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Expression and purification of Chorismate lyase (UbiC) extracted from Pseudomonas putida

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Abstract

P-hydroxybenzoic acid (PHBA) is a key component for the production of paraben preservatives and high-performance bioplastics. PHBA is currently produced through the Kolbe-Schmitt reaction based on petrochemicals. However, producing aromatic compounds, such as PHBA, by this reaction is relatively expensive due to the requirement for high temperature and high pressure and problems with byproduct formation. In addition, environmental problems and fluctuations in raw material prices result in a continued decrease in the availability of PHBAs produced by this method. Thus, the production of chemicals using renewable resources, such as microbes, will be a solution to the problems associated with Kolbe-Schmitt reaction-based production of PHBAs.

Chorismate lyase (UbiC) in the Shikimate pathway of *Pseudomonas putida* KT2440 is produced by converting the intermediate product, chorismite, into PHBA. *P. putida* is highly resistant to PHBA, and unlike other bacteria, there are few by-products, such as acetate, glycerol or ethanol, formed. We believe that PHBA production through the UbiC mechanism of *P. putida* is more economical and environmentally-efficient than the current production method. Thus, this study aimed to increase the efficiency of PHBA production by producing and purifying recombinant UbiC as a key enzyme in the production of PHBA by *P. putida*.

Keywords: *Chorismate lyase (UbiC), p-hydroxybenzoic acid (PHBA), Pseudomonas putida(P. putida), recombinant protein expression and purification*

1. INTRODUCTION

Para-hydroxybenzoic acid (PHBA) is commonly used in the chemical industry. It is a key substance in the manufacture of liquid crystal polymers (LCP) with valuable applications in the thermoplastic market[1,2]. The current production of PHBA is based on petroleum-derived chemicals through the Kolbe-Schmitt reaction. This reaction has the disadvantages of byproduct formation because the reaction requires high temperature and pressure conditions[3]. Therefore, the production of these aromatic compounds is relatively expensive. Because of environmental issues, unpredictable raw material prices, and deceased availability in the future, the development of renewable and reliable processes for PHBA production has been pursued[4,5].

The aromatic compounds and aromatic derivatives in some biological products of microbial metabolic pathways can be substituted with bio-derived ones, which has generated great interest over the past decades[6,7,8,9].

The Shikimate pathway is the key step in the synthesis of the aromatic amino acids l-tryptophan (Trp), l-phenylalanine (Phe), and l-tyrosine (Tyr), the synthesis of quinones, folates, and secondary metabolites. Many of these compounds have commercial value[11]. The pathway links carbohydrate metabolism to aromatic compound biosynthesis by converting

phosphoenolpyruvate (PEP) and d-erythrose 4-phosphate (E4P) from metabolism of the central carbon into 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP), and after a sequence of seven reactions, chorismate, a universal precursor for aromatic amino acids and other aromatic compounds, is formed[12].

Pseudomonas putida is well known for its ability to metabolize and grow fast, despite low cellular maintenance, and remains very healthy in hostile environments. Moreover, *P. putida* has a naturally high resistance to PHBA. However, we are interested in protein engineering the Shikimate pathway, rather than bioengineering the overexpression or loss of genes necessary for PHBA production in a particular bacterial strain. It would be more efficient to produce PHBA using recombinant proteins in the Shikimate pathway and be more beneficial to the chemical production industry. Therefore, we focused on chorismate lyase, which produces PHBA from glucose, using the more efficient route and produced a highly purified recombinant chorismate lyase (UbiC) from *Escherichia coli* (*E.coli*) using cloning, overexpression, and purification procedures.

2. EXPERIMENTS

2.1 Reagents

Pfu Master Mix was purchased from Biofact (Daejeon, Korea). The Plasmid Mini-prep kit and Gel Purification kit were purchased from Geneall (Seoul, Korea). DNA markers, protein markers, and His bind agarose resin were purchased from Elpis Biotech (Daejeon, Korea). Restriction enzymes (BamH1, NotI) and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Agarose, 50X Tris-acetate EDTA (TAE) buffer, Luria-Bertani (LB) medium, kanamycin, ampicillin, tetracycline hydrochloride, isopropyl β-D-1- thiogalactopyranoside (IPTG), imidazole, chloramphenicol, 10X Tris-EDTA (TE) buffer, and glycerol were purchased from LPS Solution (Daejeon, Korea). Phenylmethylsulfonyl fluoride (PMSF) and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). HiTrap Q columns, HisTrap™ Excel pre-packed columns, and Superdex 200 were purchased from GE Healthcare Biosciences (Uppsala, Sweden). *Pseudomonas putida* KT2440 and the pET-28a vector were purchased from ATCC (47504, Manassas, VA, VA) and Novagen, respectively.

2.2 Cloning of the UbiC gene

2.2-1. PCR

The PCR templates used were genomic DNA (gDNA) from *Pseudomonas putida* KT2440. DNA was amplified by PCR reactions using primers. The conditions of the PCR reaction were 10 ng gDNA, 1.25 unit/μL of Pfu master mix polymerase, and 10 pmol forward and reverse primers in distilled water. The sample was denatured at 95 ℃ for 3 minutes. PCR cycles were performed with denaturation at 95 ℃ for 30 seconds, annealing at 56 ℃ for 30 seconds, extension at 72 ℃ for 1 minute for 35 cycles, and an extra extension at 72℃ for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis and purified using a gel purification kit.

2.2-2. Insertion of the *UbiC* gene into the pET28a vector

The expression vector, pET-28a, and the UbiC gene were digested with restriction enzymes at 37 ℃ for 2 hours. The *UbiC* gene fragments were inserted into the vector using T4 DNA ligase at 23 ℃ for 20 minutes. The cloned DNA was transfected into *E.coli* (DH5α). A single colony was seeded into LB broth containing antibiotics (50 μg/mL kanamycin). Plasmids were extracted using the plasmid mini-prep kit and confirmed by DNA sequencing (Macrogen, Seoul, Korea).

2.3 Expression of His-tagged protein

The cloned genes were transfected into *E.coli* BL21(DE3) cells. A single colony was grown in LB broth containing antibiotics (50 μg/mL kanamycin) at 37 ℃ overnight. Pre-cultured cells were transferred into 500 mL LB broth and cultured at 37 ℃ until the optical density was reached to 0.6. Protein expression was induced at 37 ℃ for four hours with 0.5 mM IPTG. Competent cells were harvested by centrifugation at 4000 rpm for 30 minutes at 4 ℃ and the cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM imidazole, 100 mM PMSF) and lysed by sonication. After cell lysis, supernatant from centrifugation at 13,000 rpm for 30 minutes at 4 ℃ was used for purification.

2.4 Purification of the His-tagged protein

2.4-1. Ni^{2+}-NTA affinity chromatography of UbiC protein

Crude lysate was loaded onto a Ni^{2+}-NTA resin column that had been equilibrated with binding buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM imidazole). The column was washed using wash buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 60 mM imidazole) and the protein samples were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 M imidazole).

2.4-2. Ion-exchange and size-exclusion chromatography

After affinity chromatography, the proteins were purified using a HiTrap Q column with buffer A (20 mM Tris-HCl pH8.0) and buffer B (20 mM Tris-HCl pH 8.0, 1 M NaCl). To dilute the NaCl concentration of the elution sample, buffer A was added to the sample and the diluted sample was loaded onto a Hitrap Q column on ÄKTAPrime Plus. The hybrid protein bound to ion particles was eluted with an NaCl concentration gradient (0% - 100%). Final purification was performed by size-exclusion chromatography with a Superdex 200 column using Tris-NaCl buffer (20 mM Tris-HCl pH 8.0 and 200 mM NaCl).

November-December 2019
ISSN: 0193-4120 Page No. 2476 - 2479

3. RESULTS

3.1 Cloning and expression of chorismite lyase (UbiC) into *E.coli* BL21(DE3)

Genomic DNA was extracted from purchased *Pseudomonas putida* (*P. putida*) and the *UbiC* genes were amplified by PCR and cloned into pET-28a (Fig. 1). The DNA sequence of the cloned *UbiC* gene was confirmed by sequencing. The *UbiC* gene was transfected into *E.coli* BL21(DE3) and the protein was expressed.

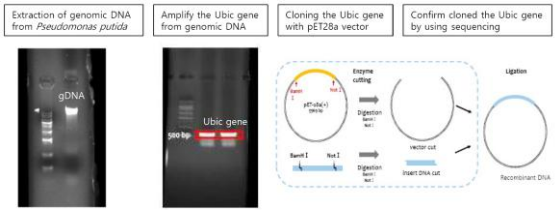

Figure 1. Scheme of chorismate lyase (UbiC) production

3.2 Purification of chorismate lyase (UbiC) protein from *E. coli* BL21(DE3)

The recombinant UbiC protein produced was disrupted by sonication and purified by affinity chromatography using Ni^{2+}-NTA. The UbiC protein, which was determined to be a consensus sequence, was eluted with 1M imidazole elution buffer and confirmed by SDS-PAGE analysis (Fig. 2).

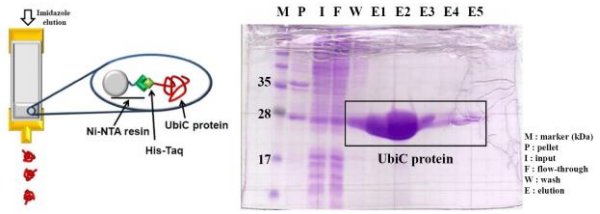

Figure 2. Purification of chorismate lyase (UbiC) using Ni-NTA

UbiC protein was overexpressed with a molecular weight of about 20 ~ 25 kDa. The UbiC protein was purified by ion-exchange chromatography with a HiTrap Q column for a secondary purification (Fig. 3). Finally, the UbiC protein was purified by size-exclusion chromatography using a Superdex 200 column. (Fig. 4).

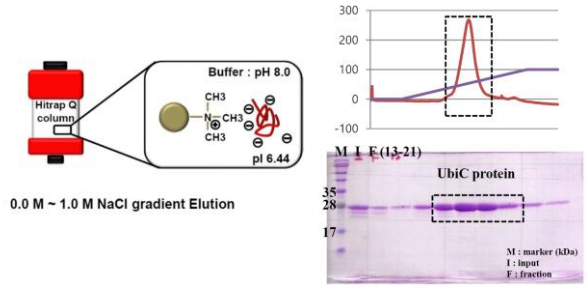

Figure 3. Purification of chorismate lyase (UbiC) using Hitrap Q

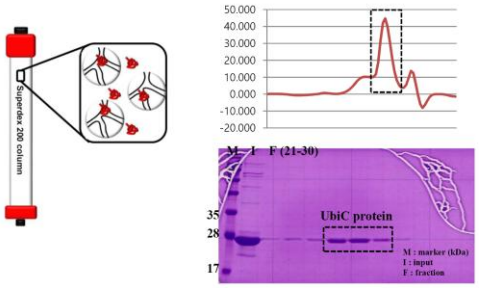

Figure 4. Purification of chorismate lyase (UbiC) using Superdex 200

4. EDITORIAL POLICY

The submitting author is responsible for obtaining agreement of all coauthors and any consent required from sponsors before submitting a paper. It is the obligation of the authors to cite relevant prior work.

5. DISSUSSION AND CONCLUSION

Chorismate lyase (UbiC) in the Shikimate pathway of Pseudomonas putida KT2440 is an important enzyme which converts the intermediate product chorismate into PHBA [13]. We believe that it would be more efficient to use a recombinant protein to produce an important enzyme within a metabolic pathway, in the bio-plastic industry than to bioengineer the important metabolic pathway in Pseudomonas putida.

In this experiment, we efficiently generated recombinant UbiC protein in *E. coli*, which was subsequently expressed and purified. The His-tag used for purification is small and, in most cases, does not affect folding of the attached protein. It has very strong reversible binding properties and can be attached to both the N- and C-terminals of recombinant protein but the optimal attachment position depends on the folding and biochemical properties of the protein. Therefore, the pET-28a vector, which has His tagging at both the N- and C-terminals, was used. Host bacteria, *E. coli* BL21 (DE3), were used for protein expression and produced a good yield of recombinant protein. After a sequential protein purification step, we succeeded in obtaining over 90 % pure protein.

In this study, we are expressed and purified UbiC protein in *E. Coli*. The yield of PHBA product could be increased if the key enzyme, as well as the UbiC protein, could be efficiently produced within the Shikimate pathway of *P. putida*. Therefore, it would be an effective way to solve environmental problems due to byproducts and relatively-expensive aromatic compounds.

November-December 2019
ISSN: 0193-4120 Page No. 2476 - 2479

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REFERENCES

1. Yu S, Plan MR, Winter G, Krömer JO.(2016) **Metabolic Engineering of Pseudomonas putida KT2440 for the Production of para-Hydroxy Benzoic Acid.** Front Bioeng Biotechnol. Nov 28;4:90
2. Ibeh C. C. (2011). **Thermoplastic Materials: Properties, Manufacturing Methods, and Applications**. Boca Raton, FL: CRC Press.
3. Yoshida T., Nagasawa T. (2007). **"Biological Kolbe-Schmitt carboxylation: possible use of enzymes for the direct carboxylation of organic substrates A2,"** in Future Directions in Biocatalysis, ed. Matsuda T., editor. (Amsterdam: Elsevier Science B.V.), 83–105.
4. Erickson B., Nelson J. E., Winters P. (2012). **Perspective on opportunities in industrial biotechnology in renewable chemicals.** Biotechnol. J. 7, 176–185.
5. Gavrilescu M. (2014). **"Biomass potential for sustainable environment, biorefinery products and energy,"** in Sustainable Energy in the Built Environment – Steps Towards nZEB: Proceedings of the Conference for Sustainable Energy (CSE) 2014, ed. Visa I., editor. (Cham: Springer International Publishing;), 169–194.
6. Wierckx N. J. P., Ballerstedt H., de Bont J. A. M., Wery J. (2005). **Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. Appl. Environ.** Microbiol. 71, 8221–8227.
7. Krömer J. O., Nunez-Bernal D., Averesch N. J. H., Hampe J., Varela J., Varela C. (2013). **Production of aromatics in *Saccharomyces cerevisiae* – a feasibility study.** J. Biotechnol. 163, 184–193.9.
8. Lin Y., Sun X., Yuan Q., Yan Y. (2014). **Extending shikimate pathway for the production of muconic acid and its precursor salicylic acid in *Escherichia coli*.** Metab. Eng. 23, 62–69.
9. Averesch N. J. H., Winter G., Krömer J. O. (2016). **Production of *para*-aminobenzoic acid from different carbon-sources in engineered *Saccharomyces cerevisiae*. Microb.** Cell Fact. 15, 1–16.
10. Curran K. A., Leavitt J. M., Karim A. S., Alper H. S. (2013). **Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*.** Metab. Eng. 15, 55–66.
11. Zhang H., Stephanopoulos G. (2013). **Engineering *E. coli* for caffeic acid biosynthesis from renewable sugars.** Appl. Microbiol. Biotechnol. 97, 3333–3341.
12. Bentley R. (1990). **The shikimate pathway – a metabolic tree with many branches.** Crit. Rev. Biochem. Mol. Biol. 25, 307–384.
13. Shi, X., Dini, A., Shao, Z., Jabarullah, N.H. & Liu, Z. (2019) Impacts of photovoltaic/ wind turbine/ microgrid turbine and energy storage system for binding model in power system, Journal of Cleaner Production, 226, 845-857.