DETERMINATION OF SARCOSINE IN URINE SAMPLES BY DIHYDROFLUORESCEIN AS A HYDROGEN PEROXIDE PROBE

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Project Topic

Metabolites impart a significant importance to the understanding of biological reactions, and consequently, to the development of diagnostic and therapeutic techniques for specific diseases. Furthermore, there has been recent interest in metabolic levels present in urine for potential noninvasive disease diagnosis. The detection of specific metabolites, however, presents certain analytical difficulties such as low or ambiguous specificity of the probe. This study investigated a new technique, utilizing oxidative, enzymatic production of hydrogen peroxide from the metabolite accompanied by dihydrofluorescein (DHF). This probe displays high selectivity towards hydrogen peroxide, and coupled with high enzymatic specificity, forms an accurate method to measure metabolite levels. Sarcosine was used as a paradigm, by treatment of sarcosine oxidase to generate hydrogen peroxide, which was exposed to DHF. This method was applied to urine samples and plotted against a calibration curve from known sarcosine solutions. The linear proportionality that resulted suggests that this is a viable technique for determination of sarcosine. Based on our best knowledge, this probing system has not been reported.

Project Details

Metabolites, as the intermediates and products of metabolic exchanges, have an intimate relationship with systemic changes in an organism. An important implication of this is that of carcinogenesis; while cancer has been traditionally seen in terms of gene regulation, affecting control and signal processes, disjoint from metabolites, it is becoming increasingly apparent that

metabolites operate as key functions in the development of several types of cancer through numerous mechanisms, such as modulating various signal transduction systems.

Effective analysis and quantification of such metabolites in applicable media may then characterize the progression of certain cancers where practical difficulties would otherwise exist. This concept was utilized in an attempted metabolomic profiling of biopsy-positive prostate cancer patients and biopsy-negative prostate control individuals that suggested a correlation between sarcosine levels and progression of the disease. Although this was later refuted, it adds stress to the importance of metabolites as novel markers for cancer diagnostics. This also illuminates the possible discriminatory nature of metabolites: prostate-specific antigen (PSA), a common marker for prostate cancer diagnostics, cannot differentiate between benign prostate conditions and prostate cancer.

Analytical methods often include combination of liquid chromatography and gas chromatography with mass spectroscopy for direct determination of metabolites from cells, plasma, or urine. However, the major disadvantages of these techniques are high instrumentation costs and the interferences from other chemicals, such as alanine, having the exact same mass as sarcosine and often co-elutes with sarcosine. Substrate-specific enzymes, however, have shown to be useful analytical reagents. One class in particular is the oxidoreductases, which often produce reactive oxygen species (ROS), such as peroxidase and hydrogen peroxide, respectively, being first reported in 1845.

One such example is the sarcosine-sarcosine oxidase enzymatic reaction. Sarcosine oxidase serves as a catalyst in the oxidative demethylation of sarcosine, an intermediate metabolite, to glycine, formaldehyde, and hydrogen peroxide. Direct determination of sarcosine

can be difficult, but indirect quantification can be achieved by this reaction. Indeed, a common analytical assay for sarcosine includes the sarcosine oxidase-mediated demethylation of sarcosine to hydrogen peroxide, which reacts with 4-aminoantipyrine and phenol to yield quinoneimine dye (Scheme 1). Colorimetric analyses can be skewed in complex matrices, such as urine and blood, however.

Scheme 1. Colorimetric assay of sarcosine via production of quinoneimine dye.

$$Sarcosine + O_2 + H_2O + Sarcosine\ Oxidase \rightarrow Glycine + Formaldehyde + H_2O_2$$

$$2H_2O_2 + Phenol + 4\text{-}aminoantipyrine + Peroxidase \rightarrow 4H_2O + Quinoneimine\ Dye$$

One solution to this is the fluorimetric determination of sarcosine. One particular subset of fluorescein derivatives is currently being recognized for its high specificity to reactive oxygen species. Many of these compounds have reported detection limits in the nanomolar concentration range, providing useful probes for oxidative stress in cellular systems. Their premise is based upon the theory that non-fluorescent fluorescein derivatives will fluoresce upon oxidation by hydrogen peroxide and other ROS, and that this fluorescence is directly proportional to the concentration of ROS present.

One of the most widely used of these fluorescein derivatives is 2',7'-dichlorofluorescein diacetate (DCFH-DA). The basis of its usage is that the nonpolar, nonionic DCFH-DA is able to cross the cell membrane, upon which it is enzymatically de-esterified by intracellular esterases to

non-fluorescent dichlorodihydrofluorescein (DCFH). In the presence of reactive oxygen species, DCFH is rapidly oxidized to highly fluorescent analog, 2',7'-dichlorofluorescein (DCF) (Figure 1).

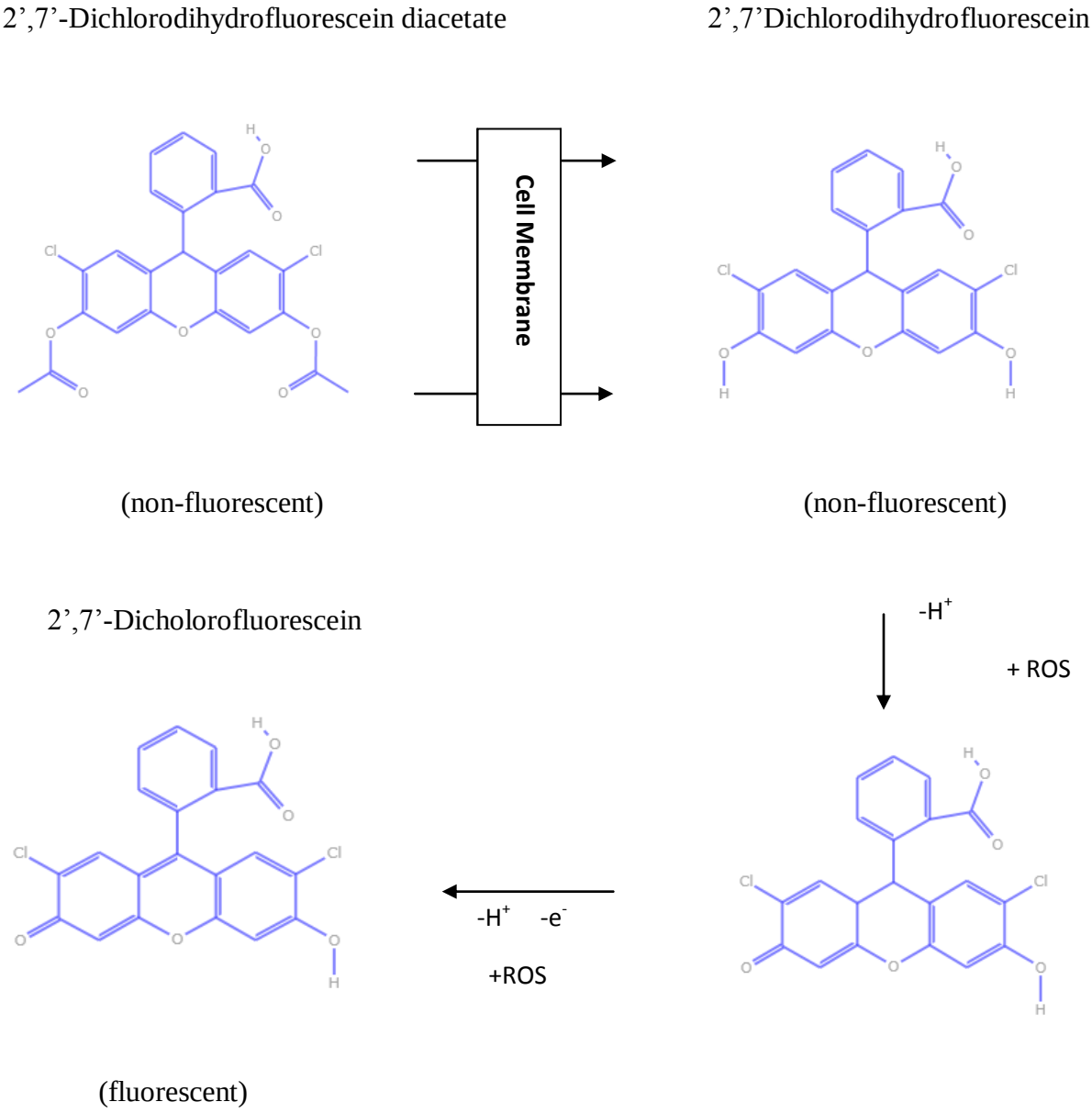

Figure 1. Proposed mechanism of DCFH-DA. DCFH-DA is de-esterified to DCFH after crossing of the cell membrane, whereby oxidation yields the fluorescent DFC.

This technique, too, has its drawbacks. Two things must occur for the above to work: (1) DCFH-DA must be deacylated to yield DCFH, (2) DCFH must be oxidized to DCF. For analytical work in media such as urine, this means that a deacylation reagent/s must be added to achieve the non-fluorescent analog. Furthermore, synthetic preparation of DCFH-DA is often complex, and direct purchase expensive.

The proposed technique directly reduces the fluorescent fluorescein to its non-fluorescent analog, DHF, bypassing the need for DCFH-DA altogether. Regeneration of fluorescein by ROS oxidation will produce a fluorescence change proportional to the amount of ROS present (Figure 2). This forms the basis of a quantifiable determination of hydrogen peroxide present. Furthermore, this approach provides a much cheaper alternative to current methods such as DCFH-DA.

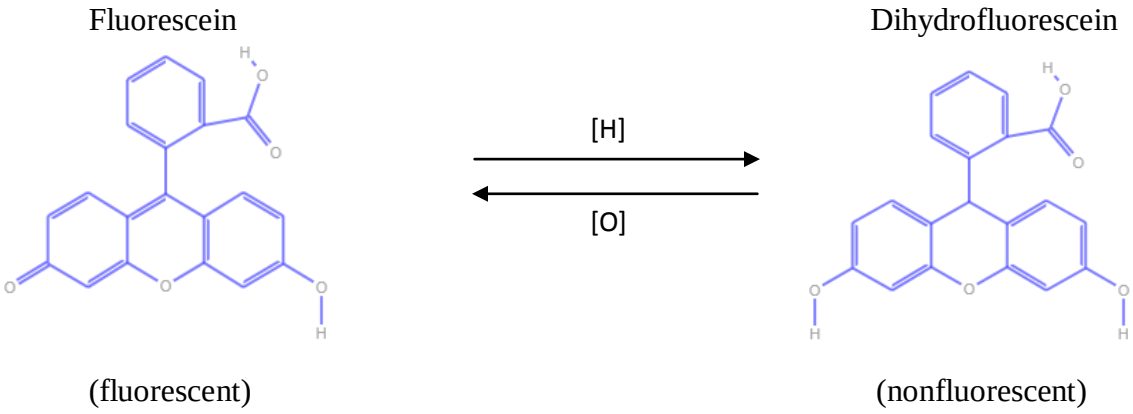

Figure 2. Reduction of fluorescein to dihydrofluorescein and subsequent regeneration of fluorescein to afford a change in fluorescence.

To generate the most cost effective probe, dihydrofluorescein (DHF), as well as several similar species, including DCFH-DA, were coupled with sarcosine oxidase and compared (Table 1). The high specificity provided by sarcosine oxidase greatly reduces the amount of probe needed, drastically cutting the cost of the technique. It was shown that DHF was, indeed, the most cost effective probe. This is due to the synthetic ease to produce it: fluorescein, a common, cheap compound, is taken into ethanol and simply reduced with zinc powder.

Table 1. Cost effectiveness of proposed technique.

Technique	Cost per sample	Relative Specificity
PSA (for prostate cancer diagnostics)	$30+	Moderate
FS-1 with sarcosine oxidase (Fluorescein disulfonate)	$0.06	High
DCFH with sarcosine oxidase (2,'7'-Dichlorodihydrofluorescein)	$0.04	High
DCFH-DA with sarcosine oxidase (2',7'-Dichlorodihydrofluroescein diacetate)	$0.05	High
Abcam Sarcosine Assay Kit	$4.55	Moderate
Antibodies-Online Sarcosine Assay Kit	$2.95	Moderate
DHF with sarcosine oxidase *(Dihydrofluorescein)*	*$0.02*	*High*

These two methods, substrate-specific enzymatic production of hydrogen peroxide coupled with subsequent fluorimetric analysis by fluorescein derivatives, supply an effective, indirect, analytical technique for metabolite-specific determinations. This concept was demonstrated by the determination of sarcosine in urine samples by determination of hydrogen peroxide formed by the enzyme sarcosine oxidase. It was shown that there does, indeed, exist a linear proportionality between amount of sarcosine and subsequent fluorescence. Thus, a cheap, simple technique is presented here for the determination of sarcosine in urine. This technique can further be easily applied to other analytes utilizing their respective oxidases.