Utilizing Intein-mediated Protein Cleaving for Purification of Uricase, A Multimeric Enzyme

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Abstract

Uric acid, a side product of nucleotide metabolism, should be cleared from blood stream since its accumulation can cause cardiovascular diseases and gout. Uricase (urate oxidase) converts uric acid to 5-hydroxyisourate, but it is absent in human and other higher apes. Yet, the recombinant form of uricase, Rasburicase, is now commercially available to cure tumor lysis syndrome by lowering serum uric acid level. Developing new methods to efficiently purify pharmaceutical proteins like uricase has attracted researchers' attention. Self-cleaving intein mediated single column purification is one of these novel approaches. Self-cleaving inteins are modified forms of natural inteins that can excise and join only at one junction site. In this study, the synthetic gene of *Aspergillus flavus* uricase, a homotetrameric protein, was cloned into pTXB1 vector as a fusion with the N-terminal of Mxe GyrA intein and chitin-binding domain (CBD) for simple purification. Expression was confirmed by western blot analysis. The fusion protein containing uricase-intein-CBD was purified on a chitin column. The cleavage was induced by adding DTT¹ as a reducing agent to release uricase. The purity of uricase and complete excision of the intein and CBD were confirmed by SDS-PAGE², while its proper folding was proved by circular dichroism and fluorescent emission studies. Isoelectric focusing further confirmed its homogeneity when a single protein band was observed at the predicted pI value. This is the first report of successful purification of a multimeric therapeutic enzyme by intein-mediated protein cleaving using a well-established and facile system.

Keywords: Intein, Uricase, Purification, *Aspergillus flavus*, pTXB

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¹ Dithiotritol

² Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Pharmaceutical proteins have many properties of ideal medications. They are highly specific; therefore, they are less prone to have side effects. They usually have high affinity for their targets and as most of them are natural human proteins, they are less likely to stimulate immune system [1].

Urate oxidase (uricase, EC 1.7.3.4) is one of the enzymes in purine destruction pathway that converts uric acid into allantoin, H_2O_2 and CO_2 in presence of oxygen [2]. Although uricase is present in the liver peroxisomes of most mammalian cells, it is absent in human and some higher primates due to some deleterious mutations [3]. Elevated amounts of uric acid in blood can lead to complications such as gout in patients who receive cyclosporine after kidney and liver transplantation [4, 5], acute renal failure in patients who receive chemotherapy drugs for leukemia and lymphoma (tumor lysis syndrome) [6, 7], and uric acid nephropathy in patients with congenital diseases such as hypoxanthine-guanine phosphoribosyl transferase deficiency [8], phosphoribosylpyrophosphate synthase superactivity [9], and type I glycogen storage disease [10]. Rasburicase, the recombinant form of *Aspergillus Flavus* uricase, is now commercially available for curing tumor lysis syndrome [11, 12]. The urate oxidase of *A. flavus* is a 135-kDa barrel-shaped homo-tetramer containing four identical active sites [13]. The enzyme is non-glycosylated and it has no intra- or inter- disulfide bond [14].

Inteins (intervening proteins) are self-splicing protein elements that can excise themselves from a host protein after transcription and translation [15]. It is possible to mutate certain amino acid residues in their N- or C-terminal splice junctions and convert them into self-cleaving peptides [16]. Self-cleaving inteins can be used with affinity tags in one step protein purification [17]. Inteins can remove the affinity tag precisely, eliminating the need for endopeptidases in

conventional affinity chromatography [16, 18]. Previously, researchers had reported the successful application of inteins for pharmaceutical protein's purification. Babu et al. reported the production of recombinant human granulocyte macrophage colony stimulating factor (hGMCSF) as a fusion with Sce VMA intein and chitin binding domain in *Pichia pastoris* [19]. Other examples of single step column purification of proteins with inteins include purifying basic human fibroblast growth factor and α -1-antitrypsin using CBD tag and Ssp DnaB and Sce VMA inteins, respectively [20]. To date, most of the intein mediated protein purifications have been used to produce monomeric proteins. There are few reports on purifying multimeric proteins. Fong et al. purified α subunit of *Escherichia coli* RNA polymerase using ELP (elastin like polypeptids)-intein [21]. In another study on repair system stimulants, intein was used for recombinant MutL purification [22]. Guan and coworkers also purified three monomeric and multimeric proteins using split intein which has some disadvantages over continuous ones [23]. Split inteins unlike continuous inteins, have two separate catalytic fragments, N- and C-inteins (I_N and I_C), and are only active when the two fragments interact. The goal of this study was to investigate the feasibility of using a facile intein-based system for purifying multimeric protein like uricase. Using huge tags like inteins might change the final protein conformation and interfere with the proper folding and association of its subunits due to steric hindrance between the tag and the target protein.

In this study, *E. coli*-codon-optimized gene of *A. flavus* uricase was synthesized and cloned in pTXB1 vector (New England Biolab). The uricase-intein-CBD fusion protein was expressed in *E. coli* BL21(DE3). The intein-mediated cleavage of uricase from CBD was done by thiol induction on a chitin column. The structural and functional properties of the purified product were determined and compared to those of conventionally purified uricase.

Materials and methods

Chemicals, bacterial strains and plasmids

H₂O₂, 4-aminoantipyrine (4-AAP), isopropyl-β-D-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Restriction endonucleases, *pfu* DNA polymerase and T4 DNA ligase were obtained from Thermo scientific (USA). Anti-CBD antibody was from New England Biolab (Ipswich, MA, USA). Molecular biology kits were from Thermo scientific (USA) and Qiagen (Hilden, Germany). Primers were supplied by Macrogen (Seoul, Korea). Unless stated differently, other chemicals were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical-grade purity.

Construction of recombinant plasmid pTXB1-uri

The synthetic gene of *A. Flavus* uricase was constructed (GeneCust, France) based on *E. coli* codon preference. The primer pairs (forward primer: 5'-C CAC CAC CAT ATG TCC GCG GTT AAA GC-3'; reverse primer: 5'-GGT GGT TGC TCT TCC GCA CAG TTT AGA TTT C) were designed for amplification of the uricase gene and incorporation of appropriate restriction sites (*Nde* I and *Sap* I, respectively). The PCR reaction conditions to amplify the uricase gene were 95 °C for 3 min, 30 cycles of 95 °C for 1min, 62 °C for 40 second and 72 °C for 1min, with a final extension at 72 °C for 7 min in an Eppendorf master cycler. The PCR product was loaded on 1% (w/v) agarose gel and purified using Qiagen gel extraction kit (Hilden, Germany) following the manufacturer's instructions. The purified fragment, digested with *Nde* I and *Sap* I, was directionally cloned into double-digested pTXB1 plasmid. DNA sequencing (MacroGen, Korea) confirmed the insertion of uricase gene in pTXB1. The resulting plasmid was called pTXB1-uri.

Protein expression and Purification

pTXB1-uri plasmid was transformed to both *E. coli* BL21 (DE3) and *E. coli* ER2566 strains as expression hosts. To select the optimum expression condition, different concentrations of IPTG (0.4 and 1 mM), induction times (3, 5 and 16 hours), culture media (Luria Bertani (LB), Terrific Broth (TB) and Yeast Extract Tryptone (2xYT)) and temperatures (25, 30 and 37°C) were tested. At last, *E. coli* BL21 (DE3) cells were selected as expression host. In order to prepare pre-culture, transformants were grown at 37 °C in Luria Bertani medium containing 100 µg/mL ampicillin with shaking at 150 rpm. The overnight preculture was inoculated (1:100) to 1.8 liter of LB containing 100 µg/mL ampicillin and incubated at 37 °C with shaking at 150 rpm until the OD₆₀₀ reached 0.5-0.6. Protein expression was induced by 0.4 mM IPTG and continued for 5 hours at 25 °C. The cell pellet from 1.8 L culture was harvested by centrifugation at 5000 g for 15 min and resuspended in 36 mL ice-cold column buffer (25 mM Tris-HCl pH 8.5, 500 mM NaCl) and sonicated on ice for 6 min (9.9 seconds pulsing and 5 seconds resting) by a SYCLON Ultra Sonic Cell SKL950-IIDN. The cell lysate was then centrifuged at 9000 g for 45 min at 4 °C and supernatant was loaded on a 3 mL chitin column pre equilibrated with column buffer. The cell extract was loaded on the column at the initial flow rate of 0.5 mL/min which was then increased to 2 mL/min. The column was washed with 20 times bed volume of column buffer before applying 3-bed volume of column buffer containing 50 mM dithiothreitol (DTT). The column was incubated at 4 °C for 40 hours with DTT. 1.5 mL fractions were collected after elution with column buffer. The buffer was exchanged via Amicon Ultra-15 centrifugal filter unit (Millipore, Billerica, MA, USA; #UFC910024, cut-off 10 kDa) to remove DTT. All protein purification steps were carried out at 4 °C.

SDS-PAGE (10 % acrylamide) was used to confirm the purity of uricase and the complete excision of intein and chitin binding domain [24]. Protein markers (Life Technologies, 26610) were used as standards. Coomassie Blue R-250 [25] and silver staining [26] were used to detect the proteins after SDS-PAGE. Protein concentration was estimated by Bradford method using bovine serum albumin as standard [27].

Western blot analysis

50 μ l of each sample was centrifuged at 15000 g for 3 min. The cell pellet was dissolved in 20 μ l of Tris-HCl buffer containing 20 mM Tris-HCl pH 8.5 and 500 mM NaCl and run on a 8 % SDS-PAGE gel. Proteins were transferred to PVDF membrane using a Trans-Blot Transfer Medium (Bio-Rad, Hercules, CA, USA) at 4 °C for 2.5 hours at a constant current of 300 A. Blocking was done with 5 % (w/v) skim milk in phosphate-buffered saline (PBS) for 1 hour. Blots were probed with anti-CBD antibody (New England BioLab, Neb) diluted 1:3300 in TBST (50 mM Tris/HCl, 150 mM NaCl, 0.1 % Tween-20) overnight at 4 °C. They were then left at room temperature for 1 hour before incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG, diluted 1:3000 in TBST, for 2 h at 25 °C. The membrane was washed three times for 5 min in TBST. Finally the protein bands were detected with ParsTous chemiluminescence detection kit (Tehran, Iran).

Enzyme activity and biochemical characterization

Uricase activity was measured spectrophotometrically at room temperature using uric acid, 4-AAP (0.96 mM) and phenol (6.8 mM) as substrates in presence of horseradish peroxidase (0.07 mg/mL). Uricase converts the uric acid into allantoin and H₂O₂. Oxidation of phenol and 4-AAP in presence of uric acid was measured by the increase in absorbance at 510 nm ($\epsilon = 6.58 \text{ mM}^{-1}$

cm⁻¹) in 20 mM potassium phosphate buffer (pH 7). All assays were performed in duplicates. The data were fitted to generate a Michaelis–Menten equation, and the apparent kinetic parameters were calculated.

Thermal stability

Thermal stability was determined at 37 °C in 20 mM Tris-HCl buffer (pH 8.5) for 72 h. Samples were removed periodically and incubated on ice. They were warmed up to room temperature to measure the residual activities. The protein concentration was 11 µg/mL in all thermal inactivations to diminish the possible effect of protein concentration on stability. Assays were performed in triplicates.

Fluorescence spectroscopy

Fluorescence emission of tryptophan residues of the purified enzyme in 20 mM Tris-HCl buffer (pH 8.5) was measured with a Perkin Elmer luminescence spectrometer LS 55. Samples were excited at 290 nm and the emission was documented between 300 and 400 nm. The excitation and emission slit were both set to 5 nm. The enzyme concentration was 60 µg/mL.

Circular dichroism (CD) spectroscopy

Far-UV circular dichroism (195–245 nm) measurements were performed using a JASCO J-715 spectropolarimeter (Tokyo, Japan). The uricase concentration was 0.2 mg/mL in column buffer. Results are expressed as molar ellipticity, $[\Theta]$ (deg cm² dmol⁻¹), assuming the average amino acid residue weight (MRW³) to be 110. The molar ellipticity was calculated from the formula $[\Theta]_{\lambda} = (\theta * 100 \text{ MRW})/(cl)$, where c is the protein concentration in mg/mL, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ .

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³ Mean amino acid residue weight

Isoelectric focusing

The pI value and homogeneity of the enzyme were determined using isoelectric focusing electrophoresis (Ettan IPGphor 3, GE Healthcare, Buckinghamshire, United Kingdom). The enzyme was dialyzed in 7M urea and 2M thiourea solution to remove ions. 100 µg of the dialyzed enzyme was mixed with the rehydration buffer containing 7M Urea, 2 M thiourea, 4 % (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS⁴), 20 mM DTT and 2 % (v/v) IPG (Immobilized pH Gradient) to the final volume of 125 µL. IPG strips (7 cm, pH 3-10) were placed in rehydration buffer with sample solution and overlaid with 1.5-3 mL mineral oil to prevent evaporation and urea crystallization. Rehydration was done at room temperature overnight. After rinsing the strip and draining excess moisture, the isoelectric focusing was performed by the following program: 100 V: 100Vh, 1000V: 1000 Vh, and 5000V: 20000Vh. The protein band was visualized by coomassie blue R250 staining.

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⁴ 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

Results and discussion

There is a high demand for therapeutic proteins and one of the best strategies for their production is recombinant protein technology. Inteins are nature gifts, which can be used to overcome the complex purification steps when their modified forms are placed between the protein sequence and an affinity tag. Modified inteins can undergo self-cleavage by changing pH or thiol-induction [28]. The pH induced cleavage can cause premature in vivo cleavage that can reduce the total protein yield [29] and thiol-induced cleavage may disrupt the disulfide bonds in the protein structure. The tetrameric uricase of *A. flavus* does not have any disulfide bonds; making it a suitable substrate for thiol induced intein cleavage. The intein containing pTXB1 vector used in this work is developed based on the Mxe GyrA intein of *Mycobacterium xenopi*, one of the smallest known naturally occurring active protein splicing elements [30]. The genetically engineered Mxe GyrA intein which is used in the pTXB1 vector, can undergo self-cleavage only in its N-terminus when induced by thiol reagents [31]. pTXB1 also contains a chitin binding domain (CBD) in C-terminus of the intein that can be used to purify the protein on chitin containing columns. CBD is cheaper and its regeneration cycles is higher than histidine tags [28]. Metal leaching and the possibility to copurify other histidine containing proteins may also happen with immobilized metal ion affinity chromatographies [32]. It is also much smaller (6 kDa) than routine affinity tags such as maltose binding protein (42.5 kDa) and glutathione S-transferase (26 kDa).

The synthetic gene of *A. flavus* uricase was designed and constructed based on NCBI entry on the enzyme's primary structure ([Q00511.3](#)) and *E. coli* codon preference. It was then cloned in *Nde* I and *Sap* I sites of the pTXB1 expression vector; making pTXB1-Uri. *Sap*I restriction site

was incorporated at the 3' end of the uricase gene in a way that no extra amino acid would be added to the purified enzyme after the self-cleavage of the Mxe GyrA intein (Fig. 1).

Both *E.coli* BL21 and ER2566 were checked as expression hosts. Varying expression host and culture conditions including different culture media, IPTG concentration, induction temperature and duration did not significantly affect the expression level. Comparing the amount of expressed proteins based on western blot analysis, the condition mentioned in materials and methods section was selected for further studies (Figure 2, Lane 3). Western blots also suggest that no *in vivo* cleavage has occurred, since all of the expressed proteins were in full length (Fig. 2).

Thiol induction by cysteine resulted in complete inactivation of the purified enzyme. Therefore, intein's self-cleavage was induced by DTT, for both 20 and 40 hours at 4 °C. 40 hours DTT incubation led to 0.2 mg/L uricase which was higher than 20 hours incubation (data not shown). The final peptide was 34 kDa (Fig. 3A), equivalent to one uricase subunit. This confirms the complete excision of intein and CBD from uricase and also its purity. As a therapeutic protein, the purity of uricase is essential since any impurity in the final drug can lead to severe side effects for patients.

Isoelectric focusing was used to study the enzyme homogeneity. Modifications such as deamination, phosphorylation, deletion and truncation can change the pI of the protein and result in multiple bands on the gel. After IEF was done, only one single protein band was observed at the predicted pI (6.8), suggesting that there was no modified form of the enzyme in the sample. Any protein aggregate or truncated form of the final product can lead to antibody production, severe side effects and reduced efficiency (Fig. 3B) [33].

The oxidation effect of uricase on uric acid was measured spectrophotometrically in a coupled reaction with HRP and phenol/4-aminoantipyrin as described in Materials and Methods. Kinetic parameters of the intein-purified uricase were compared with enzyme conventionally purified using multiple chromatographic steps including Q sepharose Ion exchange chromatography, phenyl sepharose hydrophobic interaction chromatography and a final gel filtration step using sephadex G-100. Both forms of the enzyme had k_{cat} and K_m values of $9.3 \pm 5\% \text{ s}^{-1}$ and $0.08 \pm 5\% \text{ mM}$. Fluorescence spectroscopy and far-UV CD analysis were carried on to compare the secondary and tertiary structures of the two uricases and make sure that no structural change has happened during the purification steps. CD is used to measure the percentage of α -helices and β sheets in a protein [34], while fluorescence spectroscopy determines the flexibility of proteins with respect to the conformation which may expose or bury the internal tryptophan residues [35]. Both analyses revealed minor difference between the two uricases. For the CD spectrum, intein-purified uricase exhibit a minimum at $\sim 210 \text{ nm}$, while 'conventional method purified enzyme' does not have this peak. This might suggest a slightly improved secondary structure. It is noteworthy to mention that the substrate binding sites of uricase are between first and second monomers and the third and fourth monomers [36]. Hence, when the enzyme shows activity, it means that the forth structure of the enzyme is formed correctly at least in part. This means that although a huge tag and DTT were applied to purify the enzyme, the structure of the enzyme did not change significantly.

Stability of the therapeutic proteins at 37° C significantly affects their pharmacokinetic properties. As the thermal stability studies reveal, both forms of purified uricase had the same level of stability. They maintain 50% of their activities after 12 hours of incubation at 37° C and lose their activities completely after 54 hours of incubation.

As previously mentioned, there are successful reports of purifying proteins, using inteins. Purification of human neurotrophin-3 and TNF- α derivative peptid RMPT using pTXB1 vector were described by Li and coworkers [37]. Nevertheless it seems that more studies are needed to reveal the feasibility of using this system for multimeric proteins. Guan et al. reported applying split inteins for purification of β -galactosidase (tetramer), phosphite dehydrogenase (dimer) and green fluorescent protein (monomer). In their study Zn^{2+} was added to the purification column to prevent uninduced C-terminal cleavage of the split intein. Moreover, their purified protein had the tripeptide CFN at its N-terminus which is undesirable for pharmaceutical proteins [38]. Split inteins have some other disadvantages over the simple inteins. They are more expensive and require two fragments to be expressed separately [39, 40]. In the other study on the purification of *Escherichia coli* RNA polymerase, only the α subunit of the enzyme was fused to ELP-intein and other subunits were coexpressed without any tag [21]. ELP is a large tag needing rounds of heating and cooling for purification that may be destructive for therapeutic proteins. In another report on the endonuclease activity stimulation of *Aquifex aeolicus* MutL, Mxe Gyr A intein was used for enzyme purification but no functional and structural studies considering the purification method were performed [22]. In this study, one step intein mediated purification was successfully carried out for the multimeric enzyme, uricase, using a continuous intein.

Our results demonstrate that uricase retains its homotetrameric structure during this process despite the presence of intein and somehow harsh conditions. Nevertheless, more research is needed to optimize intein system for large-scale purification of such therapeutic enzymes. Our result brings the hope that using inteins for purifying multimeric proteins could be a choice in near future.

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References:

- [1] Leader B., Baca Q.J., Golan D.E. Protein therapeutics: a summary and pharmacological classification. *Nature Reviews Drug Discovery*. (2008);7:21-39.
- [2] Keilin J. The biological significance of uric acid and guanine excretion. *Biological Reviews*. (1959);34:265-94.
- [3] Wu X., Lee C.C., Muzny D.M., Caskey C.T. Urate oxidase: primary structure and evolutionary implications. *Proceedings of the National Academy of Sciences*. (1989);86:9412-6.
- [4] Burack D.A., Griffith B.P., Thompson M.E., Kahl L.E. Hyperuricemia and gout among heart transplant recipients receiving cyclosporine. *The American journal of medicine*. (1992);92:141-6.
- [5] Baethge B., Work J., Landreneau M., McDonald J. Tophaceous gout in patients with renal transplants treated with cyclosporine A. *The Journal of rheumatology*. (1993);20:718-20.
- [6] Cairo M.S., Bishop M. Tumour lysis syndrome: new therapeutic strategies and classification. *British journal of haematology*. (2004);127:3-11.
- [7] Coiffier B., Altman A., Pui C.-H., Younes A., Cairo M.S. Guidelines for the management of pediatric and adult tumor lysis syndrome: an evidence-based review. *Journal of Clinical Oncology*. (2008);26:2767-78.
- [8] Kelley W.N., Greene M.L., Rosenbloom F.M., Henderson J.F., Seegmiller J. Hypoxanthine-guanine phosphoribosyltransferase deficiency in gout. *Annals of internal medicine*. (1969);70:155-206.
- [9] Becker M.A. Phosphoribosylpyrophosphate Synthetase Overactivity. *Encyclopedia of Molecular Mechanisms of Disease*: Springer; 2009. p. 1639-40.
- [10] Kelley W., Rosenbloom F., Seegmiller J., Howell R.R. Excessive production of uric acid in type I glycogen storage disease. *The Journal of pediatrics*. (1968);72:488-96.
- [11] Pui C.-H., Mahmoud H.H., Wiley J.M., Woods G.M., Leverger G., Camitta B., et al. Recombinant urate oxidase for the prophylaxis or treatment of hyperuricemia in patients with leukemia or lymphoma. *Journal of Clinical Oncology*. (2001);19:697-704.
- [12] Pui C., Jeha S., Irwin D., Camitta B. Recombinant urate oxidase (rasburicase) in the prevention and treatment of malignancy-associated hyperuricemia in pediatric and adult patients: results of a compassionate-use trial. *Leukemia*. (2001);15:1505-9.
- [13] Colloc'h N., Poupon A., Mornon J.P. Sequence and structural features of the T-fold, an original tunnelling building unit. *Proteins: Structure, Function, and Bioinformatics*. (2000);39:142-54.
- [14] Legoux R., Delpech B., Dumont X., Guillemot J.-C., Ramond P., Shire D., et al. Cloning and expression in *Escherichia coli* of the gene encoding *Aspergillus flavus* urate oxidase. *Journal of Biological Chemistry*. (1992);267:8565-70.
- [15] Paulus H. Protein splicing and related forms of protein autoprocessing. *Annual review of biochemistry*. (2000);69:447-96.
- [16] Mathys S., Evans T.C., Chute I.C., Wu H., Chong S., Benner J., et al. Characterization of a self-splicing mini-intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation. *Gene*. (1999);231:1-13.
- [17] Chong S., Mersha F.B., Comb D.G., Scott M.E., Landry D., Vence L.M., et al. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene*. (1997);192:271-81.
- [18] Gogarten J.P., Senejani A.G., Zhaxybayeva O., Olendzenski L., Hilario E. Inteins: structure, function, and evolution. *Annual Reviews in Microbiology*. (2002);56:263-87.
- [19] Babu K.S., Antony A., Muthukumar T., Meenakshisundaram S. Construction of intein-mediated hGMCSF expression vector and its purification in *Pichia pastoris*. *Protein expression and purification*. (2008);57:201-5.

- [20] Sharma S.S., Chong S., Harcum S.W. Intein-mediated protein purification of fusion proteins expressed under high-cell density conditions in *E. coli*. *Journal of biotechnology*. (2006);125:48-56.
- [21] Fong B.A., Gillies A.R., Ghazi I., LeRoy G., Lee K.C., Westblade L.F., et al. Purification of *Escherichia coli* RNA polymerase using a self-cleaving elastin-like polypeptide tag. *Protein Science*. (2010);19:1243-52.
- [22] Mauris J., Evans Jr T.C. Adenosine triphosphate stimulates *Aquifex aeolicus* MutL endonuclease activity. *PLoS One*. (2009);4:e7175.
- [23] Guan D., Ramirez M., Chen Z. Split intein mediated ultra-rapid purification of tagless protein (SIRP). *Biotechnology and bioengineering*. (2013);110:2471-81.
- [24] Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*. (1970);227:680-5.
- [25] Wilson C.M. [18] Staining of proteins on gels: Comparisons of dyes and procedures. *Methods in enzymology*. (1983);91:236-47.
- [26] Kerenyi L., Gallyas F. A highly sensitive method for demonstrating proteins in electrophoretic, immunoelectrophoretic and immunodiffusion preparations. *Clinica Chimica Acta*. (1972);38:465-7.
- [27] Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*. (1976);72:248-54.
- [28] Fong B.A., Wu W.-Y., Wood D.W. The potential role of self-cleaving purification tags in commercial-scale processes. *Trends in biotechnology*. (2010);28:272-9.
- [29] Elleuche S., Pöggeler S. Inteins, valuable genetic elements in molecular biology and biotechnology. *Applied Microbiology and Biotechnology*. (2010);87:479-89.
- [30] Telenti A., Southworth M., Alcaide F., Daugelat S., Jacobs W., Perler F.B. The *Mycobacterium xenopi* GyrA protein splicing element: characterization of a minimal intein. *Journal of bacteriology*. (1997);179:6378-82.
- [31] Evans T.C., Benner J., Xu M.Q. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein science*. (1998);7:2256-64.
- [32] Wong J.W., Albright R.L., Wang N.-H.L. Immobilized metal ion affinity chromatography (IMAC) chemistry and bioseparation applications. *Separation and Purification Methods*. (1991);20:49-106.
- [33] Schellekens H., Jiskoot W. Immunogenicity of therapeutic proteins. *Pharmaceutical biotechnology*: Springer; 2013. p. 133-41.
- [34] Greenfield N.J. Using circular dichroism spectra to estimate protein secondary structure. *Nature protocols*. (2006);1:2876-90.
- [35] Eftink M.R. Fluorescence techniques for studying protein structure. *Methods of Biochemical Analysis: Protein Structure Determination*, Volume 35. (2006):127-205.
- [36] Girard E., Marchal S., Perez J., Finet S., Kahn R., Fourme R., et al. Structure-function perturbation and dissociation of tetrameric urate oxidase by high hydrostatic pressure. *Biophysical journal*. (2010);98:2365-73.
- [37] Li Z.Y., Fan J.H., Yuan J.M. Single-column purification of recombinant human neurotrophin-3 (hNT3) by using the intein mediated self-cleaving system. *Biotechnology letters*. (2002);24:1723-7.
- [38] Ramirez M., Valdes N., Guan D., Chen Z. Engineering split intein DnaE from *Nostoc punctiforme* for rapid protein purification. *Protein Engineering Design and Selection*. (2013);26:215-23.
- [39] Li Y. Split-inteins and their bioapplications. *Biotechnology letters*. (2015);37:2121-37.
- [40] Müller H., Salzig D., Czermak P. Considerations for the process development of insect-derived antimicrobial peptide production. *Biotechnology progress*. (2015);31:1-11.

Legends to Figures

Figure 1: Diagram of the fusion protein construction and one step purification. Coding sequence of the uricase was inserted in pTXB1 vector. The fusion protein consists of three parts: uricase, intein and C-terminal CBD. The CBD binds to chitin coated resins and facilitates one-step purification.

Figure 2: Western blot analysis of nine different conditions for protein expression. lane 1: 25 °C, 1 mM IPTG, LB medium, BL21 strain, 5 hours of induction. lane 2: 25 °C, 0.4 mM IPTG, 2YT medium, BL21 strain, 5 hours of induction. Lane 3: 25 °C, 0.4 mM IPTG, LB medium, BL21 strain, 5 hours of induction. Lane 4: 37 °C, 0.4 mM IPTG, LB medium, ER2566 strain, 3 hours of induction. Lane 5: 30 °C, 0.4 mM IPTG, LB medium, BL21 strain, 5 hours of induction. Lane 6: 37 °C, 0.4 mM IPTG, LB medium, BL21 strain, 5 hours of induction. Lane 7: 30 °C, 0.4 mM IPTG, LB medium, BL21 strain, overnight induction. Lane 8: 37 °C, 0.4 mM IPTG, LB medium, ER2566 strain, 5 hours of induction. Lane 9: 30 °C, 0.4 mM IPTG, 2xYT medium, BL21 strain, 5 hours of induction.

Figure 3: A) SDS-PAGE analysis of the enzyme purification. lane 1 & 2- purified uricase with different concentrations. Lane 3- low molecular weight marker. B) Isoelectric focusing gel image of purified uricase. pH gradient was uniform between values 3 to 10.

Figure 4: A) Intrinsic fluorescence intensity of the purified uricase. The excitation wavelength was set at 290 nm. Enzyme concentration was 60 µg/mL in Tris buffer pH 8.5. B) Far-UV spectrum of two uricases (0.2 mg/mL) in Tris buffer pH=8.5.

Uricase purified using intein (.....) and with conventional method (____)

Figure 5: Thermal stability of differently purified uricases at 37 °C. The graph shows the percentage of remaining activity against time for uricase purified with intein (●) and uricase purified with conventional method (■).

Figure 1. Alishah et.al

Figure 2. Alishah et.al

Figure 3. Alishah et.al

A

B

Figure 4. Alishah et.al

Figure 5. Alishah et.al