Molecular Soil Biology 2016, Vol.7, No.6, 1-6
http://msb.biopublisher.ca

Research Report Open Access

Molecular Cloning, Expression, and Purification of a Urease Accessory Protein UreG of *Arabidopsis Thaliana*

Jing Kou [1], Testuo Takano [2], Shenkui Liu [1], Yuanyuan Bu [1]

1 Key Laboratory of Saline-alkali Vegetation Ecology Restoration in Oil Field (SAVER), Ministry of Education, Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, Harbin, 150040, China

2 Asian Natural Environmental Science Center (ANESC), the University of Tokyo, Tokyo 188-0002, Japan

Corresponding author email: yuanyuanbu@nefu.edu.cn

Molecular Soil Biology 2016, Vol.7, No.6 doi: 10.5376/msb.2016.07.0006

Received: 05 May, 2016

Accepted: 01 Jun., 2016

Published: 02 Jun., 2016

Preferred citation for this article:

Kou J., Takano T., Liu S., and Bu Y.Y., 2016, Molecular Cloning, Expression, and Purification of a Urease Accessory Protein UreG of *Arabidopsis thaliana*, Molecular Soil Biology, 7(6): 1-6 (doi: 10.5376/msb.2016.07.0006)

Abstract UreG is one of the accessory proteins of urease in plants. In this study, a gene encoding an UreG consisting of 363 amino acids from *Arabidopsis thaliana* was isolated. The *AtUreG* gene was expressed in Escherichia coli BL21 as a GST fusion protein. The GST-AtUreG fusion protein was induced and purified under the optimized culturing conditions of 0.2 mM IPTG and 25℃ for 5 h. Western blot analysis using an anti-GST antibody showed that the GST-AtUreG fusion protein was not degraded.

Keywords *AtUreG*; Protein expression; *Arabidopsis thaliana*

1 Introduction

Urease is a Ni-containing urea hydrolase that catalyze the conversion of urea to ammonia and carbamate, which is found in many microbes and plants (Farrugia et al., 2013). Some plants, such as soybean (*Glycine max*) (Wiebke-Strohm et al., 2016) have two urease genes, while others, including potato (*Solanum tuberosum*) (Witte et al., 2005), tomato (*Lycopersicon esculentum*) (Bacanamwo et al., 2003) and Arabidopsis (*A. thaliana*) (Bu et al., 2015) have only one. Activation of urease is best studied in *klebsiella aerogenes*. It requires the carbamoylation of a Lys residue and the incorporation of two nickel ions per active site (Farrugia et al., 2013). For in vivo urease activation, three urease accessory proteins, UreD, UreF and UreG, are absolutely necessary. Apo-urease and UreD, UreF and UreG form a complex that is competent to incorporate CO2 and nickel upon GTP hydrolysis carried out by UreG (Soriano et al., 1999).

In the previous study, comparing accessory proteins sequences from Arabidopsis and k. aerogenes shows that UreG is conserved best (42.8% identity) while UreD and UreF are only 21.8% and 19.4% identity, respectively (Witte et al., 2005). In addition, UreG mutation tests showed that any of the lysine and threonine key sites combination with nucleotide mutated would lead urease activity completely lost (Soriano et al., 1999; Soriano et al., 2000). Such as, in soybean, the urease activities and embryo-specific were lost in UreG Eu3 mutant (Freyermuth et al., 2000). In Arabidopsis, the study of Arabidopsis UreG knockout mutants showed that lacking UreG gene were unable to grow on the medium containing urea as the sole nitrogen source (Witte et al., 2005). Although UreG plays an essential role involved in urease activity, the function of the UreG protein has not been yet investigated.

In this study, we isolated an *Arabidopsis* cDNA that encodes *AtUreG* consisting of 363 amino acids, and cloned into the protein expression vector pGEX-6p-3 to form pGEX-6p-3-AtUreG plasmid. The *AtUreG* gene was expressed in *Escherichia coli* BL21 as a GST fusion protein under different concentration of IPTG, induction temperature and induction times, and purified the GST-AtUreG fusion protein with the optimized conditions, finally, a western blot to verify the stability of the recombinant protein.

2 Materials and Methods

2.1 Cloning of AtUreG cDNA fragment

The full-length cDNA sequence of *AtUreG* (1090-bp, At2g34470.2) was amplified by polymerase chain reaction (PCR) using *AtUreG* specific primers: the forward primer 5'-CTCTCGGAAATGGCATCACAC-3' and the reverse primer 5'-CATCATCACTGGCGCTTCTT-3' from the Arabidopsis cDNA.

2.2 Construction of the expression vector pGEX-6P-3-AtUreG

The 1090-bp coding region of *AtUreG* was amplified by polymerase chain reaction (PCR) using the forward primer 5'-GGATCCCTCTCGGAAATGGCATCACAC-3' (*BamHI* site underlined) and the reverse primer 5'-GTCGACCATCATCACTGGCGCTTCTT-3' (*SalI* site underlined). The PCR product was connected to the pGEX-6P-3 plasmid at the *BamHI* and *SalI* sites. Then, the pGEX-6P-3-AtUreG plasmid was transformed into *E.coli* BL21 cells, the colonies were selected on LB medium containing 100μg/ml ampicillin and further identified by PCR reaction. The expression vector pGEX-6P-3-AtUreG was used to produce GST-AtUreG cells.

2.3 Expression and purification of GST-AtUreG fusion protein

The GST-AtUreG transformed cells were incubated in the LB medium and expression of the GST-AtUreG fusion protein was induced by isopropyl β-D-thiogalactoside (IPTG). To determine the GST-AtUreG protein expression under different IPTG (0.1, 0.2, 0.5, 0.8 and 1mM/L), time (1, 2, 3, 4 and 5h) and temperature (25, 30 and 37°C) conditions, aliquots of the culture were removed at different time and different temperature, lysed in the SDS sample buffer (100 mmol/L Tris-HCl pH 6.8, 200 mmol/L DTT, 4% SDS, 2% bromophenol blue, 20% glycerol) for 5min at 100°C and analyzed by SDS-PAGE.

For great amount of GST-AtUreG fusion protein purification, the GST-AtUreG transformed cells were cultured in LB medium which containing 100 μg/ml ampicillin at 25°C and shaking at 120rpm. When the OD_{600} reached a value of about 0.6 after 5 hours culture, expression of the GST-AtUreG fusion protein was induced by adding 0.2mM IPTG for an additional 5h at 25°C. Then, the cells were centrifuged and resuspended in pre-cooled lysis buffer (100mmol/L NaCl, 100mmol/L Tris-HCl pH 8.0, 50mmol/L EDTA, 2% TritonX-100), after that, added the lysozyme to the suspension to a final concentration of 1mg/ml. The suspension was incubated on ice for 30min and mix several times, centrifuged at 15,000rpm for 30min at 4°C. After centrifugation, the supernatant containing the GST-AtUreG fusion protein was loaded on a GST resin (GE Healthcare). Non-specifically bound proteins were removed by washing buffer (0.14mol/L NaCl, 2.7mmol/L KCl, 10mmol/L Na_2HPO_4, 1.8mmol/L KH_2PO_4, 0.5% TritonX-100). The bound fusion protein with GST-tag was eluted using elution buffer (20mmol/L glutathione, 100mmol/L Tris-HCl pH 8.0). At last, the purified protein samples were analyzed by SDS-PAGE. The concentration of protein was measured by using a coomassie brilliant blue G-250 reagent, the bovine serum albumin (BSA) was used as a standard reference.

2.4 Western blot analysis of GST-AtUreG

The induced and purified proteins were separated by SDS-PAGE using a 10% gel. After electrophoresis, one gel was stained six hours by using coomassie brilliant blue R250, then removed the motley and scanned the pictures, the other gel was transferred to a Hybond-N^+ membrane (Amersham Biosciences) by using a Hoefer^{TM} TE 70 semi-dry transfer unit (Amersham Biosciences) with Tris-glycine electroblotting buffer (25mmol/L Tris-HCl pH 7.0, 10% SDS, 190mmol/L Glycine, 20% Methanol) for 1h at 100mA. After transferring, the membrane was washed by washing buffer (0.14mol/L NaCl, 2.7mmol/L KCl, 10mmol/L Na_2HPO_4, 1.8mmol/L KH_2PO_4) for 15min, blocked in 1% blocking reagent for 1h, probed with mouse anti-GST antibody (1: 3000 dilution) for 1h at room temperature, washed three times for 15min in phosphate buffer, and probed with goat anti-mouse IgG antibody labeled with alkaline phosphatase (1: 2000 dilution) for 1h at room temperature. The signals were detected by adding CDP-Star (Roche, USA) using a Luminescent Image Analyzer LAS-4000 (Fujifilm, Japan).

3 Results and Discussion

3.1 Expression of GST-AtUreG fusion protein in *E.coli*

In order to determine the expression of GST-AtUreG, the *E.coli* BL21 cells transformed with the pGEX-6P-3-AtUreG plasmid were firstly induced at different IPTG concentration conditions in 37°C for 1 hours. After induction by different concentration of IPTG (0.1, 0.2, 0.5, 0.8 and 1mM), production of GST-AtUreG (~56 kDa) fusion protein was existed (Figure 1, lane 4-8), while before induction, the protein was absent (Figure 1, lane 3). However, the production of GST-AtUreG was improved no significant effect on the IPTG concentration from 0.1-1.0mM (Figure 1). As a control, the GST protein was existed after 1 hours induced at 1mM IPTG (Figure 1, lane 2), while the protein was absent before induction (Figure 1, lane 1). Previous studies have been shown that the high concentration of IPTG may inhibit the expression of fusion proteins, and partially expressed in form of inclusion body (Massiah et al., 2015). Therefore, these results showed that 0.1-1.0mM IPTG concentration did not significantly affect the soluble GST-AtUreG protein expression level (Figure 1), we chose 0.2mM IPTG for further studies.

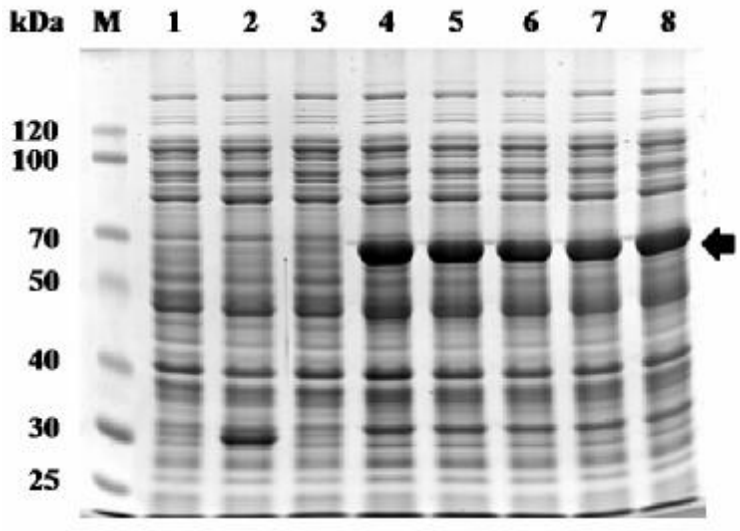

Figure 1 IPTG course of GST-AtUreG fusion protein expression

Note: Lysates were obtained from E. coli BL21 cells after expression induced at 37°C in 1h. Lane M, protein molecular weight marker; lanes 1-2, cells transformed with pGEX-6P-3 plasmid non-induced and induced; lanes 3-8, cells transformed with pGEX-6P-3-AtUreG plasmid non-induced and induced under 0.1, 0.2, 0.5, 0.8 and 1mM IPTG, respectively. Arrow indicates GST-AtUreG fusion protein

The induced temperature also had a certain effect on the expression of fusion protein (Sun et al., 2002). The effects of the culture temperatures (25, 30 and 37°C) at different induction time (1, 2, 3, 4, 5h) with 0.2mM IPTG were examined. The result shows that GST-AtUreG fusion protein was produced within a wide temperatures range (25, 30 and 37°C) (Figure 2). Along with the induced temperature elevated, the expression level of GST-AtUreG fusion protein significantly increased (Figure 2-A, B, C). Furthermore, with increasing the culture time at 25, 30 and 37°C, respectively, the expression level of GST-AtUreG fusion protein was increased at the beginning of 1h induction, however, the highest expression level of GST-AtUreG was produced after 5h induction (Figure 2-A, B, C). The higher temperatures was inappropriate for soluble protein production (Massiah et al., 2015), showing that the optimum temperature for the soluble protein was at 25°C.

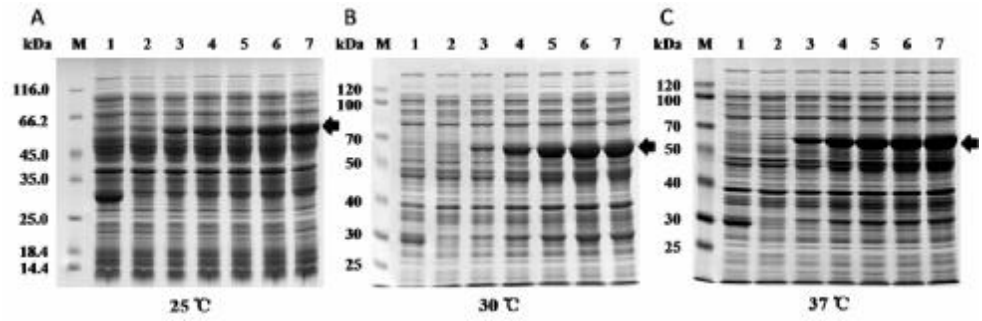

Figure 2 GST-AtUreG fusion protein expression under 25°C (A), 30°C (B) and 37°C (C) at 1, 2, 3, 4 and 5h, respectively

Note: Lysates were obtained from *E.coli* BL21 cells after expression induced. Lane M, protein molecular weight marker; lanes 1, cells transformed with pGEX-6P-3 plasmid induced; lanes 2-7, cells transformed with pGEX-6P-3-AtUreG plasmid non-induced and induced. Arrow indicates GST-AtUreG fusion protein

3.2 Purification of GST-AtUreG fusion protein in *E.coli*

In the prokaryotic expression system, the application of GST expression and purification system is more widespread, some fusion protein is soluble, some of which exist in the form of insoluble inclusion bodies. The soluble fusion protein can be purified and identified directly by the properties of GST. The key of Affinity chromatography is to select the ligand that can recognize and bind specifically to the target protein. The selected ligand should have a strong affinity to the target protein (Peng et al., 2002). To purify a large amount of GST-AtUreG fusion protein, 500ml cells transformed with the pGEX-6P-3-AtUreG plasmid culture was centrifuged, then, the obtained cell pellet was lysed with lysozyme for 30min and centrifuged. Aliquots of the supernatant were applied to a glutathione-sepharose affinity resin column which had been pre-washed with ice-cold washing buffer, after all supernatant passed through the column and washed non-specific proteins, the GST-AtUreG was eluted with 20mM glutathione concentrations at 300μL, 200μL, and 100μL, respectively (Figure 3, lane 5-7). Additionally, induced and purified GST as a control (Figure 3, lane 2), which proved experimental process was accurate.

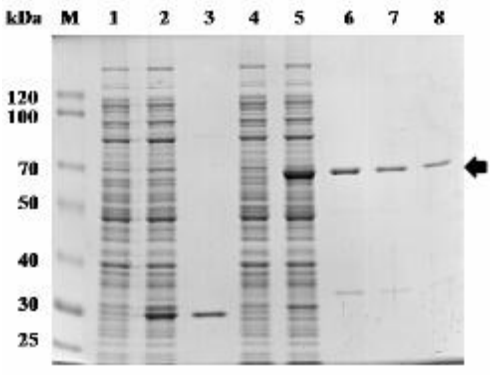

Figure 3 Effect of glutathione concentration on elution of GST-AtUreG fusion protein

Note: Cell lysates and column elutes were analyzed by SDS-PAGE and Coomassie staining. Lane M, protein molecular weight marker; lane 1-3, non-induced, induced and purified GST protein; lane 4-8, non-induced, induced and 20mM glutathione purified GST-AtUreG fusion protein. Arrow indicates the GST-AtUreG fusion protein

It has been reported that the optimal glutathione concentration to purify GST-AtUreG fusion protein was 20mM (Bo S., 2014). Two gels were prepared, one was used to dye, and the other gel was used to Western blot analysis to identify the purified protein. The result show that the fusion protein induced in the bacterial lysate and the purified

fusion protein produced single signals (Figure 4, lane 6-7), showing that the GST-AtUreG fusion protein was translated and purified correctly, and that the purified protein was not degraded.

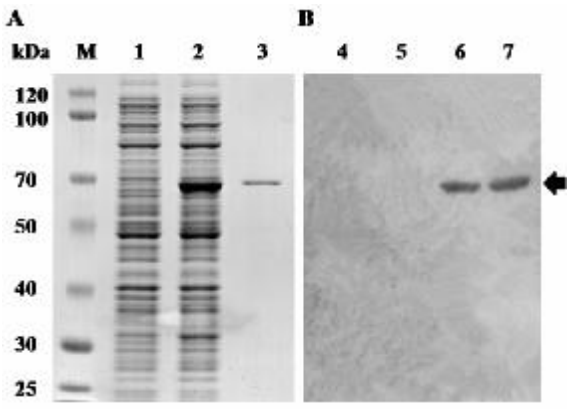

Figure 4 Western blot analysis of GST-AtUreG fusion protein

Note: Protein samples were separated on two gels. One was stained with CBB (A) and the other was transferred to a nylon membrane and probed with anti-GST antibody (B). In (A), Lane M, protein molecular weight marker; lane 1, non-induced GST-AtUreG fusion protein; lane 2, IPTG induced GST-AtUreG fusion protein; lane 3, purified GST-AtUreG fusion protein under 20 mM glutathione. In (B), lanes 6-7, the blot of GST-AtUreG fusion protein

4 Conclusions

In conclusion, we successfully expressed and purified the GST-AtUreG fusion protein in *E.coli*. The expression conditions for GST-AtUreG were optimized as 0.2mM IPTG induction at 25℃ for 5h. After the optimized method, a western blot confirmed that the GST-AtUreG fusion protein was translated and purified correctly. The obtained GST-AtUreG fusion protein provided a foundation for further studying the biological function of *AtUreG* protein and preparation of AtUreG antibody.

5 Acknowledgements

This work was supported by Heilongjiang Province foundation for Returnees (LC201405) awarded to Yuanyuan Bu. Further supported by the Program for Changjiang Scholars and Innovative Research Team in University (IRT13053) awarded to Shenkui Liu.

References

Bacanamwo M., Witte CP., Lubbers M., Polacco J., 2003, Activation of the urease of schizosaccharomyces pombe by the uref accessory protein from soybean, Molecular Genetics & Genomics,268(4), 525-34.
http://dx.doi.org/10.1007/s00438-002-0769-z PMid:12471450

Bo S., 2014, AtOxR - CSN5B interaction is regulating the AsA content helps relieve the stress of oxidation mechanism, Northeast Forestry University.

Bu Y., Kou J., Sun B., Takano T., Liu S., 2015, Adverse effect of urease on salt stress during seed germination in arabidopsis thaliana. FEBS Letters, 589(12), 1308–1313.
http://dx.doi.org/10.1016/j.febslet.2015.04.016 PMid:25907538

Farrugia MA., Lee M., Hausinger RP., 2013, Biosynthesis of the urease metallocenter, Journal of Biological Chemistry, 288(19), 13178-85.
http://dx.doi.org/10.1074/jbc.R112.446526 PMid:23539618 PMCid:PMC3650357

Freyermuth SK., Bacanamwo M., Polacco JC., 2000, The soybean eu3 gene encodes an ni-binding protein necessary for urease activity, Plant Journal, 21(1), 53-60.
http://dx.doi.org/10.1046/j.1365-313x.2000.00655.x PMid:10652150

Massiah MA., Wright KM., Du H., 2015, Obtaining soluble folded proteins from inclusion bodies using sarkosyl, triton x-100, and chaps: application to LB and M9 minimal media, Current protocols in protein science / editorial board, John E. Coligan, 84.

Peng F., Yang R., Li WX., 2002, A method for the preparation of GST fusion protein affinity chromatography. biochemical and biophysical progress, 29 (3): 362

Soriano A., Hausinger RP., 1999, GTP-dependent activation of urease apoprotein in complex with the ureD, ureF, and ureG accessory proteins.. Proceedings of the National Academy of Sciences, 96(20), 11140-4.
http://dx.doi.org/10.1073/pnas.96.20.11140 PMid:10500143 PMCid:PMC18000

Soriano A., Rp H., Gj C., 2000, Uree stimulation of GTP-dependent urease activation in the ureD-ureF-ureG-urease apoprotein complex.Biochemistry, 39(40), 12435-12440.
http://dx.doi.org/10.1021/bi001296o PMid:11015224

Soriano A., Hausinger RP., 1999, GTP -dependent activation of urease apoprotein in complex with the ureD, ureF, and ureG accessory proteins, Proceedings of the National Academy of Sciences, 96(20), 11140-4.
http://dx.doi.org/10.1073/pnas.96.20.11140 PMid:10500143 PMCid:PMC18000

Sun QM., Liu HY., Dai CB., 2002, Study on the complex and purification of -IL-6 (9-127) (.HuGM-CSF) (29-184) fusion protein. Biological Engineering Journal, 18 (3): 297 – 291.

Witte CP., Taylor MA., 2005, Analysis of two alleles of the urease gene from potato: polymorphisms, expression, and extensive alternative splicing of the corresponding mrna, Journal of Experimental Botany, 56(409), 91-9.
PMid:15533883

Witte CP., Rosso MG., Romeis T., 2005, Identification of three urease accessory proteins that are required for urease activation in Arabidopsis, Plant Physiology, 139(3), 1155-62.
http://dx.doi.org/10.1104/pp.105.070292 PMid:16244137 PMCid:PMC1283754

Wiebke-Strohm B., Ligabue-Braun R., Rechenmacher C., De Oliveira-Busatto LA., Carlini CR., Bodanese-Zanettini MH., 2016, Structural and transcriptional characterization of a novel member of the soybean urease gene family, Plant Physiology & Biochemistry Ppb, 101, 96-104.
http://dx.doi.org/10.1016/j.plaphy.2016.01.023 PMid:26874294