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On-chip enzymatic assay for chloramphenicol acetyltransferase using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

Herein, we report a chloramphenicol (CAP) acetyltransferase (CAT) activity assay based on self-assembled monolayers on gold as an alternative to conventional CAT reporter gene assay systems, which sometimes require toxic materials and complicated steps that limit their use. A CAP derivative presented on a monolayer was converted to the acetylated CAP by CAT in the presence of acetyl-CoA. The conversion was directly monitored by observing the molecular weight changes in CAP using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. CAT activity was determined under various reaction conditions by changing reaction times, CAT and acetyl-CoA concentrations. As a practical application, we identified gene expression in bacteria that were transformed with pCAT plasmid DNA. Our strategy can provide a simple and rapid assay that eliminates some commonly used but potentially detrimental steps in enzymatic assays, such as radioactive labeling and complicated separation and purification of analytes prior to detection.

1. Introduction

Genetic engineering techniques have played significant roles in the development of molecular biology and biotechnology. Particularly, the introduction of exogenous recombinant DNA into organisms has made a key contribution. As such, identifying gene expression via a reporter gene assay is an indispensable tool for monitoring the genetic regulation: for example, measuring the level of gene expression, measuring the activity of a particular promoter, and verifying transfection/transformation [1,2]. Several reporter genes have been developed [3–6] including a chloramphenicol (CAP) acetyltransferase (CAT) [7,8], β-galactosidase [9,10], green fluorescent protein [11,12], and alkaline phosphatase [13]. Among them, CAT is the first reporter gene used for the measurement of transcriptional regulation in mammalian cells [8,14]. CAP is a broad-spectrum antibiotic that is obtained naturally or by chemical synthesis [15]. However, some microorganisms show resistance to CAP through CAT-catalyzed *O*-acetylation of the substrate [16]; therefore, CAT has been effectively used for reporter gene assays. Radiolabeling [17] and fluorescent [18,19] labeling have been commonly used in the CAT reporter gene assay system. In the radiolabeling system, a radiolabeled CAP or radiolabeled acetyl-CoA is incubated with CAT enzyme-containing samples, and CAP and acetylated CAP are subsequently separated by thin-layer chromatography, followed by scintillation or autoradiography counting analysis [17]. Fluorescently labeled CAP has also been used to detect CAT activity, which simplifies chromatographic separation [18,19]. Although these methods are well established, toxic materials used and the complexity of the assay sometimes hamper their common use.

Here, we report on a simple and rapid CAT activity assay strategy that avoids the use of toxic materials and complicated steps and is based on self-assembled monolayers (SAMs) on gold using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). SAMs of alkanethiolates on gold have been widely used in various biological studies [20–25], and Mrksich's and co-workers found that they are well-suited for analysis in conjunction with MALDI-TOF MS and named this technique SAMDI MS [26–35]. SAMDI analysis yields information on molecular weights of constituents of monolayers, and thus provides not only structural information but also quantitative information with regard to the composition of the monolayers. As such, SAMDI analysis has been utilized for a broad range of enzyme activity assays [30–34]. As a typical recent example, Kornacki et al. identified a mechanism of acetyltransferase-mediated crosstalk at the biochemical level based on a peptide array and the SAMDI method [35].

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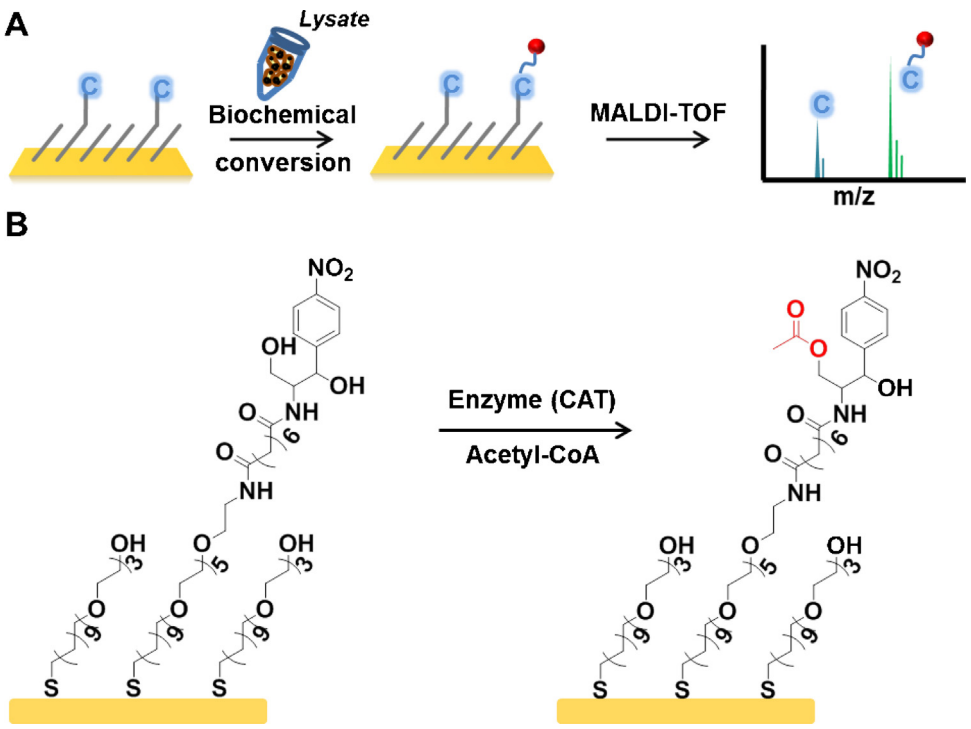

Fig. 1. (A) Schematic representation of our strategy for monitoring CAT gene expression on monolayers using MALDI-TOF MS. The monolayer presenting CAP substrate is prepared, and lysates of CAT gene-transformed bacteria are then directly treated on the monolayers with acetyl-CoA. The conversion of CAP to acetylated CAP by CAT enzyme is monitored by observing molecular weight changes using MALDI-TOF MS. (B) Structure of the CAP-presenting monolayer used in this study and its CAT-mediated conversion.

We harnessed SAMDI analysis to investigate biochemical conversions by the CAT enzyme on monolayers, as depicted in Fig. 1A. The monolayer presenting the CAP substrate is prepared on gold, and lysates of CAT gene-transformed bacteria are then directly treated on the monolayers with acetyl-CoA. The conversion of CAP to acetylated CAP is monitored by observing molecular weight changes using MS analysis, indicating CAT gene expression.

2. Materials and methods

2.1. Materials

Gold-coated slides were prepared by vacuum deposition of titanium (10 nm) followed by gold (50 nm) onto #2 glass coverslips. (1*R*,2*R*)-(−)-2-Amino-1-(4-nitrophenyl)-1,3-propanediol, 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP), and chloramphenicol acetyltransferase (CAT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The pCAT-3 reporter vector that encodes the CAT enzyme was purchased from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma–Aldrich and used without further purification. Competent *Escherichia coli* BL21 (DE3) was purchased from RBC Bioscience Corp. (Taipei, Taiwan). Luria broth (LB) was purchased from BD Biosciences (Palo Alto, CA, USA). Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Bio Basic Inc. (Markham, ON, Canada). Protease inhibitor cocktail was purchased from Roche (Mannheim, Germany).

2.2. Preparation of CAP-presenting monolayers

Gold chips were cleaned in piranha solution (sulfuric acid: hydrogen peroxide (30%) = 7:3. Caution! Extremely hot and corrosive.) before use. A gold chip was immersed in a mixed solution of CAP-terminated disulfide (0.5 mM in ethanol) and tri(ethylene glycol)-terminated disulfide (0.5 mM in ethanol) at a ratio of 50:50 for 12 h. The resulting CAP-presenting monolayer was rinsed with ethanol and dried under a stream of nitrogen.

2.3. CAT reaction on the CAP-presenting monolayer under various conditions

In general, CAT in reaction buffer (10 mM Tris–HCl with 1 mM $MgCl_2$) was mixed with acetyl-CoA, and then 5 μL of this solution was applied to the CAP-presenting monolayer. After incubation at 37 °C for various time periods from 0 to 4 h, the enzyme reaction was quenched by ethyl acetate. The monolayer was washed with water and ethanol, and analyzed by MALDI-TOF MS. For investigating the enzyme-concentration effect, CAT was used at various concentrations ranging from 0.25 to 2 U/chip with 2 mM acetyl-CoA at pH 6.0. For investigating the effect of acetyl-CoA concentration, acetyl-CoA was used at concentrations ranging from 0.25 mM to 2 mM with 2 U/chip CAT at pH 6.0.

2.4. Expression of CAT enzyme

The pCAT plasmid DNA was transformed into *E. coli* BL21 (DE3) using heat shock, and the cells were incubated at 37 °C in LB media containing 100 μg/mL ampicillin until the OD_{600} reached 0.8. Subsequently, IPTG was added a final concentration of 0.5 mM to induce protein expression. The cells were cultivated for an additional 4 h and harvested by centrifugation at 3000 × *g* for 20 min. The collected cells were resuspended in 50 mL of cold Tris–HCl buffer (10 mM, pH 6.0) supplemented with protease inhibitor cocktail. Cells were disrupted with eight sonication cycles of 3 min each, and insoluble cell debris was removed by centrifugation at 3000 × *g* for 15 min at 4 °C. Supernatant containing the CAT protein was directly used for the enzymatic reactions on the chip surface without further purification steps.