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Abstract

Numerous enzymes have been demonstrated to be active in non-aqueous solutions, yet the utility of phosphatases under such conditions has been difficult to determine. Here, we demonstrate the ability to fluorescently detect naphthol AS-MX in high percentages 1,4-dioxane with a fluorescence differential compared with naphthol AS-MX phosphate. While intensities and maximum fluorescence wavelengths changed depending on solvent conditions, these results demonstrate this system's potential for testing phosphatase activity in high amounts of dioxane.

Introduction

Phosphoric-monoester hydrolases (also known as phosphatases, EC 3.1.3) are proteinaceous enzymes that catalyze the hydrolysis of orthophosphoric acid-based substrates leading to a dephosphorylated alcohol product. Phosphatases operate in numerous biological contexts, most notably in signaling and regulatory roles, and thus are typically found in aqueous solutions. Certain enzymes, however, have been previously shown to catalyze reactions in non-aqueous media, as reviewed elsewhere [1] [2] [3] [4] [5] [6] [7] [8]. In addition to providing detailed insights into the fundamental role of water in enzyme-mediated reactions, the practical utility of non-aqueous enzymology has been demonstrated in applications such as enhancing substrate specificity [3], reversing common reactions [8], and catalysis on water-insoluble substrates [8]. The use of specific enzymes, such as phosphatases, in non-aqueous (including aprotic) environments can lead to beneficial gains for specific industries, such as in the petroleum industry where the controlled removal of phosphates can save time and money [9]. While phosphatases have shown promise in environments containing a small amount of non-aqueous solvents [10] [11] [12] [13] [14], without a feasible activity detection system, they have been historically intractable to study using low percentages of water.

Aqueous *in vitro* detection of phosphatase activity is typically conducted using polar, colorimetric indicators that do one of the following: become colored upon dephosphorylation, lose color upon dephosphorylation, or change color upon binding a newly liberated inorganic phosphate. These colored compounds, however, are not soluble in aprotic solutions and thus are not suitable for work in low water environments. While not the most common phosphatase substrate, in combination with azo dyes, phosphorylated naphthalen-2-ol derivatives have been used for decades for histologically detecting phosphatase activity *in vivo* [15] [16] [17] [18]. These phosphorylated compounds have subsequently been used to also work without coupling dyes in blood serum [19] and other aqueous environments [20], relying on the fluorescence behavior of their dephosphorylated products. Indeed, Albert Weller studied the fluorescence of naphthalen-2-ol in detail in the 1950's [21]. In the present study, a method for fluorescence detection of one naphthalen-2-ol derivative will be extended to binary solvent mixtures containing 1,4-dioxane and aqueous tris(hydroxymethyl)aminomethane (Tris) buffers. This derivative, 3-hydroxy-2',4'-dimethyl-2-naphthanilide, is often referred to as naphthol AS-MX and is soluble in both aqueous and organic solutions, allowing it to be used in mixtures containing high amounts of 1,4-dioxane. Furthermore, its fluorescence differential when compared with a phosphorylated naphthol AS-MX, as described here, makes it a feasible substrate for phosphatase detection.

Objective

The objective is to demonstrate differential fluorescence between naphthol AS-MX and naphthol AS-MX phosphate in high percentages of dioxane, leading to a feasible phosphatase detection system in organic media.

a

Figure Legend

Figure 1. Emission spectra for naphthol AS-MX when excited at 388 nm.

Panels (A) and (B) depict fluorescence spectra of 2.5 mM naphthol AS-MX in 70:30 (red), 75:25 (orange), 80:20 (green), 85:15 (blue), and 90:10 (purple) 1,4-dioxane:Tris buffer (v:v) ratios. Solutions were made using pH 7.0 (panel A) or pH 8.0 (panel B) Tris buffer.

Panel (C) depicts the enzymatic conversion of 0.23 mM naphthol AS-MX phosphate to naphthol AS-MX after a 3 h incubation with (red) or without (blue) without 0.91 mg/mL (of >0.4 units/mg) wheat germ acid phosphatase (WGAP) in 64:36 1,4-dioxane:Tris (pH 7.0) mixtures as tracked by increased fluorescence.

In all panels, vertical error bars represent standard deviation (n=6) and fluorescence instrumentation parameters were held constant (see Methods Section).

Results & Discussion

Fluorescence intensities of naphthol AS-MX were measured under various conditions over an emission range of 400–600 nm in order to show the feasibility of using naphthol AS-MX phosphate for detecting phosphatase activity in non-aqueous environments. Specifically, 5 aprotic: protic ratios (70:30, 75:25, 80:20, 85:15, and 90:10 of 1,4-dioxane: Tris buffer) were tested, with the protic component prepared as 0.1 M Tris at one of two different pHs (7.0 or 8.0).

It is known that the maximum fluorescence intensity (λ_{max}) of naphthol AS-MX in largely aqueous solutions is 512 nm when excited at 388 nm [20]. As illustrated in figure 1, the λ_{max} is indeed within the range of 500 and 550 nm for all conditions with well-defined maxima. For each set of data that resulted from using the same buffer, the λ_{max}

value changed as a function of aprotic: protic solvent ratio, with a redshift occurring at higher amounts of aprotic solvent (1,4-dioxane). Interestingly, it has been previously identified that in aqueous solutions, naphthalen-2-ols have a fluorescence that is redshifted upon deprotonation [22], yet here λ_{max} values are near identical across the pH range tested with the largest changes occurring in higher dioxane percentages. pK_a 's of compounds, however, have been known to increase with the addition of non-aqueous solvents [23], and thus this effect may be playing a role in fluorescence intensity.

Naphthol AS-MX has the highest fluorescence intensity in 70% dioxane with the 30% Tris buffer protic component composed using Tris buffer at pH 8.0 (with a λ_{max} value at approximately 515 nm). Another noteworthy finding revealed that as the percentage of dioxane increased, the fluorescence decreased in intensity (with a less defined blueshifted λ_{max}). Specifically, for both Tris buffer pHs used, naphthol AS-MX had the lowest intensity in mixtures corresponding to samples containing 90% dioxane. This decrease in intensity, however, did not appear to be linearly dependent on the aprotic: protic (dioxane: Tris buffer) ratio and larger differences can be at lower dioxane amounts.

As would be expected different buffers used as the protic component of the solvent mix affects results substantially, thus the aqueous buffers used in the present study contained the same amount of the same buffer system (0.10 M Tris(hydroxymethyl)aminomethane). When comparing all of the results, the highest intensity values (emission at 512 nm) corresponded to the more alkaline protic component (i.e., pH 8.0) across each individual solvent ratio. Fluorescence changes from pH differences in the protic component of the solvent may be directly due to protonation state of the compound itself (naphthol AS-MX's hydroxyl pK_a is 7.021) or to changing interactions with the solvent. Thus, additional studies are necessary to further delineate the exact contributions to the observed changes.

Fluorescence of phosphorylated naphthol AS-MX was also investigated under each condition. The results from these experiments are tabulated in supplementary table 1. Importantly, two-tailed Student's *t*-tests between naphthol AS-MX and naphthol AS-MX phosphate indicate clear fluorescence differential for all conditions at emission wavelengths between 500 and 600 nm (*p*-values below 0.05). Also, one-sample, two-tailed Student's *t*-tests indicate that naphthol AS-MX phosphate did not fluoresce at all when excited at 388 nm (*p*-values were above 0.6 for all conditions). Therefore, any detectable fluorescence emission in a reaction starting with naphthol AS-MX phosphate will indicate dephosphorylation to naphthol AS-MX.

Previously, soluble phosphatases have never been shown to have activity in solutions made up of more than 25% organic solvent [10] [11] [12] [13] [14]. Figure 1C, however, shows that in this study wheat germ acid phosphatase (WGAP) was able to convert naphthol AS-MX phosphate into naphthol AS-MX after 3 h in a 64:36 dioxane: aqueous Tris (pH 7.0) mixture, as indicated by fluorescence emission with a maximum around 515 nm (when excited at 388 nm) only when WGAP was added to the naphthol AS-MX. This emission intensity is much lower here than is seen in (Fig. 1) panels A-B due to the use of a lower concentration of naphthol AS-MX and a different fluorimeter. Despite this, *p*-values of less than 0.05 when using a two-samples of unequal variance, two-tailed Student's *t*-test to compare the samples with or without wheat germ acid phosphatase clearly indicate activity. These data, therefore, display proof of principle that the methodology described in this paper can be used for detection of phosphatase activity in high percentages of organic media.

Conclusions

Presented in this paper is a method for detecting the compound naphthol AS-MX compared with its phosphorylated precursor in dioxane: aqueous buffer mixtures using fluorescence spectroscopy in a 96-well format. The methods include a description of the types of plastic/glassware used, the chemicals and concentrations that worked and suggested starting spectrometer parameters. Additionally, it was found that increasing the

pH of the protic component of the mixtures or the v:v percentage of the protic component increases the fluorescence of naphthol AS-MX at 512 nm when excited at 388 nm. For any given pH used for the protic component of the solvent mixture, increasing the amount of dioxane (the aprotic component) redshifted the λ_{max} , with a more pronounced shift when using a lower pH protic component. Results from the present study demonstrate the feasibility of using naphthol AS-MX phosphate as a substrate for phosphatase activity testing in high percentages of dioxane. Furthermore, the plate/film combination described has a more general application, allowing for additional fluorescence studies in other organic solvents. Finally, dephosphorylation activity of wheat germ acid phosphatase was detected using a 64:36 1,4-dioxane: aqueous Tris (pH 7.0) mixture, opening the door for additional studies of phosphatases in high amounts of aprotic solvents.

Limitations

Dioxane evaporated from the wells of 96-well plates when left uncovered, yet clouds up the films after about an hour when covered, which is not enough time to show a distinct enzymatic time course plot. Therefore, the enzymatic reaction was shown in this study using the spectra at a single time point after enough time had lapsed to see a distinct increase in fluorescence. Obtaining time course enzymatic data may be possible in the future using cuvettes where both evaporation and film clouding will be less of an issue.

Alternative Explanations

Conjectures

This work shows the feasibility of fluorescently detecting naphthol AS-MX compared with naphthol AS-MX phosphate in high amounts of 1,4-dioxane, and as such will allow us to test different phosphatases in dioxane to better understand the water requirements for activity.

Additional Information

Methods

For these studies, homogenous aprotic: protic binary mixtures (70:30 or 90:10 [v:v] dioxane: Tris buffer) were made using 0.10 M Tris Base (Dot Scientific) in water titrated with hydrochloric acid (Fisher) until pH 7.0 or 8.0 was achieved and spectroscopic grade 1,4-dioxane (Acros Organics). Naphthol AS-MX phosphate (Sigma) or naphthol AS-MX (Tokyo Chemical Industry) was subsequently added to make concentrations of 2.5 mM and left to dissolve overnight at temperatures between 20°C and 30°C. Subsequently, 75:25, 80:20, and 85:15 (v:v) mixtures were prepared by mixing appropriate ratios of the original 70:30 and 90:10 mixtures just described. To obtain accurate volumes and eliminate evaporation issues, all dioxane and dioxane mixtures were mixed using a repeater pipette (Eppendorf), all samples were made and stored in amber-tinted glass vials with F217/polytetrafluoroethylene-lined lids (Qorpak), and the lid-bottle junction of stored samples was also wrapped in Parafilm M (Bemis NA). Aqueous pHs were determined using an Accumet pH probe (Fisher Scientific).

Exactly 300 μL of each sample, including mixture blanks, were plated in sextuplicate into wells of black polypropylene 96-well plates (Greiner Bio-One) using positive displacement pipettes (Gilson). Upon plating, polypropylene sealing film (Dot Scientific) was used to seal off the wells and prevent evaporation. Sealed plates were immediately read spectroscopically at room temperature using a Varian Cary Eclipse Fluorescence Spectrophotometer fitted with a microplate adaptor attachment (Agilent) at a photomultiplier tube voltage of 600 V and an averaging time of 0.1 seconds. The data described in

this paper were collected using an excitation wavelength of 388 nm with an excitation slit width of 10 nm and a 250–396 nm excitation filter. Emission spectra (400–600 nm) were collected, using a slit width of 10 nm and no emission filter.

For the enzymatic spectra, mixtures of 64:36 dioxane: 0.1M Tris buffer (pH 7.0) with 0.23 mM (final concentration) naphthol AS-MX phosphate were made both with and without 0.91 mg/mL of >0.4 units/mg wheat germ acid phosphatase (Sigma) and in amber vials. They were subsequently incubated without shaking at room temperature for 3 h. These solutions were then pipetted (330 μ L) into each of 4 wells of a black 96-well plate and sealed, as described above. Fluorescence emission spectra were determined using an excitation wavelength of 388 nm on a SpectraMax M2 instrument (Molecular Devices). Samples were blanked to identical solvent mixtures without wheat germ acid phosphatase or naphthol AS-MX phosphate present.

Arithmetic means and standard deviations were calculated for each set of 6 replicate wells. The means of the corresponding dioxane: buffer mixture blanks were subtracted from the naphthol AS-MX or naphthol AS-MX phosphate means to yield the reported fluorescence intensities. Standard deviations were propagated using standard error propagation methodology. Significances of pairwise differences were determined using unpaired, two-tailed Student's *t*-tests and determined significant at *p*-values of less than or equal to 0.05.

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Ethics Statement

Not Applicable.

Citations

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