CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and instruments

The laboratory materials were sourced, and experimental preparations were carried out in the laboratory of Asst. Prof. Dr. Rung-Yi Lai and the Center for Science and Technological Equipment at Suranaree University of Technology. The fluorescence probe was synthesized by the research group of Assoc. Prof. Dr. Anyanee Kamkaew. All chemicals were obtained from Sigma-Aldrich or TCI Chemicals. Absorption spectra were measured using a UV-Vis spectrophotometer (Agilent Technologies Cary 300). Fluorescence spectra were recorded with a fluorescence spectrophotometer (PerkinElmer LS55).

3.2 Plasmid construction of pET30-EcNfsB

The *NfsB* gene was amplified from *E. coli* NEB5alpha genomic DNA using Q5 high-fidelity DNA polymerase (New England Biolabs). The pET30-*Ec*NfsB plasmid was constructed through Gibson assembly, incorporating the PCR-amplified gene into a modified pET30 plasmid (Gibson et al., 2009). The overexpressed *Ec*NfsB protein includes an N-terminal His-tag, followed by the NfsB sequence (underlined label).

MSSHHHHHHSSGENLYFQGGGMDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPW
HFIVASTEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVDQEDADGRFATPE
AKAANDKGRKFFADMHRKDLHDDAEWMAKQVYLNVGNFLLGVAALGLDAVPIEGFDAAILDAEFGL
KEKGYTSLVVVPVGHHSVEDFNATLPKSRLPQNITLTEV

3.3 Overexpression and purification of *Ec*NfsB

Plasmid pET30-*Ec*NfsB was transform in *E. coli* BL21DE3 (less than 5% of competent cell) using heat shock method (Froger and Hall, 2007). The mixture was placed on ice for 20-30 min, then incubated at 42 °C for 45 seconds (heat shock) and incubated on ice for 2 min. After that, Luria-Bertani (LB) media was added to the mixture and incubated with the shaking speed of 200 rpm at 37 °C for 1 hour. The cell culture was spread on LB agar plate containing 50 µg/mL kanamycin and incubated at 37 °C overnight.

A single colony of *E. coli* BL21(DE3) harboring pET30-*Ec*NfsB was inoculated into 10 mL of LB broth containing 50 µg/mL kanamycin and cultured with the shaking speed of 200 rpm at 37 °C for overnight. Ten milliliters of overnight culture was inoculated in 1 L of LB and incubated with the shaking speed of 200 rpm at 37 °C until its OD_{600} reached about 0.5-0.7. Isopropyl-β-*D*-1-thiogalactopyranoside (IPTG) was added to a final concentration of 200 µM to induce the protein expression, then the culture mixture was grown with the shaking speed of at 200 rpm at 20 °C for overnight. The cells were harvested via centrifugation at 5000 rpm and 8 °C for 25 minutes. The collected cell pellets were resuspended in a lysis buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, and 10 mM imidazole (pH 8.0). Cell lysis was performed using sonication (1.5-second cycle, 50% duty) with a BANDELIN SONOPULS HD 2070 homogenizer for 30 seconds in three repetitions. Following lysis, the sample was centrifuged at 12000 rpm and 4 °C for 40 minutes. For purification, the resulting supernatant was loaded into a Ni-NTA column (QIAGEN) and washed with lysis buffer, and adsorbed protein was eluted by the stepwise concentration of imidazole in elution buffer (20, 50, 100, 150, 250, and 500 mM imidazole with 300 mM NaCl, and 50 mM NaH_2PO_4, pH 7.8). After elution, the purity of the protein fraction was analyzed using SDS-PAGE. The protein was incubated with excess flavin mononucleotide (FMN). The unbound FMN was removed by a 10-DG column (BioRad) pre-equilibrated with 100 mM Tris–HCl buffer, 20% glycerol, pH 7.5. The purified protein concentration was determined based on the

extinction coefficient of FMN at 445 nm (ε = 12500 $M^{-1} \cdot cm^{-1}$). Finally, the purified protein was aliquoted and stored at -80 °C.

3.4 *Ec*NfsB activity assay and kinetic constant determination

Every assay was conducted in a total volume of 2 mL. Each reaction mixture contained a varied concentration of IND-NO_2 in the presence of 0.5 μM *Ec*NfsB and 100 μM NADH in PBS buffer (pH 7.4) at 37 °C. The reaction was initiated by the addition of NADH. The concentration of IND-OH in the reaction was determined by fluorescence spectroscopy according to the calibration curve of the product (IND-OH). For kinetic constant determination, every reaction was analyzed every 5 seconds for a total reaction time of 5 min. The initial rate was assessed based on the rate of product formation. Additionally, the initial velocities for each probe concentration were measured in triplicate. The kinetic parameters (K_m and V_{max}) were determined by fitting the reaction rate against substrate concentration using nonlinear regression of Michaelis–Menten curves in GraFit 5.0 (Erithacus Software, Horley, Surrey, UK). The apparent k_{cat} was calculated by dividing V_{max} by the final enzyme concentration.

3.5 HPLC analysis of the reduction of probe catalyzed by *Ec*NfsB

HPLC analysis were performed on an Agilent HPLC 1100 with a ZORBAX Eclipse XDB-C18 (4.6 mm x 150 mm, 5 μm ID) column. The solvents were solvent A (100% 20 mM KPi buffer, pH 5.5), and solvent B (100% acetonitrile). The linear gradient at a flow rate of 1.0 mL/min was 0 min (90% A and 10% B), 12 min (20% A and 80% B), and 15 min (90% A and 10% B) detected by a DAD detector at 254 nm and 430 nm. The reaction solution of probe (10 μM), NADH (50 μM), and *Ec*NfsB (0.5 μM) was incubated in PBS buffer (10 mM PBS, pH = 7.4) for 20 min. Afterward, the reaction was quenched by the addition of acetonitrile, then centrifuged to precipitate protein. The supernatant was subjected to HPLC analysis.

3.6 Specificity test of probe IND-NO_2

IND-NO_2 (10 μM) was added to the mixture of different metabolites in 10 mM PBS buffer (pH 7.4) with 3% Tween-80, including NADH (200 mM), glucose (1 mg/mL), vitamin C (1 mg/mL), BSA (1 mg/mL), DTT (1 mM), GSH (1 mM), sodium sulfide (H_2S, 1 mM), sodium bisulfite (HSO_3^-, 1 mM) and 0.5 μM *Ec*NfsB with 50 μM NADH (NTR full reaction). All reaction mixtures were incubated at 37 ºC for 20 min. After incubation, the fluorescence emission of each reaction mixture at 564 nm was gathered with the excitation of 520 nm.

3.7 Test bacteria

The bacterial strains used in this study included the Gram-positive *Staphylococcus aureus* TISTR1466 and the Gram-negative *Escherichia coli* TISTR780 and *Pseudomonas aeruginosa* TISTR781. All strains were sourced from the Thailand Institute of Scientific and Technological Research (TISTR).

3.8 Preparation of bacterial cells

An inoculum of test bacteria was cultured in 10 mL of Luria- Bertani (LB) broth and grown at 37 ºC and 200 rpm overnight. 2.5 mL of bacterial inoculum was transferred into 250 mL of LB and incubated at 37 ºC until the OD_{600} reaches 0.5–0.7. The bacterial cells were then harvested by centrifugation at 8 ºC and 5000 rpm for 25 min. The cell pellets were washed with sterile 10 mM PBS (pH 7.4) three times to remove any traces of the culture medium. Bacterial cells were suspended in 10 mM PBS (pH 7.4) to make the final OD_{600} of 2.0.

3.9 NTR detection in bacterial cells by IND-NO_2

One milliliter of bacterial suspension (OD_{600} = 2.0) was incubated with 10 μM of probe **IND-NO_2** compared with two control samples. One is without **IND-NO_2** addition, and another is with addition of 0.4 mM dicoumarol, which is a NTR inhibitor. All samples were incubated at 37 ºC for 12 h. Following incubation, the biochemical reduction of the probe was stopped by sonication to lyse the cells, followed by centrifugation at 12,000 rpm and 25 ºC for 1 min. The resulting supernatant was transferred to a 96-well

black/clear bottom plate (Thermo Scientific™), and fluorescence intensity was measured using a fluorescence microplate reader (Thermo Scientific Varioskan LUX) at λ_{em} = 564 nm (λ_{ex} = 520 nm).

3.10 Statistical analysis

The statistical comparisons were determined based on the one-way ANOVA using the SPSS 25 for mac OSX (SPSS, Chicago, IL, USA). All results were presented as means±standard deviations. Statistical significance was accepted when the P values were less than 0.05 *(*P < 0.05, **P < 0.01, ***P < 0.001).*

3.11 References

Froger, A., and Hall, J. E. (2007). Transformation of plasmid DNA into E. coli using the heat shock method. *J Vis Exp*(6), 253.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods. 6*(5), 343-345.