Enzyme and Microbial Technology 118 (2018) 76–82

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Mutational analyses for product specificity of YjiC towards α-mangostin mono-glucoside

Tae-Su Kim[a,b,1], Tuoi Thi Le[a,1], Hue Thi Nguyen[a], Kye Woon Cho[a], Jae Kyung Sohng[a,b,*]

[a] Department of Life Science and Biochemical Engineering, SunMoon University, 70 Sun Moon-ro 221, Tangjeong-myeon, Asan-si, Chungnam, 31460, Republic of Korea
[b] Department of BT-Convergent Pharmaceutical Engineering, SunMoon University, 70 Sun Moon-ro 221, Tangjeong-myeon, Asan-si, Chungnam, 31460, Republic of Korea

ARTICLE INFO

Keywords:
Product specificities
Glycosyltrnasferase
α-mangostin
3-O-β-D-glucopyranoside
Mutagenesis
YjiC

ABSTRACT

Glycosyltransferases (GTs) are key enzymes for the post-modification of secondary metabolites in drug development processes. In our prior research, an one-pot enzymatic system produced α-mangostin 3,6-di-O-β-D-glucopyranoside (Mg1) at a higher proportion using wild-type glycosyltransferase (YjiC) but α-mangostin 3-O-β-D-glucopyranoside (Mg2) exhibited markedly higher anti-bacterial activities. This study focuses on a *Bacillus licheniformis*-originated flexible glycosyltransferase by mutagenesis to examine the active site residues involved in glycosylation for a product specificity towards Mg2. The generated H298A, H298S, and H298C mutants of YjiC exhibited a regiospecificity towards glycosylated product (Mg2) and were targeted in this study. The production pattern of Mg1 decreased to 63 (H298A), 85 (H298S) and 95% (H298C) yields compared to the wild-type YjiC. The increase of uridine 5′-diphosphate (UDP) leading to the inhibition of enzyme activity and production of uridine 5′-diphosphate glucose (UDP-glucose) in overall system was critical for the specific glycosylated product formation rate. H298A, H298S, and H298C mutants and YjiC exhibited 244, 251, and 186% increases in Mg2 production yields, respectively. And also H298A, H298S, and H298C showed 281, 279, and 251% increases in yield of Mg3 compared with wild type YjiC, respectively. There was improved conversion of both mono-glucosides product (Mg2a and Mg3) than di-glucosides products. The H298 mutants were found to overcome the limitation of the wild-type YjiC for regioselective synthesis of Mg2 by an enzymatic system.

1. Introduction

α-Mangostin is a natural prenylated xanthone from *Garcinia mangostana* Linn. It has notable biological properties such as anti-fungal, anti-bacterial, anti-viral, anti-oxidant, anti-inflammatory, anti-allergic, and anti-cancer activity [1–9]. This compound has been extensively studied, particularly with respect to human cancer cell lines such as leukemia, colon cancer, small-cell lung cancer, breast cancer, melanoma cancer, pancreatic cancer, and prostate cancer [10,11]. However, only a chemical synthesis method has been used to modify the molecular structure of α-mangostin to improve its biological properties [12,13].

A glycosylation is a reaction catalyzed by glycosyltransferases (GTs) which adds carbohydrates to molecules such as proteins, lipids, and secondary metabolites. Most approved biological activity or investigational natural products isolated from plants and microbes are glycosylated compounds which are either currently used as drugs or drug leads [14,15]. Glycosylation greatly influences the physiological and physicochemical properties of lipophilic compounds, improves chemical stability, and lowers chemical toxicity, including an increased water solubility and altered pharmacokinetic features [16,17]. YjiC belongs to the GT1 protein family characterized by a GT-B fold and a reaction mechanism based on an inversion that has been of interest to many researchers because wild-type glycosyltransferase is involved in the modification of terpenes, anthocyanins, co-factors, steroids, peptide antibiotics, macrolides, and other important structures [8–10]. YjiC has been used to synthesize novel glucosides of flavonols, isoflavonols, anthraquinone, and polyketides [18–24]. Although it has demonstrated a high flexibility towards various acceptor and donor substrates [19,20,22,23,25,26] making it an attractive and promising enzyme for the development of new drugs derived from natural products. However, it has never been engineered for improvements in substrate specificity and enzyme activity.

In our previous research, Mg2 exhibited a enhanced anti-bacterial

* Corresponding author at: Department of Life Science and Biochemical Engineering, SunMoon University, 70 Sun Moon-ro 221, Tangjeong-myeon, Asan-si, Chungnam, 31460, Republic of Korea.
E-mail address: sohng@sunmoon.ac.kr (J.K. Sohng).
[1] These authors are equally contributed.

https://doi.org/10.1016/j.enzmictec.2018.08.001
Received 29 March 2018; Received in revised form 31 July 2018; Accepted 2 August 2018
Available online 03 August 2018

activity than α-mangostin against particular Gram-positive bacteria *Micrococcus luteus* [25]. Since α-mangostin possesses multiple hydroxyl groups, YjiC conjugates sugar to the most part non-regiospecifically, producing multiple glycosides. This creates difficulties in forming the Mg2 (target compound) which necessitates engineering YjiC to glycosylate at a regiospecific hydroxyl position and to synthesize a high-value α-mangostin glycosylated product. The active site cavity of YjiC was examined to decipher the primary amino acid residues involved in the product specificity of glycosylation reactions by mutagenesis and ITC (isothermal titration calorimetry) studies. In our mutational study, we increased the UDP inhibition activity of YjiC and at the same time enhanced the Mg2 yield in a one-pot enzymatic system.

2. Materials and methods

2.1. Chemicals and reagents

Flavonoids (flavone: apigenin and chrysin, flavonol: 3-hydroxyflavone, isoflavone: genistein) and xanthone (α-mangostin) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). UDP-α-D-glucose and UDP were purchased from GeneChem (Daejeon, Republic of Korea). A Quik-Change II site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile and water were purchased from Mallinckrodt-Baker (Phillipsburg, NJ, USA). All the other chemicals used were of high analytical grade and commercially available.

2.2. Culture conditions, wild-type, mutant enzymes' expressions, and purification

Wild-type YjiC and its variants were expressed in *E. coli* BL21 (DE3). The recombinant strains were cultured in LB-media supplemented with ampicillin (100 μg/mL), at 37 °C until the OD_{600} value reached 0.6. A concentration of 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) was added for induction of protein expression and the culture was incubated at 20 °C for 18 h. The cells were harvested by 3000 rpm centrifugation at 4 °C and washed twice with a buffer (50 mM Tris-HCl of pH 7.5 containing 10% glycerol). Crude extracts were prepared by sonicating the cell suspension in 5 mL of 50 mM Tris-HCl (pH 7.5) containing 1 mM 10% glycerol and centrifugation with 12,000 rpm for 30 min at 4 °C.

The recombinant proteins were purified by using TALON metal-nickel resin (Takara Bio, Shiga, Japan). Protein was eluted by using 250 mM imidazole (buffer containing 50 mM NaH_2PO_4 and 300 mM NaCl). The fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S1) and the buffer was removed with Amicon Ultra-15 filters (Millipore, 30 K NMWL device, Milford, MA, USA). The purified proteins were stored in a buffer containing 50 mM Tris-HCl, pH 7.5. The protein concentrations were determined with the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Inc., CA, USA).

2.3. Site-directed mutagenesis study

The Quick-Change II site-directed mutagenesis kit (Stratagene) was used to introduce mutations in the *yjiC* gene at specific sites. The previously reported pET302/NT-His-YjiC construct served as a template DNA for mutagenesis reactions [19]. All processes were performed according to the manufacturers' instructions. The presence of a desired mutation was confirmed by DNA sequence analysis (Genotech, Daejeon, Republic of Korea). The mutagenesis primer is summarized in Table S1.

2.4. CD spectroscopy

The purified YjiC and mutant samples in 100 mM potassium phosphate buffer (pH 8) were used in CD measurements. The CD spectra were assessed with a J-710 spectropolarimeter (JASCO, Tokyo, Japan) and a cylindrical quartz cell with a 1-mm path length. The data scan rate was 200 nm/min and the value represents the average of three scans. A secondary structure analysis was based on CD multivariate SSE (version 2.01.00).

2.5. Measurement of the UDP-binding affinity in an isothermal titration calorimetry experiment

The ITC measurement was performed with a UMP (MicroCal iTC 200 system, GE Healthcare, USA) at the Korea Basic Science Institute (Ochang, Republic of Korea). Mutant proteins (100 μM) were prepared with 100 mM potassium phosphate buffer (pH 8) in the 200 μL ITC cell and 2 mM UDP was added to the protein, which has an approximately 20-fold higher concentration than protein. The titration steps were initially composed of 2 μL injections (19 times).

2.6. In vitro reactions

The enzyme activity of all mutants was tested in 50 μL reactions containing a 100 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 2.0 mM UDP-α-D-glucose, 1.0 mM acceptor substrate (apigenin, chrysin, genistein, 3-hydroxy flavone, and α-mangostin), and 100 μg/mL of YjiC and mutant enzymes. The reactions were incubated at 37 °C for 1 h.

The reverse reaction was carried out with Mg2 at 1 mM in the presence of 4 mM UDP with a 50 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, and 5 μg of the enzyme which was added to 50 μL of the reaction. The reaction was incubated at 37 °C for 5 h.

The one-pot enzymatic reaction applied for the synthesis of α-mangostin glycosides was described in a previous paper [25]. Briefly, the one-pot reaction mixture (200 μL) for the synthesis of α-mangostin glucosides was prepared using a substrate dissolved in DMSO or methanol, 20 mM $MgCl_2$, 150 mM acetyl phosphate, 50 mM glucose-1-phosphate, 1 mM ATP, 2 mM UMP, 5 mM α-mangostin, and 100 μg/mL each of the crude enzymes (UMK, ACK, GalU) and purified enzymes (YjiC and mutants). The reactions were incubated at 37 °C for 2 h.

All reactions were stopped by adding 490 μL methanol and 10 μL of the enzyme reaction sample. The samples were centrifuged and filtered by a Whatman Filter (0.2 μm) before HPLC analysis. The peak areas of all glycoside products were compared to each other.

2.7. Conditions for the HPLC-PDA and UPLC/ESI-mass

Glycosylated α-mangostin samples were analyzed using HPLC (Shimadzu, Kyoto, Japan) on a reversed-phase column (Shim-pack GIS ODS C_{18} column, 250 × 4.6 mm, 5 μm) at a UV absorbance of 254 nm based on a previous report [27]. The binary mobile phase was composed of solvent A (0.025% TFA in HPLC-grade water) and solvent B (100% acetonitrile, CH_3CN). The total flow rate was maintained at 1 mL/min for the 22-min program. The solvent conditions were as follows: 0–4 min 20–50% acetonitrile, 4–8 min 50–100% acetonitrile, 8–12 min 100% acetonitrile, 12–22 min 100–20% acetonitrile, and 22–25 min 20% acetonitrile. The exact mass of the glycosylated α-mangostin products was analyzed by HR-QTOF ESI/MS (high-resolution quadrupole-time-of-flight electrospray ionization mass spectrometry) [ACQUITY UPLC/SYNAPT G2-S (Waters, Milford, MA)] in the positive ion mode.

3. Results and discussion

3.1. Mutational study of YjiC to change product specificities

Although Mg2 showed a higher anti-bacterial activity compared to the Mg1, Mg3 and α-Mangostin against particular Gram-positive bacteria such as *M. luteus* [25]. When UDP-glucose was added at a 30 mM concentration as a donor substrate to the in vitro reaction mixture (not