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)

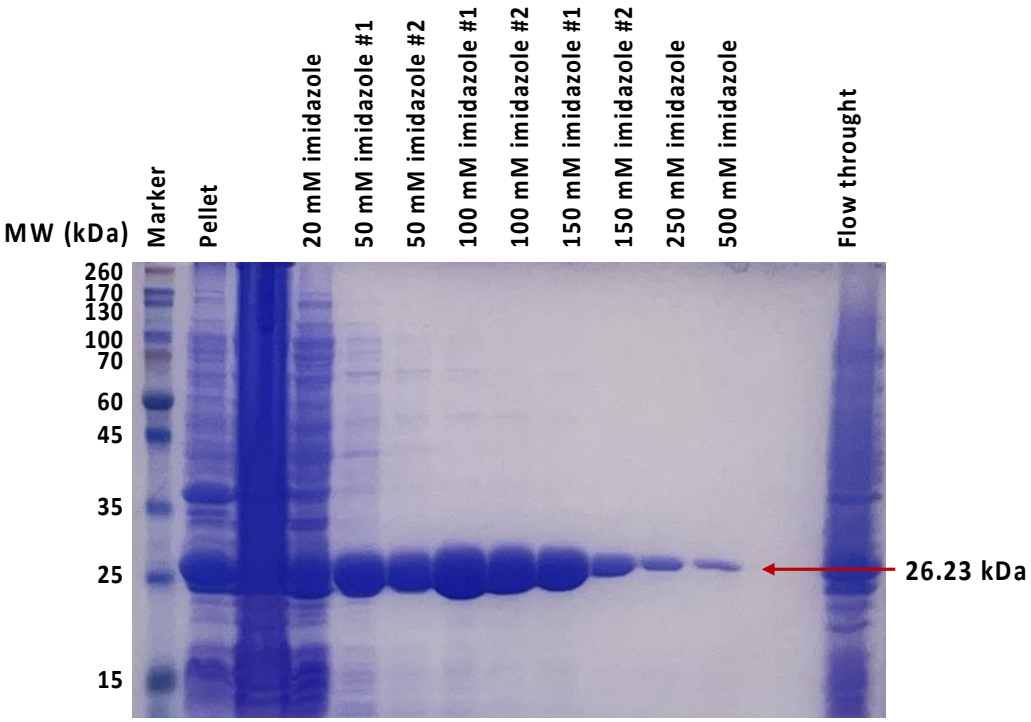

Figure 4.1 The SDS-PAGE analysis of *Ec*NfsB purification by Ni-NTA chromatography. The pure fractions were collected.

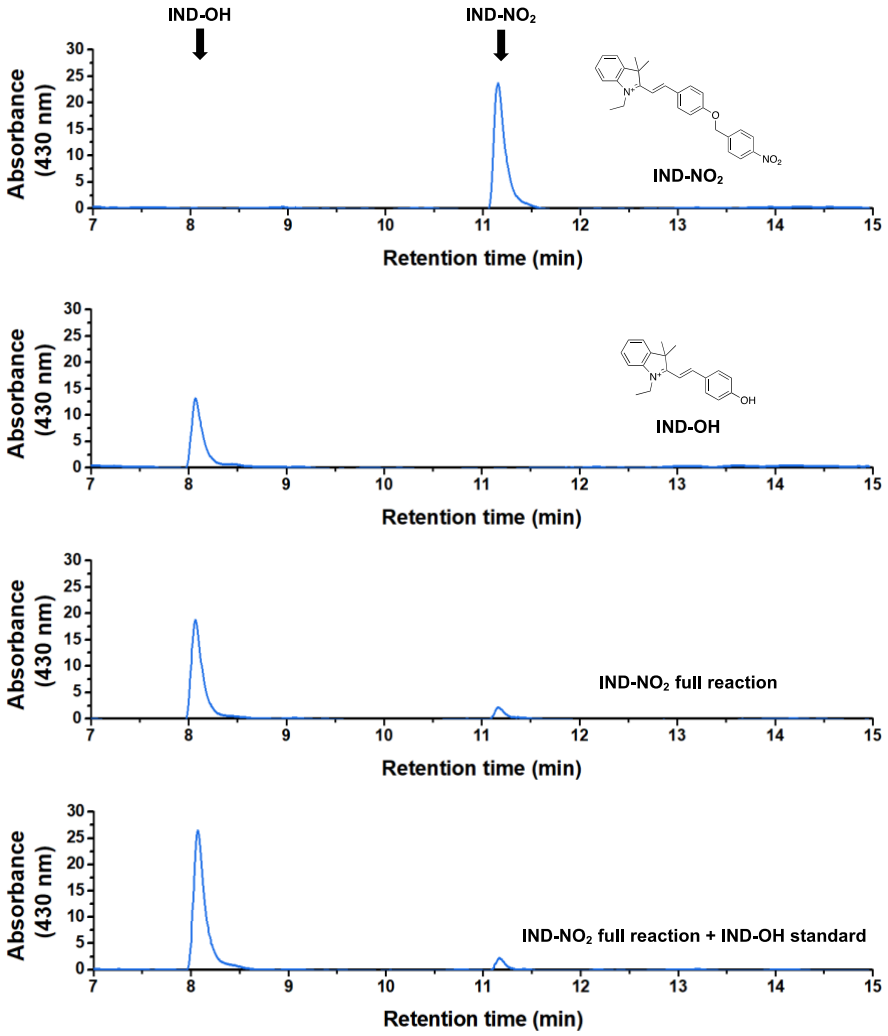

Figure 4.2 HPLC analysis of probe IND-NO_2 reduction catalyzed by *Ec*NfsB.

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.

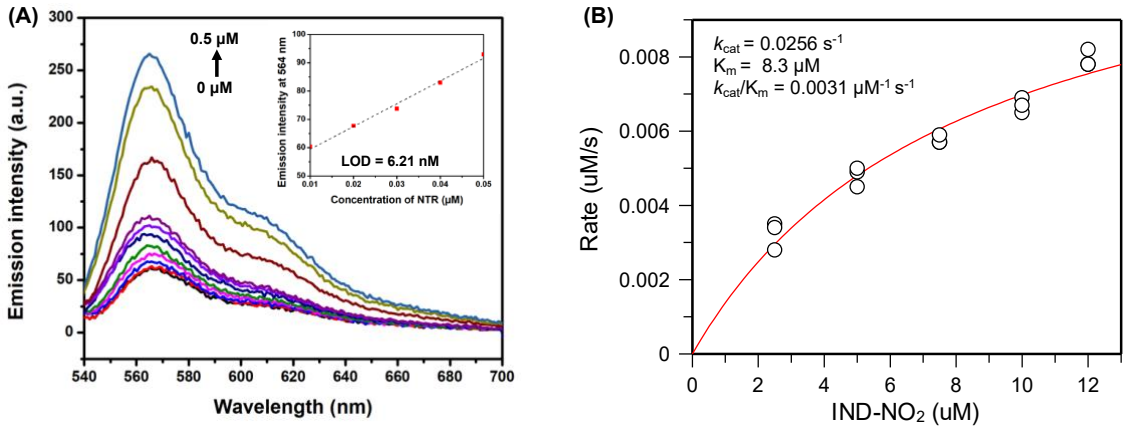

Figure 4.3 (A) Determination of limit of detection (LOD) value. 10 μM IND-NO_2 and 50 μM NADH were incubated with different concentrations of *Ec*NfsB in PBS buffer (pH 7.4) with 3% Tween-80 at 37 °C for 20 min. Each reaction was analyzed by fluorescence spectroscopy (λ_{ex} = 520 nm) to determine its 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.

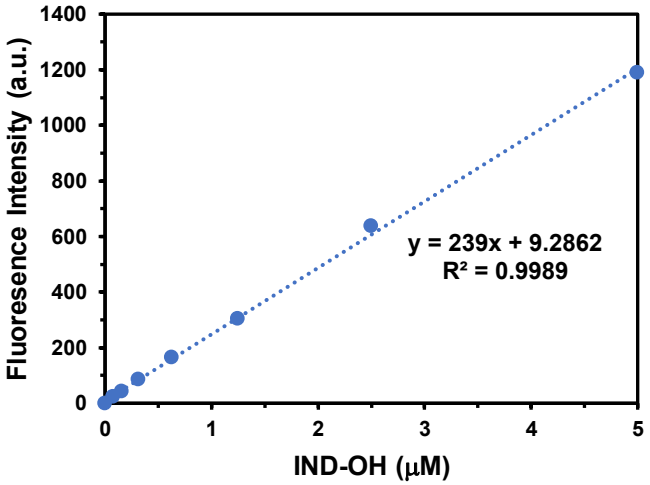

Figure 4.4 The calibration curve of IND-OH determined by fluorescence spectroscopy (Excitation: 520 nm. Emission: 564 nm).

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).

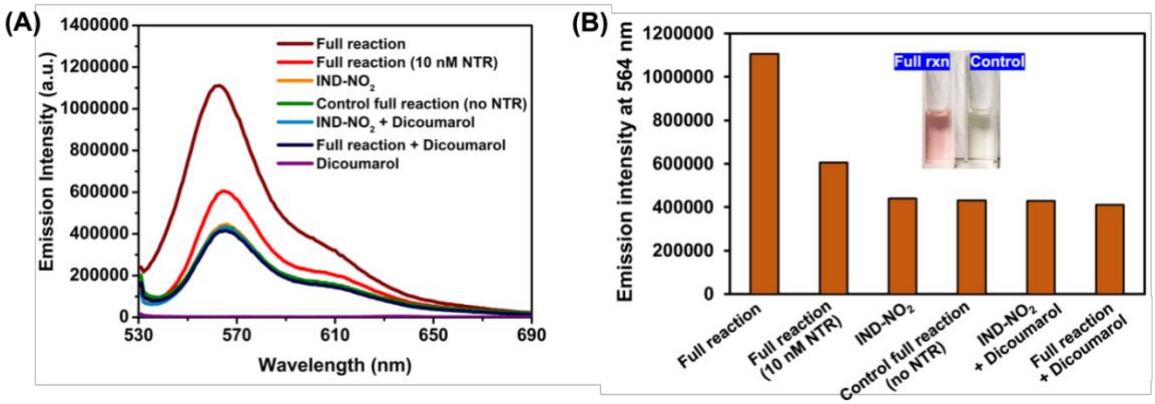

Figure 4.5 Fluorescence turn-on behavior of IND-NO_2 in the NTR-catalyzed reaction (*Ec*NfsB + NADH). (A) Fluorescence spectra and (B) fluorescence intensities at 564 nm. Only a full reaction containing *Ec*NfsB and NADH can activate the fluorescence signal compared with other controls. Furthermore,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

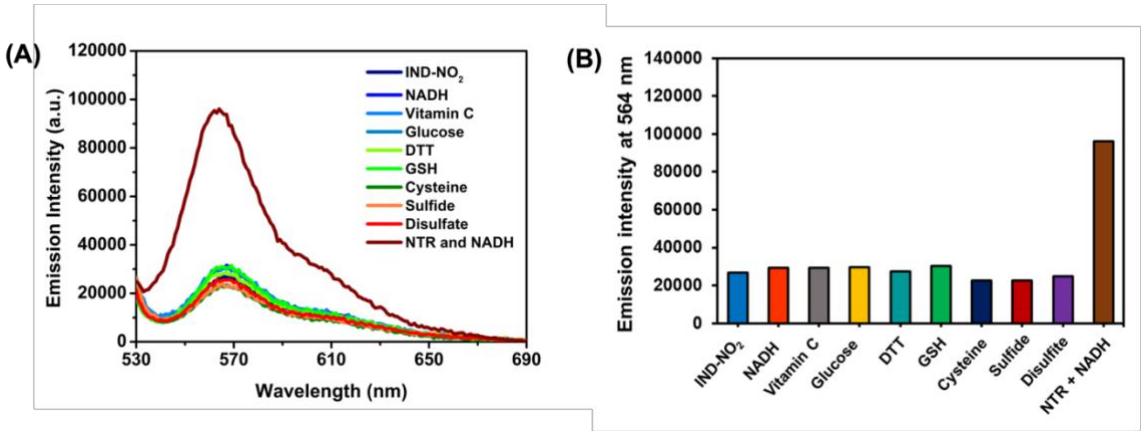

Figure 4.6 Fluorescence analysis of IND-NO_2 incubated with different biological molecules compared with the NTR-catalyzed reaction (*Ec*NfsB + NADH). (A) Fluorescence spectra and (B) fluorescence intensities at 564 nm. The results showed that only the NTR-catalyzed reaction displayed the highest fluorescence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

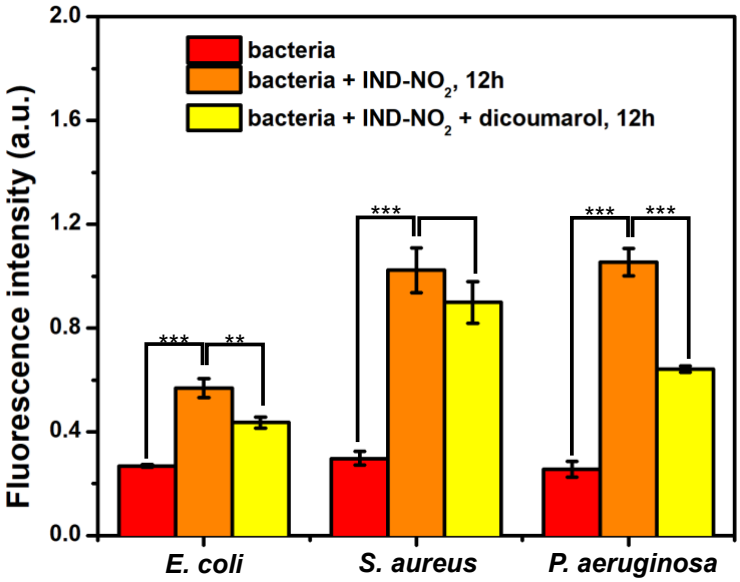

Figure 4.7 NTR detection in different bacteria using IND-NO_2 as probe. The fluorescence intensity was determined at the wavelength 564 nm (λ_{ex} = 520 nm) after the bacterial cells were treated with 10 μM IND-NO_2 with and without dicoumarol (0.4 mM), incubated at 37 °C for 12 h. All experiments were 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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