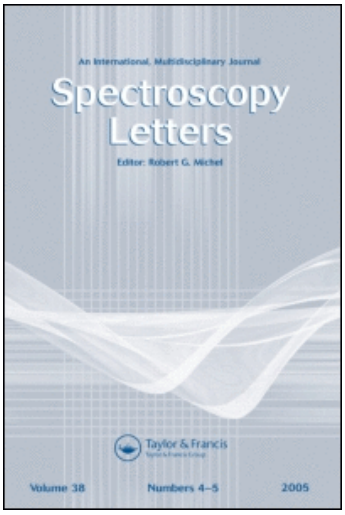

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Determination of NAD(P)H by A Bioluminescent Enzyme Fiber Optic Probe

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DETERMINATION OF NAD(P)H BY A BIOLUMINESCENT ENZYME FIBER OPTIC PROBE

Key Words: NAD(P)H, bioluminescence, fiber optic, bacterial luciferase, chemiluminescence.

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ABSTRACT

A fiber optic probe was interfaced to a photon counting system for the determination of nicotinamide adenine dinucleotide reduced [NAD(P)H] by a bioluminescence method. The reagents employed in a bacterial luciferase/flavin mononucleotide /decanal system were optimized. Attempts were made to increase the quantum yield of the system. Dodecanal, tridecanal, and tetradecanal were evaluated as alternative aldehyde reagents for decanal, and hydrogen peroxide was added to the system. Neither attempt increased the quantum yield of the system. However, a relatively low detection limit of 1.6×10^{-9} M for NAD(P)H was obtained with a linear dynamic range of 3.8 orders of magnitude. These results demonstrate the sensitivity of this instrumentation and assay.

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INTRODUCTION

Bioluminescence (BL) methods have the advantages of high sensitivity with relatively simple inexpensive instrumentation (1). A number of these methods have been developed for nicotinamide adenine dinucleotide reduced [NAD(P)H] because of its role in biosynthetic processes (1-14). Two enzymes are necessary for the production of light in the presence of NAD(P)H. NAD(P)H oxidoreductase catalyzes the oxidation of NAD(P)H to $NAD(P)^+$ while reducing FMN (flavin mononucleotide) to $FMNH_2$. Bacterial luciferase utilizes $FMNH_2$, oxygen, and a long chain aldehyde (typically decanal) to produce the carboxylic acid, FMN, and 490 nm light. Detection limits in the picomolar to attomolar concentration levels have been reported.

Fiber optic probes offer potential for BL analyses because of their simplicity, durability, and potential for remote monitoring (15, 16). However, the sensitivity is degraded compared to conventional optical design, presumably because of reduced collection efficiency.

The goal of this project was to improve the detection limit of NADH using a BL fiber optic probe using two approaches in order to increase the quantum yield of the reaction. First, the substitution of alternative aldehydes for decanal was investigated. Second, hydrogen peroxide was added to the system.

EXPERIMENTAL

The photon counting system employed for this project was constructed from a borosilicate glass cell, a fiber optic cable (Oriel #77525), a photomultiplier tube (Thorn EMI # 9789QB), an amplifier/discriminator (Princeton Applied Research #1120), a photon counter (Princeton Applied Research #1112), a 386 IBM compatible computer, and an interface between the photon counter and the computer (Intel #8255A). Since the photon counter was not designed to store and

record data for more than one cycle of operation, software was written to control the counter and collect data for 20 six second cycles for a total of two minutes. A magnetic stirrer was used to agitate the reaction mixture constantly. The cell was capped by a rubber stopper through which the fiber optic was inserted into the solution. All reagents except for NAD(P)H were introduced into the cell with mechanical pipettes. The analyte was introduced using a gas-tight 50 μL syringe that was inserted through the rubber stopper. The cell was lowered onto the magnetic stirrer which was housed in a light-tight box with the detection system. A detailed description of the instrumentation is given by Jocoy (1).

NAD(P)H, bovine serum albumin (BSA), FMN, and bacterial luciferase from *Vibrio fisheri* and *Vibrio harveyi* were obtained from Sigma Chemical. Aldrich Chemical supplied the aldehydes employed in this work. Because the aldehydes trimerized within a few hours of storage, they were purified by vacuum distillation prior to use.

RESULTS AND DISCUSSION

The first portion of the experiment work was the optimization of the standard assay, including the temperature and concentrations of the various reagents employed (14). The optimum temperature for our system was 24°C, which was comparable to the literature values which vary between 20°C and 30°C (2-7, 9-13, 15). Bovine serum albumin (BSA) was added to the enzyme solution in order to stabilize the enzyme. Makemson and Hastings (17) reported that high concentrations of BSA reduced the bioluminescence intensity because BSA competed with bacterial luciferase for the aldehyde. We observed an optimal BSA concentration of 3.7×10^{-5} M, which is comparable to the literature values.

Figure 1 shows an optimization curve of bacterial luciferase. The enzyme from *Vibrio fisheri*, rather than from *Vibrio harveyi*, was used because it provided a higher quantum yield. The bioluminescene reached a maximum value at

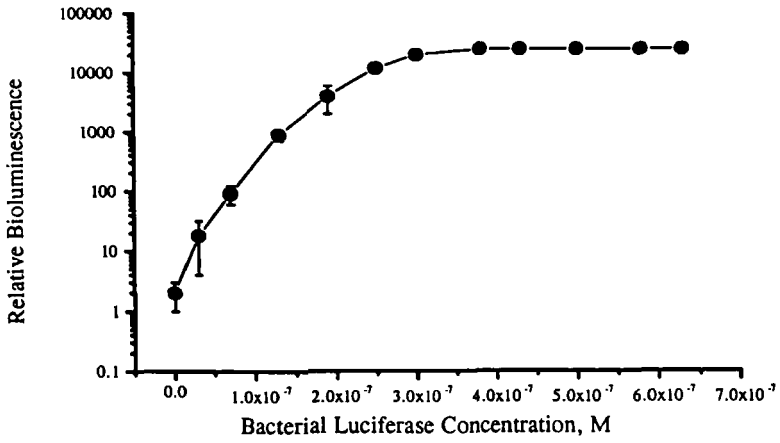

Figure 1. Optimization of bacterial luciferase.

approximately 3 x 10^{-7} M and remained constant at higher concentrations. At the higher concentrations, the bioluminescence was produced as a relatively short burst, so that little light was collected at the end of the two minute acquisition time. However, at the lower concentrations on the plateau, the bioluminescence signal occurred throughout the entire acquisition period. Consequently, the optimal concentration was selected as the lowest value that gave the maximal bioluminescence signal. A concentration of 3.8 x 10^{-7} M was selected as the optimum.

Although the presence of FMN is required for the bioluminescent reaction, it absorbs the luminescence and therefore its concentration must be carefully controlled. Figure 2 shows our optimization study, demonstrating significant reduction in luminescence at concentrations higher and lower than the optimized value of 2.1 x 10^{-6} M.

The standard assay employs decanal as the aldehyde. Decanal has relatively low solubility in water, and we found that better precision was obtained

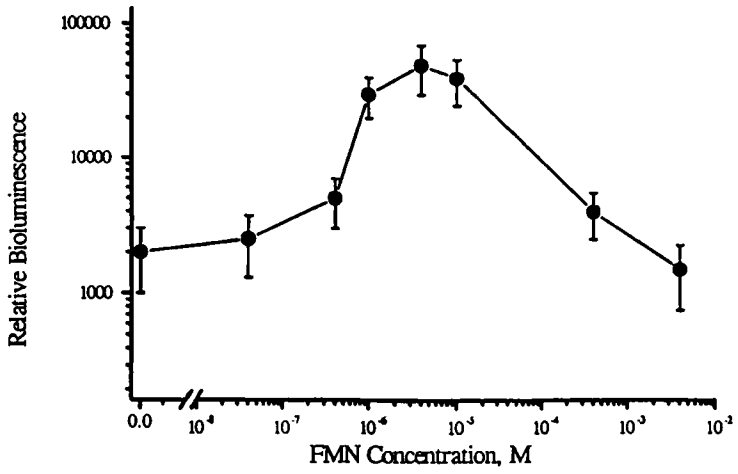

Figure 2. Optimization of FMN.

by dissolving the aldehyde in ethanol before introduction in the reaction mixture. The optimization graph for decanal is shown in Figure 3. The bioluminescence intensity increases up to approximately 2.6×10^{-4} M decanal, which was the optimized concentration used in future experiments.

The use of other long chain aldehydes was investigated in order to improve the sensitivity of the assay. Dodecanal, tridecanal, and tetradecanal were obtained for this study, but preliminary results showed that tridecanal and tetradecanal trimerized rapidly, and hence only dodecanal was investigated in detail. Unlike decanal, whose optimization graph reached a plateau at high concentrations (Figure 3), dodecanal induced a maximum value at 7×10^{-6} M, with lower and higher concentrations reducing the bioluminescence intensity (Figure 4). Comparison of the signals produced by the two aldehydes showed that there was no statistical difference at the optimized values. However, decanal was chosen as the superior reagent because of its longer storage time and its lower sensitivity to variation in concentration.

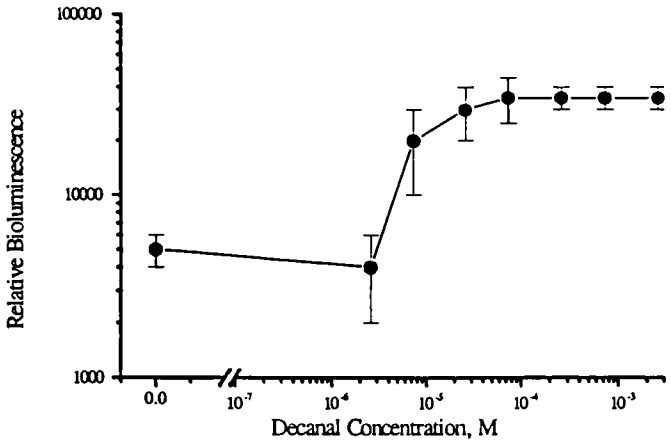

Figure 3. Optimization of decanal.

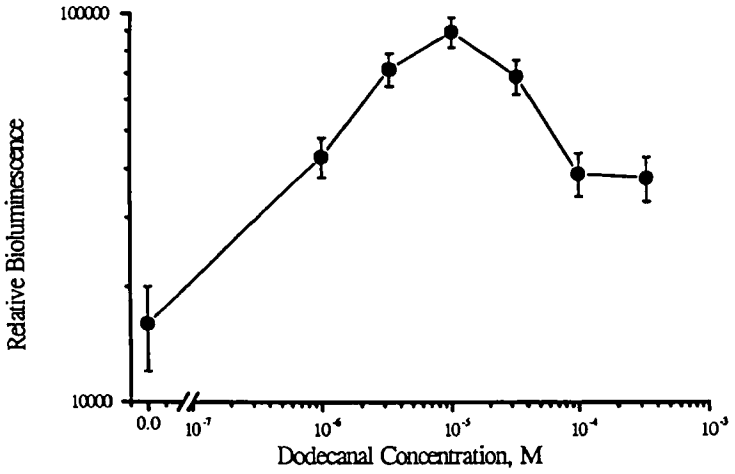

Figure 4. Optimization of dodecanal.

TABLE 1. Optimized Conditions and Analytical Figures of Merit for NAD(P)H

OPTIMIZED PARAMETERS	
Parameter	Value
Bacterial luciferase	3.8×10^{-7} M
BSA	3.7×10^{-5} M
Decanal	2.6×10^{-4} M
FMN	2.1×10^{-6} M
Temperature	24°C
ANALYTICAL FIGURES OF MERIT	
Slope of calibration graph	0.9
Limit of detection	1.6×10^{-9} M
Linear dynamic range	3.8 orders of magnitude

The final aspect of the optimization study was the addition of hydrogen peroxide in order to increase the bioluminescence signal (18). No significant increase in signal was observed in our study, and hence this reagent was not employed in our optimized assay. However, after this work was completed, Watanabe et al. (19) reported that maximum light output was obtained at 25-50 mM hydrogen peroxide.

The results of our optimization and analytical figures of merit for the optimized assay are shown in Table 1. A slope of 0.9 was observed in our system, instead of the predicted value of 1. The degradation in the slope is probably due to the reduced efficiency of the fiber optic detection system, as previously reported by Gautier et al. (4). A limit of detection of 1.6×10^{-9} M was obtained with a linear dynamic range of 3.8 orders of magnitude. These figures of merit are comparable with previously reported assays using the bacterial luciferase bioluminescence system, demonstrating the potential of instrumentation for high sensitivity analysis for NAD(P)H.

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