (°C)	Thermo-stability	Genbank accession No.	References
<i>P. aeruginosa</i> Ps43	<i>E. coli</i>	15	–	–	KJ718888	Shaaban et al. (2015)
<i>C. utilis</i>	<i>H. polymorpha</i>	25.13	–	–	P78609	Chen et al. (2008)
<i>C. utilis</i>	<i>E. coli</i>	38.4	37	37 °C, 24 h, 40%	P78609	Liu et al. (2011)
<i>Microbacterium</i> sp. Strain ZZJ4-1	–	5.32	30	65 °C, 30 min, 100%	AEY68606	Kai et al. (2008)
<i>A. flavus</i>	<i>P. pastoris</i>	11.6	–	–	CAA43895	Fazel et al. (2014)
<i>A. flavus</i>	<i>E. coli</i>	27	30–37	–	CAA43895	Li et al. (2006)
<i>B. firmus</i> DWD-33	–	9.58	50	60 °C, 60 min, 100%	–	Kotb (2015)
<i>K. marxianus</i>	<i>E. coli</i>	50.54	42	40 °C, 90 h, 79.75%	BAP70065	This study

Fig. 4 Optimal pH and pH stability of the purified KmUOX

Fig. 5 Optimal temperature and thermostability of purified KmUOX. **a** Optimal temperature and thermostability (incubation for 15 min). **b** Thermostability of KmUOX when it was incubated at 40, 45, or 50 °C for 90 h

then incubated at 40, 45, or 50 °C for a longer period. The activity of tag-free KmUOX decreased significantly during the first 15 min and then became relatively stable. After 90 h of incubation, the retained activities were 79.75%, 52.11%, and 23.65%, respectively (Fig. 5b).

Consequently, KmUOX is a thermostable uricase. Although there are several other thermostable uricases (reported in the literature), their specific activities are relatively low. The uricase from *Microbacterium* sp. strain ZZJ4-1 retains 100% of activity after incubation at 65 °C for 30 min, but its specific activity is only 5.32 U mg⁻¹ (Kai et al. 2008) (Table 2). A uricase from *B. firmus* DWD-33 is more stable and retains approximately 100% of the activity after incubation at 60 °C for 60 min, but its specific activity is 9.58 U mg⁻¹ (Kotb 2015) (Table 2). Enzymatic activity of a thermostable uricase from a novel *B. thermocatenuatus* strain is not lost noticeably after incubation at 75 °C for 45 min (Lotfy 2008), but this enzyme has not been purified and cloned, and it is difficult to compare it with other uricases. On the other hand, uricases with high specific activity are not so thermostable. The uricase from *C. utilis* has 25.13 or 38.4 U mg⁻¹ specific activity depending on the literary source (Table 2). Nevertheless, the activity decreases by nearly 50% in 36 h after incubation at 37 °C (Liu et al. 2011). The retained activity of uricase from *B. subtilis* is ~50% after incubation at 37 °C for 12 h (Pfrimer et al. 2010). Only 15% of uricase activity from *A. flavus* is preserved after 10-min incubation at 40 °C (Imani and Shahmohamadnejad 2017). In the present study, the specific activity of KmUOX was 50.54 U mg⁻¹, which is rather high relative to the reported uricases. Furthermore, it retained nearly 80% of activity after incubation at 40 °C for 90 h, suggesting that KmUOX is a stable enzyme with a high potential for future application.

Effects of metal ions and chemicals

The influence of ions and chemicals on KmUOX activity was also determined (Table 3). Almost all the tested ions reduced the activity of KmUOX, and the activity was completely inhibited by Fe³⁺ (Table 3). Only 19.82% of activity was retained when KmUOX was incubated with 1 mM Cu²⁺. The mechanism of action of Cu²⁺ on uricase is complex. Some uricases have Cu²⁺-binding sites, and mutation of a His residue in the Cu²⁺-binding site (H-X-H-X-F) leads to the loss of enzymatic activity (Chu et al. 1996). In contrast, uricases such as *B. subtilis* UOX and KmUOX, which have no Cu²⁺-binding sites remain active. Some reports indicate that Cu²⁺ enhances uricase activity (Ravichandran et al. 2015); however, a uricase with a Cu²⁺-binding site is still inhibited by Cu²⁺ (Suzuki et al. 2004). KmUOX was weakly activated by acetone, DMSO, or n-hexane. EDTA activated KmUOX too: a 10% improvement in activity was detected.

Table 3 Effect of ions and chemicals on KmUOX

Ion or chemical	Relative activity
No	100%
Mg ²⁺	87.99%
Ca ²⁺	95.32%
Fe ²⁺	6.07%
Fe ³⁺	–
K ⁺	88.69%
Na ⁺	90.92%
Ni ²⁺	84.77%
Cu ²⁺	19.82%
Methanol	84.55%
Ethanol	71.97%
Acetaldehyde	–
Acetone	108.42%
n-Hexane	102.21%
DMSO	103.19%
DTT	94.93%
PEG4000	68.04%
β-Mercaptoethanol	62.98%
Tween 80	95.26%
EDTA	110.57%
H ₂ O ₂	68.68%

Other tested chemicals inhibited the enzyme, especially acetaldehyde, which inhibited KmUOX completely. In addition, the activity of KmUOX was reduced by 31.32% by 20 μM H₂O₂.

Kinetic constants of purified KmUOX

The kinetic constants of recombinant tag-free KmUOX were determined next. Recombinant KmUOX showed that K_m and V_{max} values of 67.60 μM and 56.35 μM/(min mg), respectively, and K_{cat} and K_{cat}/K_m values were found to be 32.74 S⁻¹ and 484.32 S⁻¹ mM⁻¹, respectively.

Discussion

Uricase can serve as a therapeutic protein or detection enzyme in the clinical analyses; thus, a tag-free product is preferred. The traditional chromatography has also been used to obtain tag-free uricase (Li et al. 2006; Liu et al. 2011). Because of the high expression of recombinant uricase, the efficiency of the traditional method is not low, and more than 60% of enzyme can be recovered. Nonetheless, at least three steps with three different columns are necessary to obtain pure uricase; this procedure requires more labor and has a high material cost. Inteins are self-cleaving proteins that can splice junctions in a host protein. Thus, a

self-cleaving intein can be employed with an affinity tag for one-step protein purification. In this study, the intein system was applied to obtain tag-free KmUOX through incubation of the matrix (cellulose) and bound KmUOX at 40 °C for 9 h. Tag and intein were adsorbed on cellulose, and finally, tag-free KmUOX was released into the buffer and recovered (Fig. 3). Although not all KmUOX was rid of the CBM3 tag, the tag-fused KmUOX stayed adsorbed on cellulose and did not affect quality of the purified tag-free enzyme. An extra step for noncleaved-protein removal is not needed.

A high recovery rate (yield) is important for purification of recombinant proteins. A high recovery rate (92.34%) was easily obtained via the RAC adsorption of the CBM3 tag protein (Table 1). The final yield of 77.11% of pure tag-free KmUOX is high in comparison with the other reports (Legoux et al. 1992; Shaaban et al. 2015). A method that combines magnetic beads and a nonspecific protease recovers 67% of the enzymatic activity of a recombinant uricase (Legoux et al. 1992). A recombinant uricase, which has originally been derived from *P. aeruginosa* Ps43, when expressed in *E. coli*, retains 72% of its enzymatic activity after purification on a Ni²⁺ Sepharose column; however, its expression level is low (2.14 U ml⁻¹), and specific activity of the pure enzyme is only 15 U mg⁻¹ (Shaaban et al. 2015). In the present study, because of the high adsorption ability of RAC (over 90%) and the high cleavage efficiency, as much as 77.11% of KmUOX could be recovered.

To achieve a high purification yield in this study, several important points had to be taken care of. First, the spontaneous cleavage of intein during expression and purification is a negative factor for the yield. Although the cleavage of intein is induced by switching to a buffer with pH 6.5, self-cleavage during expression is almost unavoidable. In this study, a temperature of 37 °C and short induction time were chosen to reduce the self-cleavage. Thus, the spontaneous cleavage was well controlled. Second, purification should be started as soon as possible after the cells are collected and disrupted. Spontaneous cleavage of intein is uncontrollable even at low temperature during storage (e.g., 4 °C); this phenomenon may reduce the recovery rate. To avoid the loss of uricase during purification, RAC was added immediately after the lysate was obtained. Finally, not all CBM3-intein-fused uricase was cleaved from CBM3-intein, as depicted in Fig. 3. In this study, to balance thermal inactivation and the yield, the cleavage was conducted at 40 °C for 9 h, and 89.67% of KmUOX was cleaved from CBM3-intein and recovered (Supplementary Fig. S3). The concentration of tag-free KmUOX is easily improved by the method proposed in this study. Here, the routine procedure of cleavage was conducted in 40 ml of cleavage buffer with a 300 ml starting volume of the culture for protein expression. Because the cleavage was induced by adjusting pH, the volume could be reduced to increase the concentration of

recovered KmUOX. In our study, 0.78 mg ml⁻¹ tag-free pure KmUOX was obtained by reducing the volume of cleavage buffer to 10 ml (data not shown).

Cellulose is a stable and economically efficient matrix for KmUOX purification. During the purification of recombinant healthcare-related proteins, the cost of the matrix is one of the key factors that need to be considered. The price of cellulose analyzed in this study can be as low as ~2 US cents/g at the laboratory scale and as low as 0.02 US cents/g when large-scale manufacturing is implemented (Hong et al. 2008b). Because several 100 mg of a fusion protein can be bound to 1-g RAC, the protein purification cost based on RAC alone could be as low as ~10 US cents per gram of purified protein (Hong et al. 2008a). Furthermore, cellulose is stable under the protein purification conditions. The buffer, temperature, pH, and even high-speed centrifugation may not change its structure substantially and may not affect specific adsorption of a protein. Compared to the use of chitin and DTT during one-step protein purification for recombinant uricase (Alishah et al. 2016), low cost and high stability under various conditions make cellulose an ideal matrix for uricase purification according to this study.

Our research group seems to be the first to express a thermostable uricase cloned from *K. marxianus* in *E. coli*. The tag-free uricase was efficiently purified by means of a self-cleaving intein and a CBM3 tag with high specific affinity. The inexpensive, stable matrix used for the purification reduced the cost and simplified the purification process. The specific activity of KmUOX was 50.54 U mg⁻¹, which is high as compared with the uricases reported in the literature. Furthermore, 79.75% of the activity of the uricase was still retained after incubation at 40 °C for 90 h, indicating that KmUOX is a thermostable enzyme.

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Compliance with ethical standards

Conflict of interest The authors are aware of the ethical responsibilities and have no conflicts of interest.

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