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論　文　内　容　の　要　旨

氏　名　（　Tran Thi My Duyen　）	
論文題名	Development of biosensors for monitoring of antibiotics in water environment　（環境水中抗菌性物質検出用バイオセンサーの開発）

論文内容の要旨

The overuse and misuse of antibiotics, particularly in developing countries, have resulted in antibiotic residue in the water environment, which can stimulate the occurrence and spread of antibiotic-resistant bacteria. Because antibiotic resistance is becoming a serious threat to global human health, there is an increasing need to obtain comprehensive information on residual antibiotics in environmental water samples. This information is crucial for further studies, in terms of understanding the factors contributing to the occurrence of antibiotic-resistant bacteria and helping to prevent their spread.

Currently, monitoring of antibiotics is often performed in laboratories that in most cases use conventional techniques such as liquid chromatography coupled with mass spectrometry, requiring expensive equipment, specialized personnel, and time-consuming sample treatment procedures. These drawbacks have made wide application of these methods difficult in developing countries, where overuse and misuse of antibiotics is much more serious than in developed countries. Therefore, developing a simple and low-cost detection method appropriate for preliminary screening for antibiotics in the water environment in a low-resource setting is an urgent need. Biosensors are promising alternative tools because they may provide rapid, simple, and reliable methods that would be highly useful for preliminary screening prior to using more costly techniques. The main objective of this thesis was to study the development of biosensors for monitoring the two main categories of antibiotics: sulfonamides (SAs), which inhibit folate biosynthesis, and antibiotics that inhibit bacterial protein synthesis.

To develop a biosensor for sulfonamides, a dihydropteroate synthase (DHPS) enzyme-based biosensor was investigated. DHPS is a crucial enzyme in the folate biosynthesis pathway, which catalyzes the reaction of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHP-PP) with *para*-aminobenzoic acid (pABA) to yield 7,8-dihydropteroate and pyrophosphate (PPi). Because of the structural similarity between SAs and pABA, SAs can serve as an alternative substrate of DHPS, causing production of non-functional dihydropterin-sulfonamide adducts and PPi. Therefore, the DHPS enzyme has potential for use as a biosensor recognition element, through detection of PPi. To construct the biosensor, three components were required: (i) a substrate of DHPS, DHP-PP; (ii) a recombinant DHPS enzyme; and (iii) a method for detecting PPi. First, a DHP-PP substrate was synthesized through the bioconversion of dihydroneopterin to DHP-PP, using two recombinant enzymes in the folate synthesis pathway, dihydroneopterin aldolase and dihydropterin pyrophosphokinase. Then, a mixture of the resulting DHP-PP, recombinant DHPS enzymes, conversion reagent for PPi, and ATP-dependent luciferase was freeze-dried into pellets for subsequent addition to a water sample. After a water sample containing SAs is added to the freeze-dried reaction pellet, the amount of SAs present can be estimated by measuring the luminescence intensity. The biosensor could detect a class-specific group of SAs (sulfadimidine, sulfadoxine, sulfadimethoxine, sulfadiazine, sulfathiazole, and sulfamethoxazole) with a limit of detection (LOD) of approximately 20 ng/mL. Moreover, the freeze-dried enzyme reaction pellets were found to retain 80% sensitivity after storage at 4°C for one week. In short, the enzyme-based biosensor is a simple-to-prepare tool that shows promise for rapid, easy, and low-cost detection of SAs in water environments.

To develop a biosensor for detecting antibiotics that inhibit bacterial protein synthesis, such as

aminoglycosides, tetracyclines, chloramphenicol, and macrolides, the author hypothesized that an *in vitro* transcription/translation (IVTT) system reconstituted from the purified recombinant components necessary for *Escherichia coli* translation could serve as a suitable biological recognition element. The concept of the biosensor is that it detects the anti-translational activity of antibiotics that inhibit bacterial protein synthesis through the color changes induced by β-galactosidase (β-GAL) synthesis on paper discs. In the absence of antibiotics, β-GAL is synthesized and hydrolyzes a colorimetric substrate, changing the color of the paper disc from yellow to purple. In the presence of antibiotics, inhibition of β-GAL synthesis occurs, resulting in inhibition of the color change. The color change can be evaluated by the naked eye or quantified using a desktop scanner and image analysis software. Concentration-dependent color variation in the paper discs was observed by the naked eye; the LODs for paromomycin, tetracycline, chloramphenicol, and erythromycin, were estimated to be 0.5, 2.1, 0.8, and 6.1 μg/mL, respectively. The effects of temperature and pH differences on the response of the biosensor were also investigated, and the color change was confirmed to occur between 15–37°C and pH 6–10. In addition, the paper-based biosensor could be used to detect 0.5 μg/mL paromomycin, spiked in actual environmental water samples, by the naked eye. Although the sensitivity of the biosensor needs to be improved to enable detection of the concentration of antibiotics commonly detected in water environments, the author provides a novel concept for paper-based, simple, rapid, easy-to-use, and easy-to-dispose biosensors that are appropriate for the preliminary screening of antibiotics in water environments.

To improve the sensitivity of the paper disc–based colorimetric biosensor to antibiotics, the following four refinements were added: (i) patterned papers instead of paper discs, (ii) a high-sensitivity luciferase (NanoLuc) instead of ß-GAL, (iii) a different IVTT system (PURExpress 2.0 instead of PURExpress), and (iv) a double-translation cascade instead of a single translation system. In the IVTT reactions mentioned so far, the DNA template containing the reporter genes is under the control of the T7 promoter, which is recognized by T7 RNA polymerase. To improve the sensitivity of the biosensor, a cascade containing two translation steps was designed: mRNA encoding a T3 RNA polymerase is translated, and then the resulting RNA polymerase activates the T3-mediated transcription and translation of NanoLuc, leading to light emission. In the presence of antibiotics, the transcription/translation of T3-mediated NanoLuc is doubly inhibited through two sequential steps: (i) the inhibition of translation of T3 RNA polymerase, which is required for transcription of NanoLuc mRNA, and (ii) the inhibition of transcription/translation of T3-mediated NanoLuc itself, suppressing light emission. Light emission was detected using a digital camera and quantified using image analysis software. The paper-based biosensor with this double-translation cascade offered a more sensitive response to paromomycin (an approximately 10-fold improvement in the LOD) compared to the response of the normal reporter template. The LOD for other antibiotics did not improve. Furthermore, freeze-dried printed papers embedded with the IVTT reaction mixture functioned after one week of storage at 4°C.

Overall, the author presents novel concepts for the development of simple, rapid, easy-to-use, and easy-to-dispose (in the case of the paper-based biosensor) biosensors that are appropriate for the preliminary screening of antibiotics contaminating water environments. These biosensors show great potential to provide important information for future studies that can help to prevent the occurrence of resistant bacteria through comprehensive monitoring of residual antibiotics in water environments.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

東南アジアにおいて脅威となっているESBL（Extended Spectrum βLactamase）産生菌等の多剤耐性菌の高頻度な出現は、現地での畜水産業における抗生物質の無秩序な使用が主原因である。博士論文「Development of biosensors for monitoring of antibiotics in water environment」では、多剤耐性菌の出現防止に向けて、こういった抗生物質の使用実態を迅速かつ安価にモニタリングできるバイオセンサーの開発を目指すものであり、東南アジアの畜水産業において大量に使われている作用機序が異なる２群の抗生物質について、新規の抗生物質検出機構に基づくバイオセンサーの基盤技術の開発に成功した。

具体的には、主に畜産業に用いられるサルファ剤について、葉酸代謝経路の特定の酵素（DHPS）を当該抗生物質が特異的に阻害することを利用して、この阻害反応により抗生物質濃度依存的に生成されるPPi量を蛍光強度に変換して高感度で検出できるバイオセンサーの新規基盤技術を開発した。次に、アミノグリコシド系抗生物質等のタンパク質合成阻害作用を有する抗生物質について、人工的な転写・翻訳システムを小型のろ紙上に提示し、抗生物質による翻訳系の阻害作用の強さを呈色反応の阻害、さらには蛍光の減弱化を指標として定量的に検出するバイオセンサーの新規基盤技術を開発した。

本研究の成果は、今後東南アジア等の発展途上国において使用される当該抗生物質に対する実用的な環境モニタリングシステムの開発につながる有用な基盤技術を確立したことにあり、また抗生物質の作用機序を利用した点は学術的価値も高い。以上、これらの点についての審査により、博士（薬科学）の学位論文に値するものと認める。