INVESTIGATION OF NITROREDUCTASE DETECTION

BY A FLUORESCENCE PROBE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biochemistry and Biochemical Technology

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การตรวจจับไนโตรรีดักเตสโดยโพรบฟลูออเรสเซนต์

นางสาวธวัลยา คำทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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INVESTIGATION OF NITROREDUCTASE DETECTION BY A FLUORESCENCE PROBE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ธวัลยา คำทอง : การตรวจจับไนโตรรีดักเตสโดยโพรบฟลูออเรสเซนต์ (INVESTIGATION OF NITROREDUCTASE DETECTION BY A FLUORESCENCE PROBE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รุง-ยี ไลย์, 37 หน้า

คำสำคัญ: ไนโตรรีดักเตส; ตัวตรวจวัดเรืองแสง; การตรวจหาจุลชีพกลุ่ม ESKAPE; การเรืองแสงตามแอคติวิตี้

ไนโตรรีดักเตส (NTR) เป็นเอนไซม์ที่พบได้ในแบคทีเรียหลากหลายชนิด โดยมีบทบาทสำคัญในการกำจัดความเป็นพิษของสารประกอบที่มีหมู่ไนโตร ด้วยคุณสมบัตินี้จึงทำให้เอนไซม์ไนโตรรีดักเตสเป็นเป้าหมายสำหรับใช้ในการตรวจหาเชื้อแบคทีเรีย ในงานนี้ได้ทำการพัฒนาโพรบเรืองแสงที่ตอบสนองต่อ NTR (IND-NO_2) เพื่อใช้ในการตรวจจับกิจกรรมของ NTR ในจุลชีพโดยเฉพาะ โดยใช้เอนไซม์ NTR จาก *Escherichia coli* (*Ec*NfsB) เป็นเอนไซม์ต้นแบบในการทดลองเบื้องต้น เมื่อเอนไซม์ทำปฏิกิริยากับโพรบ IND-NO_2 ในสภาวะที่มี NADH หมู่ไนโตรของโพรบนี้จะถูกรีดิวซ์โดย *Ec*NfsB เกิดเป็นหมู่อะมิโน ส่งผลให้เกิดความเข้มของการเรืองแสงที่ความยาวคลื่น 564 นาโนเมตรที่เพิ่มขึ้นอย่างมีนัยสำคัญ การเกิดปฏิกิริยาดังกล่าวถูกวิเคราะห์ด้วยเครื่องวัดการเรืองแสงของสาร (fluorescence spectroscopy) และเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) เพื่อยืนยันว่าสารผลิตภัณฑ์ที่ได้คือ IND-OH นอกจากนี้ยังมีการศึกษาค่าพารามิเตอร์จลนศาสตร์สำหรับการรีดิวซ์ของโพรบด้วย เมื่อทำการศึกษาความจำเพาะของโพรบต่อ NTR และสารรีดิวซ์ทางชีวภาพต่าง ๆ พบว่าโพรบนี้แสดงความจำเพาะสูงต่อ NTR และไม่มีถูกรีดิวซ์โดยสารรีดิวซ์ทางชีวภาพต่าง ๆ นอกจากนี้ โพรบยังสามารถตรวจจับกิจกรรมของ NTR ในแบคทีเรีย ได้แก่ *Escherichia coli* TISTR780, *Pseudomonas aeruginosa* TISTR781 และ *Staphylococcus aureus* TISTR1466 ซึ่งเป็นตัวแทนของเชื้อก่อโรคในกลุ่ม ESKAPE โดยตรวจวัดการเรืองแสงด้วยเครื่องวัดการเรืองแสงของสาร และสามารถสังเกตได้ด้วยตาเปล่า แสดงให้เห็นถึงศักยภาพในการตรวจหาเชื้อแบคทีเรียแบบเรียลไทม์ของโพรบนี้

สาขาวิชาเคมี
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ลายมือชื่อนักศึกษา ธวัลยา คำทอง
ลายมือชื่ออาจารย์ที่ปรึกษา Rung-Yi Lai

TAWANYA KAMTHONG : INVESTIGATION OF NITROREDUCTASE DETECTION BY A FLUORESCENCE PROBE. THESIS ADVISOR : ASST. PROF. RUNG-YI LAI, Ph.D. 37 PP.

Keywords: Nitroreductase; Fluorescent probe; ESKAPE pathogens detection; Activity-based fluoresecence

Nitroreductase (NTR), found in a variety of bacteria, plays a crucial role in the detoxification of nitro-containing compounds, making it a valuable target for bacterial detection. In this study, we developed an NTR-responsive fluorescent probe (IND-NO_2) for the selective detection of NTR activity in microorganisms. *E. coli* NTR (*Ec*NfsB) was used as a model enzyme in preliminary experiments. The probe's nitro group was reduced to an amino group by *Ec*NfsB in the presence of reduced nicotinamide adenine dinucleotide (NADH), resulting in a marked fluorescence increase at 564 nm. The reaction was characterized by fluorescence spectroscopy and high-performance liquid chromatography (HPLC) to confirm the product identity, IND-OH. *Ec*NfsB kinetic parameters for the probe's reduction were also determined. Furthermore, the probe was demonstrated to show high NTR specificity, while the control experiments showed no reduction by various biological reductants. Furthermore, the probe successfully detected NTR activities in the bacteria of *Escherichia coli* TISTR780, *Pseudomonas aeruginosa* TISTR781, and *Staphylococcus aureus* TISTR1466, which represent ESKAPE pathogens, by fluorescence spectroscopy and observation with naked eyes demonstrating its potential for real-time bacterial detection.

School of Chemistry
Academic Year 2024

Student's Signature ธัญญพา ค่ำทอง
Advisor's Signature Rung Yi Lai

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Tawanya Kamthong

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DTT	Dithiothreitol
FMN	Flavin mononucleotide
GSH	Glutathione
HPLC	High-performance liquid chromatography
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kDa	Kilodalton
MW	Molecular weight
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NfsB	Oxygen-insensitive NAD(P)H nitroreductase
NTR	Nitroreductase
OD_{600}	Optical density of a sample measured at a wavelength of 600 nm
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
UV-Vis	Ultraviolet-Visible
°C	Degrees Celsius
ε	Extinction coefficient
λ	Wavelength
Φ	Quantum yield

CHAPTER I

INTRODUCTION

1.1 Background and significance

Nitroreductases (NTRs) are FMN-dependent enzymes that can reduce a range of nitro-containing compounds in the presence of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agent (Parkinson et al., 2000; Peter F. Searle et al., 2004). In general, nitroreductase enzymes are found in various bacteria species, including ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) that are common causes of life-threatening nosocomial infections amongst critically ill and immunocompromised individuals due to their potential drug resistance mechanisms (Santajit and Indrawattana, 2016).

In recent years, the development and utilization of fluorescent probes have emerged as powerful tools for studying nitroreductase activity with high sensitivity and selectivity (Jia et al., 2021; Wang et al., 2023). Previously, Wang and colleagues developed a two-photon fluorescent probe, TP-NO_2, with simple synthesis steps and high yield. Their probe has been successfully applied for NTR detection in cells under simulated hypoxia conditions (Wang et al., 2020). Wang and colleagues also developed NFP-NTR a benzoindole-based fluorescent probe with excellent selectivity and satisfactory sensitivity for detecting nitroreductases in live Hela cells under hypoxic conditions with low cytotoxicity (Wang et al., 2022). However, the development of NTR probes to enhance their efficacy is still attractive. In this study, a NTR-responsive fluorescent probe was tested to detect NTR in microorganisms (Figure 1.1). The reduction of the probe's nitro group was demonstrated by *E. coli* nitroreductase (*Ec*NfsB) catalyzed *in vitro* reaction to form an amino group, that generates the fluorescent signal. Therefore, we detected NTR activities in bacteria by using the probe, which could be a promising tool for the detection of ESKAPE pathogen infection.

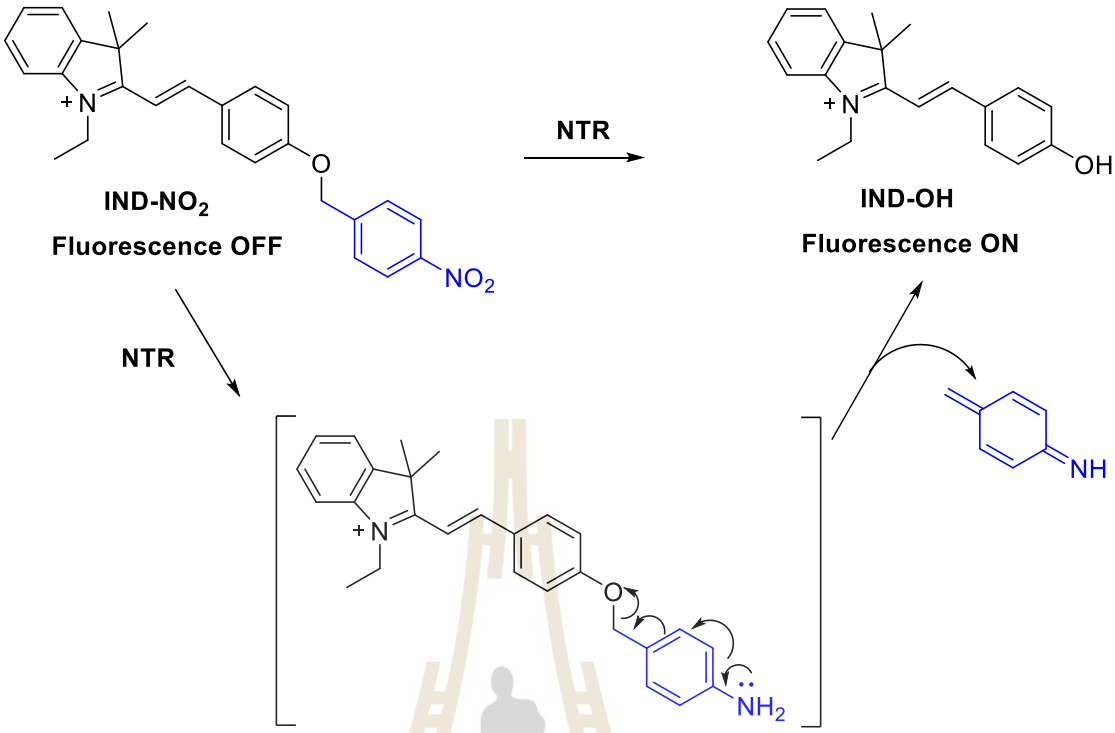

Figure 1.1 Proposed reaction mechanism of the probe IND-NO_2 reduced by NTR to generate the fluorescent IND-OH.

1.2 Research objective

1) To conduct *in vitro* assays using *E. coli* NTR as a model enzyme to reduce the fluorescence probe.
2) To test the probe in different microorganisms

1.3 Scope and limitations

In this work, *E. coli* NTR (*Ec*NfsB) was chosen to be a model enzyme to show that the probe's nitro group can be reduced by NTR to form an amino group, that generates the fluorescence signal. The reaction was confirmed by fluorescence spectroscopy and HPLC. The enzyme kinetics for the probe were determined. The reduction specificity of the probe was tested by conducting control experiments in the presence of various biological reductants. Lastly, the IND-NO_2 probe was tested in a few microorganisms,

including the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacteria *Escherichia coli* TISTR780 and *Pseudomonas aeruginosa* TISTR781.

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CHAPTER II

LITERATURE REVIEW

2.1 ESKAPE pathogens

The ESKAPE pathogens are a group of six bacterial pathogens known for their high resistance to antibiotics, making them major concerns into public health. The term "ESKAPE" is an acronym for the following bacteria: E stand for *Enterococcus faecium*, S stand for *Staphylococcus aureus*, K stand for *Klebsiella pneumoniae*, A stand for *Acinetobacter baumannii*, P stand for *Pseudomonas aeruginosa*, and E stand for *Enterobacter spp.* These pathogens are frequently linked to many clinical syndromes such as bloodstream infections, urinary tract infections (UTI), pneumonia, and other healthcare-associated Infections (HAIs) (Luo et al., 2024). Their capacity to develop resistance to several antibiotics, including drug uptake limitation, drug target site modifications, enzyme-mediated drug inactivation, and active drug efflux, make them were identified as critical multidrug-resistant bacteria (Singh et al., 2024). Table 2.1 shows the overview of ESKAPE pathogens including their antibiotic resistance and major clinical syndrome.

Table 2.1 Overview of ESKAPE pathogens (Luo et al., 2024; Miller and Arias, 2024).

Bacterial species	Antibiotic resistance	Major clinical syndromes
Enterococcus spp.	Vancomycin, linezolid, ciprofloxacin, nitrofurantoin	Bloodstream infection, infective endocarditis, intra-abdominal infection, UTI
S. aureus	Methicillin, oxacillin, penicillin	Bloodstream infection, infective endocarditis, ABSSSI, CAP, HAP/VAP, bone and joint infection
K. pneumoniae	Carbapenem, cephalosporin, aminoglycoside, fluoroquinolone, polymyxin, tigecycline	Bloodstream infection, UTI, CAP, HAP/VAP, intra-abdominal infection
A. baumannii	Carbapenem, β-lactams, aminoglycoside, fluoroquinolone	HAP/VAP, bloodstream infection, UTI
P. aeruginosa	Carbapenems, piperacillin-tazobactam, fosfomycin	HAP/VAP, bloodstream infection, UTI
Enterobacter spp.	Carbapenems, β-lactams, polymyxins, cefotaxime	Bloodstream infection, UTI, HAP/VAP, intra-abdominal infection

ABSSSI, acute bacterial skin and skin structure infection; CAP, community-acquired pneumonia; HAP/VAP, health-care-associated pneumonia/ventilator-associated pneumonia; UTI, urinary tract infection

2.2 Structure and Mechanism of Nitroreductases (NTRs)

Nitroreductases (NTRs) are a family of flavoenzymes expressed in bacteria and a few eukaryotes (Boddu et al., 2021). These enzymes can reduce a variety of nitro-containing compounds into amino groups by the reduced flavin cofactor at the active site, which is reduced by NADH or NADPH (Haynes et al., 2002).

Nitroreductase can be classified into two functional classes based on their sensitivities to oxygen: oxygen-insensitive (Type I) and oxygen-sensitive (Type II) reductases. There are two possible ways for NTR enzymes to reduce nitro groups, as seen in Figure 3. Type I nitroreductase uses a two-electron transfer mechanism to reduce the nitro group into nitroso, hydroxylamine, and amine. Nitro compounds, hydroxylamine, and amine metabolites are stable, whereas nitroso intermediate is unstable because the second and third two-electron transfer is faster than the first two-electron reduction. Consequently, the nitroso intermediate can react with biomolecules, leading to the formation of toxic and mutagenic products. Conversely, Type II nitroreductase uses a single electron transfer mechanism to carry out reduction reactions, resulting in an unstable nitro radical anion that reoxidizes to a starting nitro compound and produces a superoxide anion with a futile cycle under aerobic conditions. In the absence of oxygen, two nitroradical anions create the initial nitro compound and the nitroso derivative. It is thought that this is how nitroso compounds, which are observed in biological systems, can be formed (Rice et al., 2021). In *Escherichia coli,* there are two genes of oxygen-insensitive (Type I) nitroreductase, NfsA and NfsB. NfsA uses NADPH as an electron donor, whereas NfsB can use either NADH or NADPH as a source of reducing equivalents (Whiteway et al., 1998).

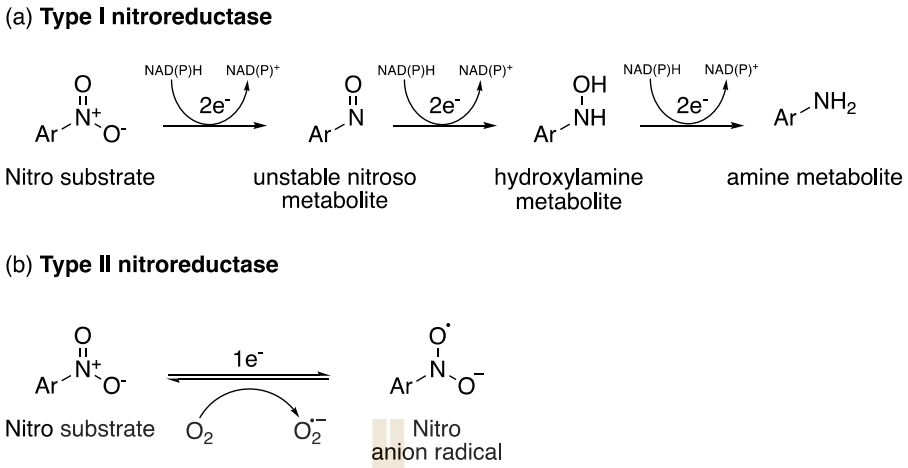

Figure 2.1 Reduction of a nitro group catalyzed by Type I (a) and Type II (b) nitroreductases, respectively. (Modified from Pimviriyakul et al., 2023).

2.3 Detection of nitroreductase activity

In pathogenic bacteria, Nitroreductases can be involved in the inactivation of antibiotics or the detoxification of nitro-containing compounds by a redox process that permanently degrades or neutralizes antibiotics (Thomas and Gwenin, 2021). It is also used in the activation of nitro-containing prodrugs such as Tretazicar (CB1954), which is an anticancer prodrug, and an antibacterial diazeniumdiolate prodrug (Hibbard and Reynolds, 2019; P. F. Searle et al., 2004). Detecting nitroreductase activity in pathogenic bacteria can provide insights into their virulence mechanisms and aid in the development of novel antimicrobial strategies. In cancer cells, it was presumed that hypoxia can promote overexpression of the intracellular reductases such as NTR (Nitroreductase), AzoR (Azoreductase) and DTD (DT-diaphorase). Therefore, nitroreductase is attractive for evaluating the regions with low oxygen levels in tumors, which are generally termed as hypoxia (Janczy-Cempa et al., 2021; Kumari et al., 2019).

Recently, many researchers have developed methods for detecting nitroreductase activity in bacteria or hypoxic cells by turn-on-off fluorescence probes, which could be promising tool for detecting pathogen infection or cancer cells. Because nitroreductases catalyze the reduction of nitro-containing compounds in the presence of NAD(P)H, the development of nitroreductase turn-on fluorescence probe has gained much attention. Wang and colleagues developed a benzoindole-based fluorescent

probe, NFP-NTR, for the detection of nitroreductase (NTR). This probe selectively reacts with NTR in the presence of NADH, facilitating the cleavage of the *p*-nitrobenzyl ether bond and generating a fluorescence signal. The proposed sensing mechanism of NFP-NTR for NTR is depicted in Figure 2.2, where NTR catalyzes the reduction of the electron-withdrawing nitro group (–NO_2) to an electron-donating amino group (–NH2). This transformation induces self-immolation ether cleavage, ultimately releasing the fluorophore NFP-1, resulting in a significant increase in fluorescence intensity(Wang et al., 2023). Additionally, NFP-NTR exhibited a detection limit of 17 ng/mL and demonstrated exceptional selectivity and sensitivity. The probe has also proven effective for imaging live HeLa cells in hypoxic conditions with minimal cytotoxicity, highlighting its potential for further biological applications related to NTR activity.

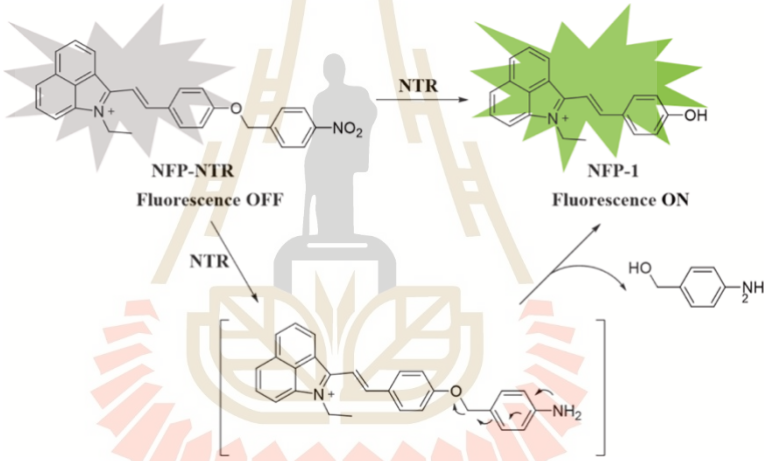

Figure 2.2 Proposed mechanism of the fluorescence signal turn-on of probe NFP-NTR catalyzed by NTR (Wang et al., 2022).

In 2023, Yan and colleagues introduced TCF-Nitro, a red-emitting NTR sensor with a long-wavelength emission at 560 nm, designed for detecting NTR activity in bacteria. This sensor is based on a modified **2**2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) scaffold, incorporating an NTR-responsive nitro locking group (Figure 2.3). TCF-Nitro enables the detection of NTR and facilitates the monitoring of NTR levels in *E. coli* as well as other clinically significant bacterial species (Yan et al., 2023).

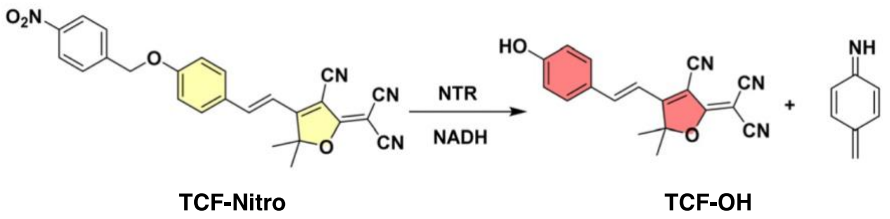

Figure 2.3 NTR catalyzes the reduction of the nitro group in TCF-Nitro to an amine, initiating a fluorescence "turn-on" response as a result of TCF-OH release (Yan et al., 2023).

2.4 Fluorescence

Fluorescence is the process in which a substance absorbs light or other electromagnetic radiation at a specific wavelength and subsequently emits light at a longer wavelength. The molecules that show their fluorescent properties are called fluorophores. The emitted light is typically in the visible spectrum, even if the absorbed light is in the ultraviolet (UV) range (Lichtman and Conchello, 2005) . The Jablonski diagram illustates how a fluorophore emits light after absorbing the light (Figure 2.4). When the fluorophore do not absorb energy or absorb excitation light, electrons are confined in the ground state **(S_0).** After electrons were excited by interacting with a wavelength of photons or excitation wavelength (λ_{ex}) as shown in blue, the interaction between photons and fluorophores promoted the transition from the ground state (S_0) to higher energy states. Electrons at the higher excited states move to the S_1 state through heat emission and other non-radiative processes. When electrons fall from S_1 to S_0, the photons of light are released as shown in the green arrow, which makes the molecules fluorescent (Llères et al., 2007). The fluorescence wavelength is also called as emission wavelength (λ_{em}).

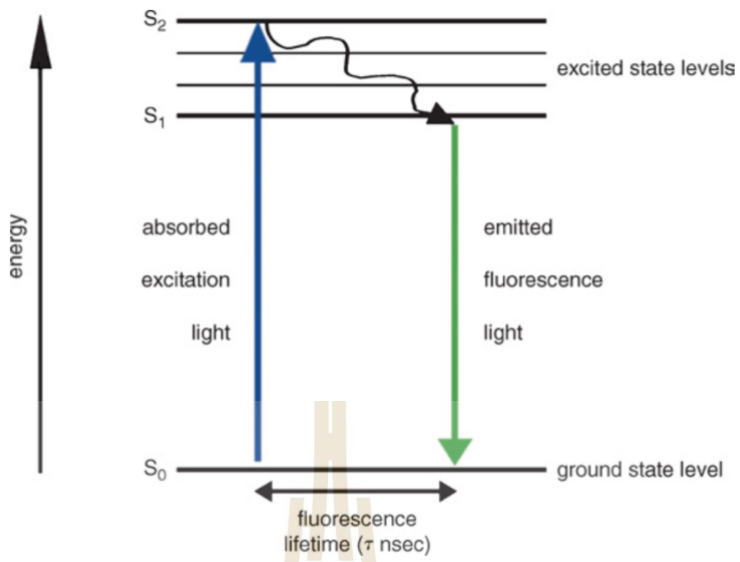

Figure 2.4 The Jablonski diagram. (Llères et al., 2007)

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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

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CHAPTER IV
RESULTS AND DISCUSSION

4.1 Detection of the nitroreductase activity using IND-NO_2

To investigate if IND-NO_2 is an NTR substrate, *E. coli* nitroreductase (*Ec*NfsB) was chosen as a model enzyme because its enzymatic studies have been reported (Prosser et al., 2010). It was overexpressed in *E. coli* BL21(DE3) containing a plasmid encoding *Ec*NfsB with an *N*-terminal His-tag controlled by the T7 promoter. After that, *Ec*NfsB was purified by Ni-NTA chromatography. The molecular weight of *Ec*NfsB is 26.23 kDa, which is consistent with SDS PAGE analysis. (Figure 4.1). The purified *Ec*NfsB was applied to the NTR assay containing IND-NO_2 and NADH. The assay was monitored by fluorescence spectrometry to detect the generation of IND-OH proposed in Figure 1.1. The fluorescence signal at 564 nm increased along with the reaction time compared with the control experiments without the addition of *Ec*NfsB or NADH. To characterize the product's identity as the proposed sensing reaction in Figure 1.1, the full reaction mixture was analyzed by HPLC monitored at 430 nm, since IND-NO_2 and IND-OH absorb at this wavelength. The retention times of the IND-NO_2 and IND-OH standards were 11.2 and 8.1 min, respectively (Figure 4.2). The chromatography of the full reaction showed a new peak at about 8.1 min. To further confirm the product's identity, the standard of IND-OH was spiked into the full reaction mixture to show the comigration of the peak with a larger peak area. Therefore, *Ec*NfsB can catalyze the reduction of IND-NO_2 in the presence of NADH to generate IND-OH, which has a fluorescence signal at 564 nm using the excitation wavelength of 520 nm in PBS buffer (pH 7.4)

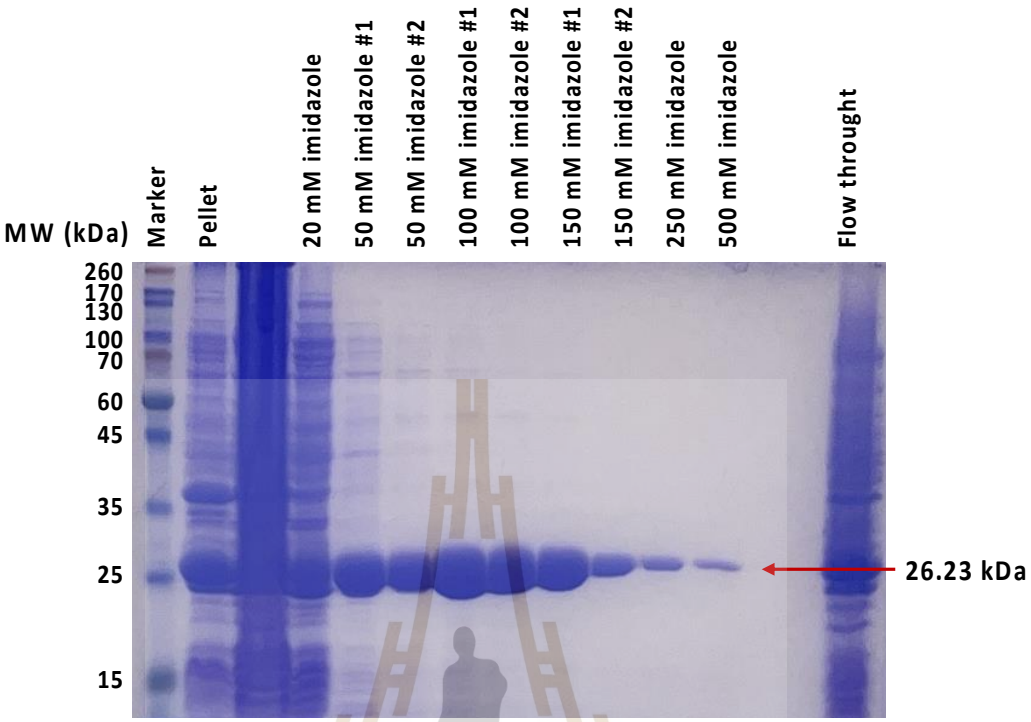

Figure 4.1 The SDS-PAGE analysis of *Ec*NfsB purification by Ni-NTA chromatography. The pure fractions were collected.

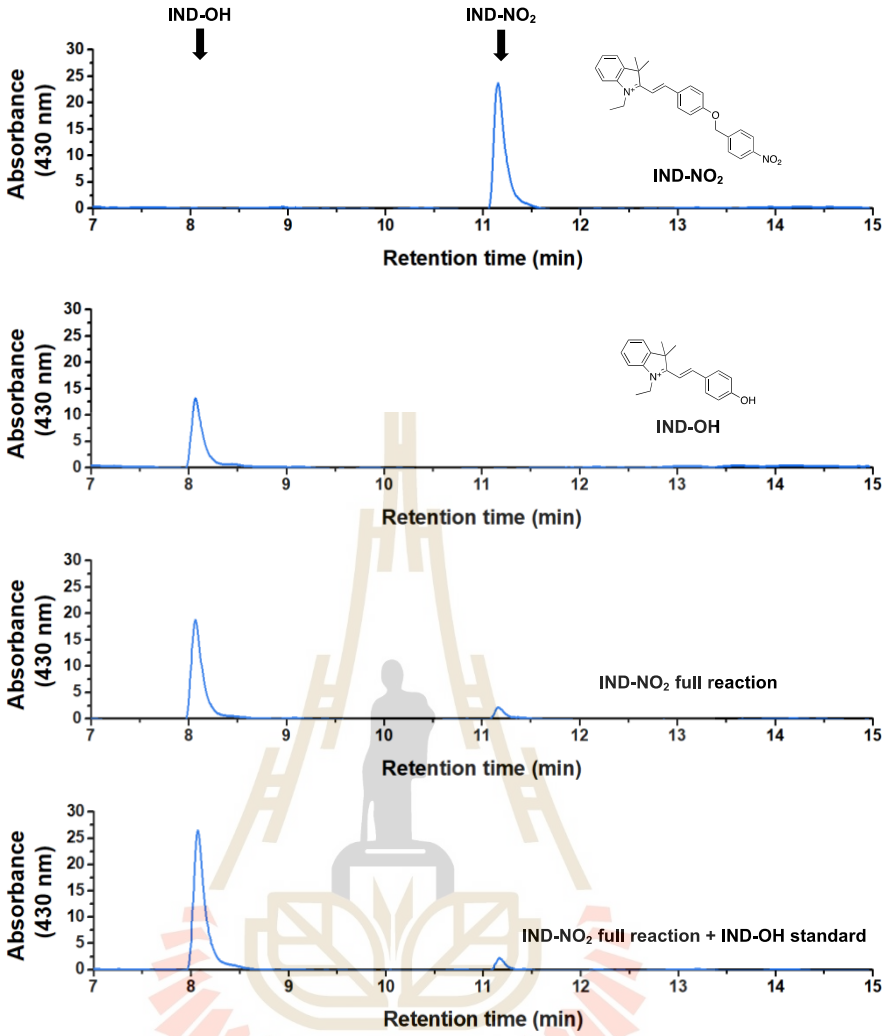

Figure 4.2 HPLC analysis of probe IND-NO_2 reduction catalyzed by *Ec*NfsB.

A linear relationship between the fluorescence intensity and *Ec*NfsB concentration was observed in the abovementioned investigation. Therefore, its limit of detection (LOD) for *Ec*NfsB was determined by fluorescence spectroscopy for an example. A fixed concentrations of 10 μM IND-NO_2 and 50 μM NADH, various concentrations of *Ec*NfsB were added in PBS buffer containing fixed concentrations of 10 μM IND-NO_2 and 50 μM NADH followed by 20 min incubation followed by determining fluorescence intensity at 564 nm. (Figure 4.3A). According to the principle of 3**σ**/slope, the determined LOD value was 6.21 nM (0.16 μg/mL), which is lower than other reported *E. coli* NTR probes (Guo et al., 2013; Kim et al., 2021; Li et al., 2023). Furthermore, endogenous NTR

concentrations in a few microorganisms have been reported, ranging from 0.78 μg/mL in *P. aeruginosa* to 4.81 μg/mL in *E. coli* (Zhang et al., 2024). This implied that our probe is suitable to detect NTR activity in living organisms.

To gain more insight into *Ec*NfsB-catalyzed IND-NO_2 reduction, its enzyme kinetic parameters were determined by a continuous assay of IND-OH generation monitored by fluorescence spectroscopy (excitation: 520 nm, emission: 564 nm). The concentration of IND-OH in each assay was calculated according to the calibration curve of IDN-OH (Figure 4.4). Various concentrations of IND-NO_2 were added into the mixture of 0.5 μM *Ec*NfsB and 100 μM NADH to determine the generation rates of IND-OH. Then, the generation rates of IND-OH and the substrate IND-NO_2 concentrations were plotted as Figure 4.3B to fit the Michaelis-Menten kinetic equation. Therefore, the apparent kinetic parameters of k_{cat} and K_m were 0.0256 s^{-1} and 8.3 μM, respectively, which are similar to the reported NTR probe (Brennecke et al., 2020). However, our probe's synthesis is simpler.

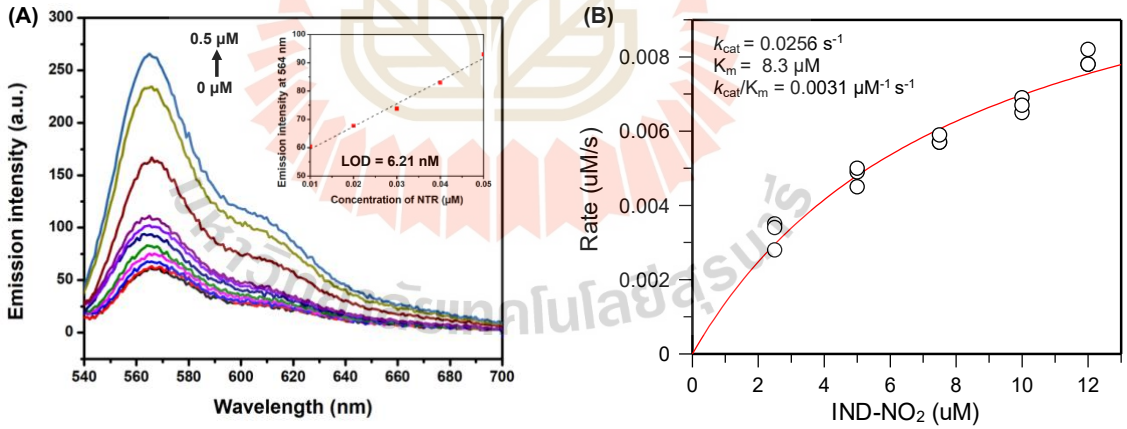

Figure 4.3 (A) Determination of limit of detection (LOD) value. 10 μM IND-NO_2 and 50 μM NADH were incubated with different concentrations of *Ec*NfsB in PBS buffer (pH 7.4) with 3% Tween-80 at 37 °C for 20 min. Each reaction was analyzed by fluorescence spectroscopy (λ_{ex} = 520 nm) to determine its fluorescence intensity at 564 nm. The intensities were plotted for LOD determination according to the principle of 3σ/slope. (B) The Michaelis–Menten plot of IND-NO_2 reduction catalyzed by *Ec*NfsB. The generation rate of IND-OH was determined by incubating various concentrations of IND-

NO_2 with the fixed concentrations of 0.5 μM of *Ec*NfsB and 100 μM NADH. The reaction rate for each IND-NO_2 concentration was determined in triplicate experiments.

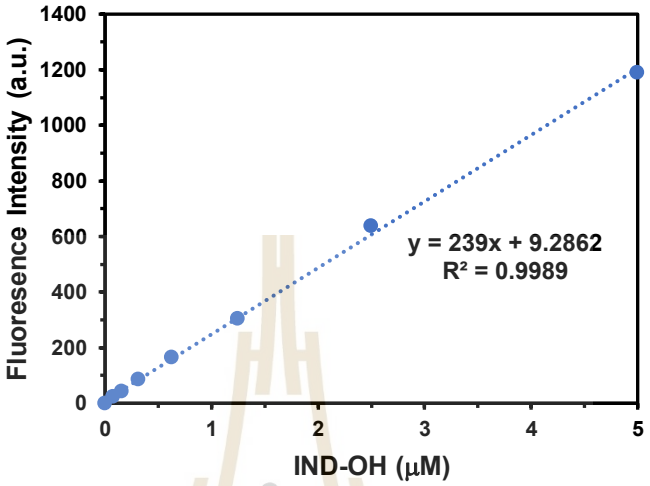

Figure 4.4 The calibration curve of IND-OH determined by fluorescence spectroscopy (Excitation: 520 nm. Emission: 564 nm).

4.2 Selectivity of IND-NO_2

Since IND-NO_2 can be reduced to produce IND-OH in the *Ec*NfsB-catalyzed reaction, as demonstrated by the aforementioned tests, IND-NO_2's specificity for the NTR reaction was further examined. Figures 4.5A and 4.5B demonstrate that only the full reaction containing substrate (IND-NO_2), cofactor (NADH), and enzyme (*Ec*NfsB) can activate the fluorescent signal, even at a very low enzyme concentration of 10 nM (near the limit of detection). Conversely, when the NTR inhibitor, dicoumarol, was introduced to the full reaction prior to IND-NO_2, the fluorescent signal did not enhance compared to the other controls (IND-NO_2, IND-NO_2 + NADH, and IND-NO_2 + dicoumarol). This indicates that IND-NO_2 is only reduced in the NTR-catalyzed reaction (*Ec*NfsB + NADH).

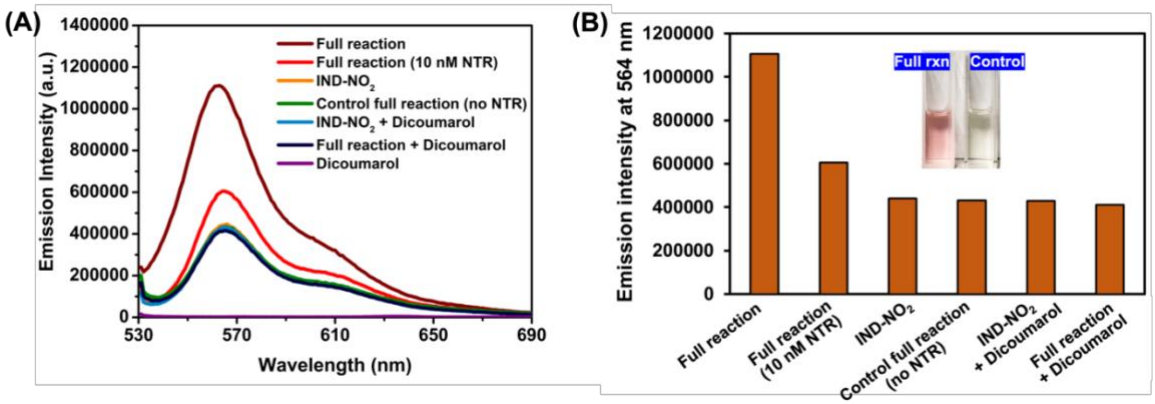

Figure 4.5 Fluorescence turn-on behavior of IND-NO_2 in the NTR-catalyzed reaction (*Ec*NfsB + NADH). (A) Fluorescence spectra and (B) fluorescence intensities at 564 nm. Only a full reaction containing *Ec*NfsB and NADH can activate the fluorescence signal compared with other controls. Furthermore, the full reaction using 10 nM *Ec*NfsB (near the limit of detection) generated a noticeable fluorescence signal enhancement.

The abovementioned studies have shown that IND-NO_2 can be reduced to generate IND-OH in the *Ec*NfsB-catalyzed reaction. However, there are various metabolites and proteins in the cellular environment. Therefore, the specificity of IND-NO_2 to the NTR reaction was required to test. Different biological molecules, especially the ones with reducing abilities, were individually incubated with 10 μM IND-NO_2 in PBS buffer with 3% Tween-80 to monitor the IND-OH generation by fluorescence spectroscopy. The selected molecules are reduced nicotinamide adenine dinucleotide (NADH), glucose, vitamin C (Vc), a protein sample (bovine serum albumin, BSA), free thiol compounds (cysteine, GSH (reduced glutathione), and DTT), and reduced sulfur compounds (S^{2-} and HSO_3^-). After incubation, each mixture was analyzed by fluorescence spectroscopy monitoring the signal of 564 nm (λ_{ex} = 520 nm), which corresponds to IND-OH formation compared with the full reaction (NTR and NADH), Figure 4.6A. Figure 4.6B shows that only the full reaction has the high fluorescence signal at 564 nm, while reactions with the other molecules showed the background signals from IND-NO_2 itself. The results suggested that all tested biological molecules

do not reduce IND-NO_2. Furthermore, IND-NO_2 incubated with NADH alone did not generate IND-OH, confirming that IND-NO_2 is a nitroreductase-activated probe.

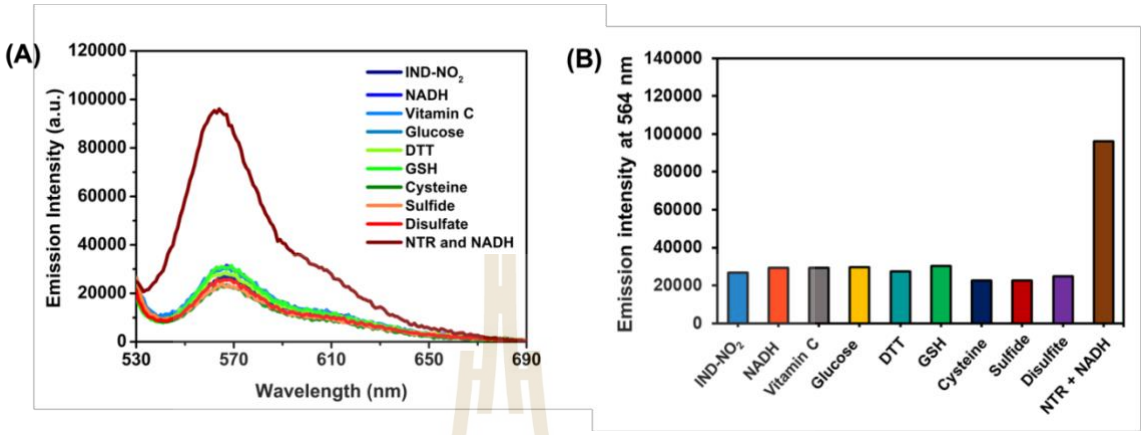

Figure 4.6 Fluorescence analysis of IND-NO_2 incubated with different biological molecules compared with the NTR-catalyzed reaction (*Ec*NfsB + NADH). (A) Fluorescence spectra and (B) fluorescence intensities at 564 nm. The results showed that only the NTR-catalyzed reaction displayed the highest fluorescence signal at 564 nm compared to other samples, which showed the background signals comparable to IND-NO_2.

4.3 Detection of bacterial NTR and inhibitory test

All *Ec*NfsB enzymatic results suggested that IND-NO_2 is a promising probe to detect NTR. Therefore, the IND-NO_2 probe was applied to detect NTR in bacteria. *Escherichia coli* TISTR780, *Pseudomonas aeruginosa* TISTR781, and *Staphylococcus aureus* TISTR1466 were used to represent Gram-negative and positive microorganisms. *E. coli*, *S. aureus,* and *P. aeruginosa* were individually cultivated in LB (Luria-Bertani) medium at 37 ºC till OD_{600} was 0.5 to 0.7. The cells were harvested and washed with PBS buffer three times. Lastly, the cells were resuspended in PBS buffer to make the OD_{600} of 2.0. The probe IND-NO_2 was added to each solution to make a final concentration of 10 μM. The solution was incubated at 37 ºC for 12 hours. After lysing cells by sonication followed by centrifugation, the resulting supernatant was analyzed by fluorescence spectrometry at 564 nm (λ_{ex} = 520 nm) corresponding to IND-OH. In

Figure 4.7, every sample containing bacteria treated with IND-NO_2 (orange bar) exhibited higher fluorescence intensities than those without IND-NO_2 (red bar), indicating that the reduction occurred inside bacterial cells. Furthermore, the bacterial cells were treated with the probe with the addition of an NTR inhibitor, dicoumarol (Johansson et al., 2003) to confirm the specificity of IND-NO_2 toward the NTR activity of bacteria. The fluorescence signals (yellow bar) were lower than the signals from those bacteria treated with IND-NO_2 alone (orange bar).

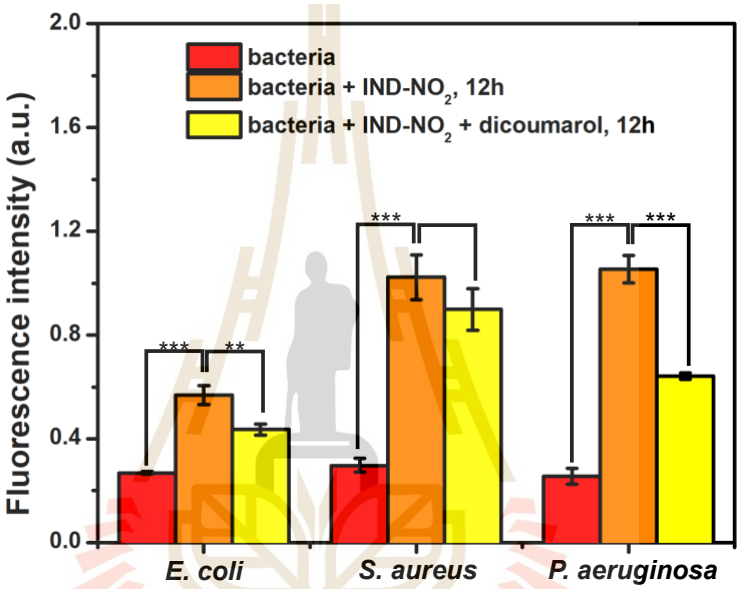

Figure 4.7 NTR detection in different bacteria using IND-NO_2 as probe. The fluorescence intensity was determined at the wavelength 564 nm (λ_{ex} = 520 nm) after the bacterial cells were treated with 10 μM IND-NO_2 with and without dicoumarol (0.4 mM), incubated at 37 °C for 12 h. All experiments were performed with 3 replicates. Statistical analysis is based on one-way ANOVA *(*P < 0.05, **P < 0.01, ***P < 0.001)*

4.4 References

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CHAPTER V

CONCLUSIONS

In summary, we designed a novel fluorescence turn-on probe IND-NO_2 (NTR probe) to detect nitroreductase (NTR) in bacteria cells. Synthesis of the IND-NO_2 probe was completed in a few steps. To test its NTR-activated property, *Ec*NfsB was used as a model enzyme. The enzymatic studies showed that *Ec*NfsB catalyzes the reduction of IND-NO_2 in the presence of NADH to generate IND-OH, which was characterized by fluorescence spectroscopy and HPLC analysis. The fluorescence turn-on signal resulted from IND-OH with the fluorescence maximum at 564 nm (λ_{ex} = 520 nm). In addition, IND-OH displayed pink color, which can be observed by naked eyes. The photophysical properties of IND-OH made it promising in practical applications. Furthermore, the reduction of IND-NO_2 is specific to the NTR-catalyzed reaction while all tested biological reductant molecules did not reduce the probe. The LOD value for *Ec*NfsB was determined as 6.21 nM, which is lower than other *E. coli* NTR probes. In bacteria tests, the IND-NO_2 probe was incubated with the in-house bacteria, *E. coli, S. aureus,* and *P. aeruginosa* representing ESKAPE pathogens, to show the higher fluorescence signals compared with the decreasing signals in reaction of the same bacteria containing an NTR inhibitor, suggesting the NTR-catalyzed turn-on signal was resiponsible for the change in fluorescence. Overall, the designed probe was demonstrated as a potential tool for identifying ESKAPE pathogen infections.

APPENDICES

APPENDIX A

SUPPORTING INFORMATION

A.1 Probe synthesis and characterization

IND-OH based on an indolium fluorophore and linked with 4-hydroxybenzene was chosen as a simple and convenient fluorescent reporter for NTR detection. Subsequently, 4-nitrobenzene, commonly referred to as the classical moiety reduced by NTR, was conjugated with IND-OH using an ester linker to synthesize IND-NO_2, which is non-fluorescent due to loss of donor-acceptor structure (Figure A.1).

To synthesize IND-NO_2, compound 1 was primarily synthesized by the substitution reaction of 4-hydroxybenzaldehyde and 4-nitrobenzyl bromide.(Rostami and Hamidi Zare, 2019) Then, the reaction between indolium, prepared as previously described,(Khaikate et al., 2024; Usama et al., 2018) compound 1 was carried out to generate the corresponding IND-NO_2 with a moderate yield (55%). Meanwhile, the condensation of indolium and 4-hydroxybenzaldehyde was also performed to afford the corresponding IND-OH in a moderated yield (72%). This synthesis was done by Asst. Prof. Dr. Onnicha Khaikate.

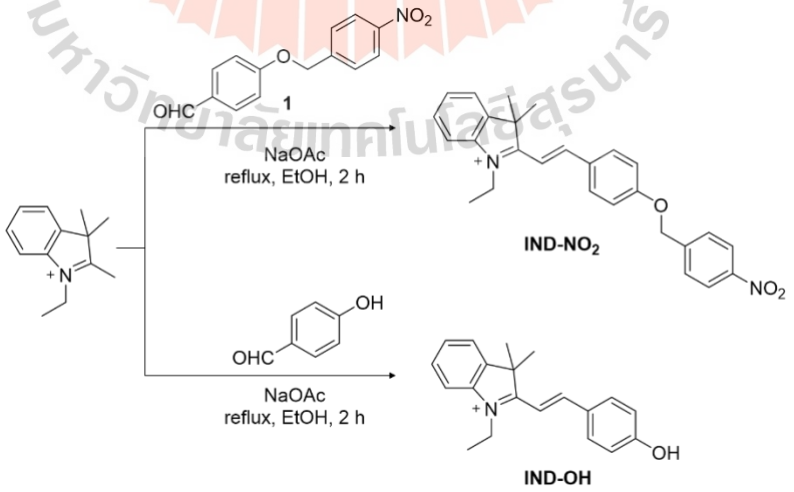

Figure A.1 Synthetic routes for IND-NO_2 and IND-OH.

A.2 Photophysical properties of IND-NO_2 and IND-OH

Firstly, the photophysical properties of IND-NO_2 in different solvents, including THF, DMSO, MeOH, and PBS buffer (pH 7.4), were determined by Asst. Prof. Dr. Onnicha Khaikate, and the results are summarized in Table A.1. As illustrated in Figure A.2A, the absorption spectra of IND-NO_2 in all tested solvents exhibited negligible changes, with absorbance maximum ranges from 418 to 432 nm. Weak fluorescence emission of IND-NO_2 was detected in various solvents, as displayed in Figure A.2B. Nonetheless, the outcomes met our need for a turn-on fluorescent probe. Next, the photophysical properties of reporter IND-OH, containing a typical D-**π**-A structure with an electron push–pull effect, were measured in different solvents (Figures A.2C–D). In all solvents, the absorption region is well-extended to the visible area. The maximum absorbance of IND-OH is between 459 and 548 nm in all tested solvents (Figure A.2C). Furthermore, IND-OH exhibited a fluorescence increase in protic solvents, peaking at 545–567 nm (Figure A.2D), with quantum yields ranging from 0.0003 to 0.0013 (Table A.1). This might be due to the hydrogen bonding between the reporter IND-OH and the solvents, resulting in the stabilization of excited-state species (Krystkowiak et al., 2006).

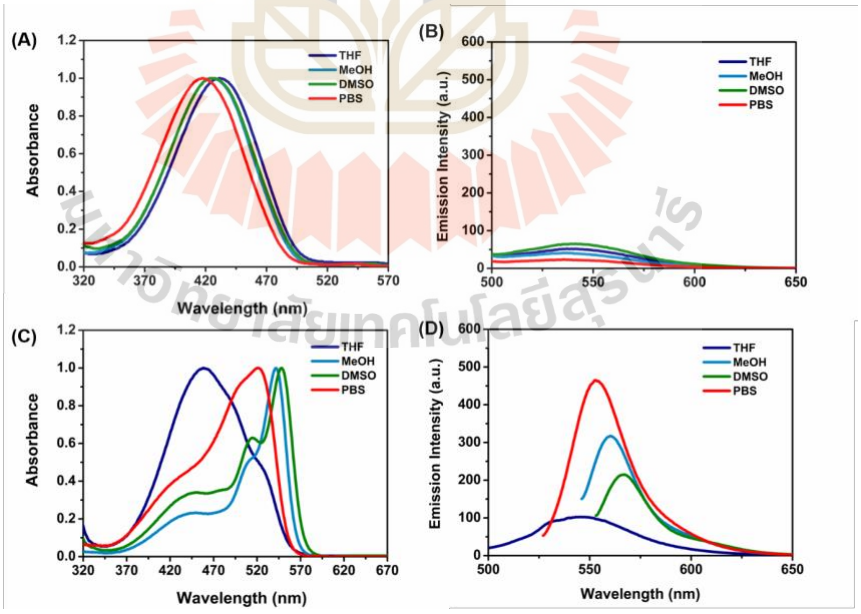

Figure A.2 (A) Absorption spectra and (B) fluorescence spectra (excited at λ_{max} in each solvent) of IND-NO_2 (10 μM) in different solvents. (C) Absorption spectra and (D) fluorescence spectra (excited at λ_{abs} in each solvent) of IND-OH (10 μM) in different solvents.

Table A.1 Photophysical properties of IND-NO_2 and IND-OH (10 μM) in different solvents.

Photophysical properties						
Probes	**Solvents**	**[a]λ_{abs} (nm)**	**[b]ε ($M^{-1}cm^{-1}$)**	**[c]λ_{em} (nm)**	**$\Delta\lambda$ (nm)**	**[d]Φ_f**
IND-NO_2	THF	432	35,200	Non-fluorescence		
	MeOH	425	34,200	Non-fluorescence		
	DMSO	426	31,800	Non-fluorescence		
	PBS	418	32,900	Non-fluorescence		
IND-OH	THF	459	25,900	545	86	0.0004
	MeOH	542	49,100	560	18	0.0003
	DMSO	548	41,600	567	19	0.0013
	PBS	521	32,600	553	32	0.0006

[a]λ_{abs} = absorption maximum wavelength. [b]ε = molar absorptivity. [c]λ_{em} = emission maximum wavelength (Excitation wavelength at λ_{abs}). [c]$\Delta\upsilon$ = stokes shifts (λ_{em} - λ_{abs}). [d]Φ_f = fluorescence quantum yields calculated using quinine sulfate in 0.1 M H_2SO_4 was used as a standard (Φ_f = 0.54).

A.3 The photographs of *Ec*NfsB catalyze reduction of IND-NO_2 to generating IND-OH

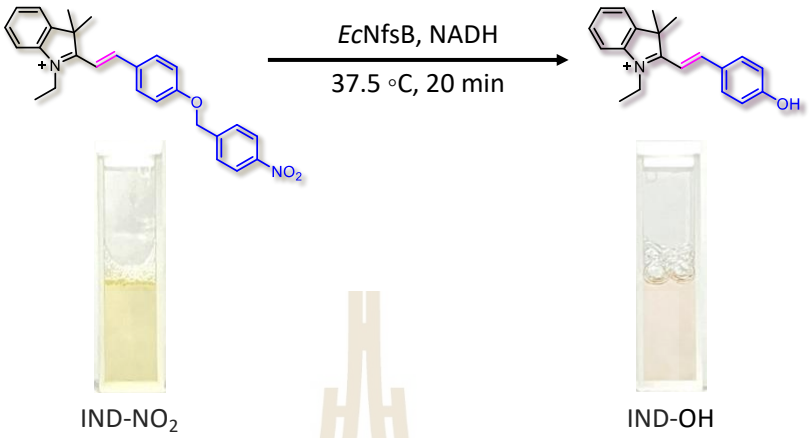

Figure A.3 The photographs of *Ec*NfsB catalyze the reduction of IND-NO_2 to generate IND-OH. The reaction containing 10 μM IND-NO_2 incubated with 0.5 μM *Ec*NfsB and 50 μM NADH in PBS buffer pH 7.4 at 37 °C for 20 min. The generation of IND-OH displayed a pink colour which can be observed by the naked eyes.

A.4 References

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APPENDIX B

PRESENTATIONS

List of poster presentation

Tawanya Kamthong, Onnicha Khaikate, Anyanee Kamkaew, and Rung-Yi Lai (February 2024). Improvement of esterification catalyzed by carboxylic acid reductase and Investigation of nitroreductase detection by a fluorescence probe. **Science Postgrad Annual Research Conference: SPARC 2024,** Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Tawanya Kamthong, Onnicha Khaikate, Anyanee Kamkaew, and Rung-Yi Lai (August 2024). Investigation of nitroreductase detection by a fluorescence probe. **The 18th International Symposium of the Protein Society of Thailand**, Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

Abstract submitted in Science Postgrad Annual Research Conference: SPARC 2024

Science Postgrad Annual Research Conference: SPARC 2024
Institute of Science, Suranaree University of Technology
23 February 2024

Improvement of esterification catalyzed by carboxylic acid reductase and Investigation of nitroreductase detection by a fluorescence probe

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Abstract

There are two enzymatic projects in this report. The first project is to improve esterification catalyzed by carboxylic acid reductases (CAR). The native reaction of CAR is to reduce carboxylic acid to aldehyde by using ATP and NADPH in the presence of Mg^{2+}. Because of its potential of biocatalyst application, CAR was developed to catalyze esterification of carboxylic acid in the presence of alcohol without NADPH in the reaction. However, its model esterification reaction of cinnamic acid and methanol was reported about 45%. To improve its yield, ATP regeneration catalyzed by polyphosphate kinase and pyrophosphate degradation catalyzed by pyrophosphatase were systematically applied in the CAR-catalyzed esterification. The conversion and yield of the optimized reaction were 90% and 65%, respectively. The second project is to investigate a fluorescence probe for the detection of nitroreductase (NTR) in bacteria. *E. coli* NTR was chosen to be a model enzyme to show that the probe's nitro group can be reduced by NTR to form an amino group, that generates the fluorescence signal. The reaction was confirmed by fluorescence spectroscopy and HPLC. The enzyme kinetic for the probe was further determined. Lastly, the reduction of the probe was only catalyzed by NTR compared with the control experiments using various biological reductants.

Keywords: Biocatalysts; Carboxylic acid reductase; Esterification; Nitroreductase; Fluorescent probe

Abstract submitted in the 18th International Symposium of the Protein Society of Thailand

PP075

Investigation of nitroreductase detection by a fluorescence probe

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ABSTRACT

Nitroreductase (NTR) in a wide range of bacteria plays a vital role in the detoxification of nitro-containing compounds. Therefore, nitroreductase detection could be applied in detection of bacteria. In this work, we reported an NTR-responsive fluorescent probe (OK-54) to detect NTR in few microorganisms. In the preliminary tests, *E. coli* NTR was chosen to be a model enzyme. The probe's nitro group can be reduced by NTR to form an amino group in the presence of nicotinamide adenine dinucleotide (NADH), resulting in a significant increase of fluorescence signal at 564 nm. The reaction was characterized by fluorescence spectroscopy and HPLC to confirm the product identity. The enzyme kinetic for the probe's reduction was further determined. In addition, to confirm the sensing specificity, the results showed that the reduction of the probe is only catalyzed by NTR compared with the control experiments using various biological reductants. Lastly, the probe was successfully used to detect bacterial NTR activity *in vivo*.

The 18th International Symposium of the Protein Society of Thailand. Convention Center, Chulabhorn Research Institute, Bangkok, Thailand. Aug 28-30, 2024.

CURRICULUM VITAE

Tawanya Kamthong was born on March 15th, 2000, in Sisaket, Thailand. In 2022, she graduated with a Bachelor of Science in Biology (with first-class honors) from Institute of Science, School of Biology, Suranaree University of Technology (SUT), Thailand. Since 2015, the Development and Promotion of Science and Technology Talents Project (DPST, Thailand) has awarded her a scholarship. Under the guidance of Assoc. Prof. Dr. Sineenat Siri, she carried out senior research on rapid one-step synthesis of silver nanoparticles using tea and coffee wastes during her bachelor's degree. In 2022, she conducted her master's degree program in Biochemistry and Biochemical Technology at the School of Chemistry, SUT under the supervision of Asst. Prof. Dr. Rung-Yi Lai with DPST scholarship. Her master's thesis focuses on the investigation of nitroreductase detection by a fluorescence probe. This work has been reported in the 18th international symposium of the Protein Society of Thailand (listed in APPENDIX B) in August 2024. In the end of 2024, she received the Japanese government (monbukagakusho:MEXT) scholarship as a research student to conduct her second master's study in Biochemistry at the Graduate School of Agriculture, Hokkaido University, Japan, under the supervision of Assoc. Prof. Dr. Wataru Saburi.