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A Simultaneous Coupling Azo-dye Method for the Quantitative Assay of Esterases: Biochemical Characterization

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Abstract

Background Enzyme activity is a subject of continuous research. Comparison of data obtained from various quantitative methods needs standardization of techniques in order to verify the results of histochemical and biochemical assays utilized in the study of tissue enzyme activity.

Objective Establishment of a biochemical method for the quantification of enzyme activity in α-naphthyl acetate esterases (ANAE) containing solution using hexazotized pararoseaniline (HP) as a coupling agent.

Methods Wavelength of maximum absorbance (λ_{max}) of coupled HP in solution was analyzed spectrophotometrically based on the simultaneous-coupling method of ANAE demonstration.

Results λ_{max} of the coupled HP was found to be at 425 nm. The relationship between the optical density of the final reaction product (FRP) and the enzyme concentration was linear with the use of azo dye in solution.

Conclusion Data obtained from the biochemical assay of ANAE activity was in agreement with those documented by the histochemical methods in use. Thus, characterization of enzyme activity may be standardized when studying tissue sections and tissue homogenates.

Keywords Esterases, Biochemical assay, Histochemistry, Spectrophotometry

Introduction

The activity of enzymes is regulated at different cellular levels. Many variables determine the activity of an enzyme, which may be localized at one region of the cell but not the other. This concept of integrating the location and function of enzymes has revitalized their study during the last decade, although such functions of enzymes do not necessarily correlate with their location [(1)].

In analysis of enzyme activity, it is often important to compare the results obtained with different quantitative methods. Work with esterases (EC 3.1.1.x), a diverse group of hydrolases catalyzing the reaction on ester bonds in the metabolic pathways [(2)], has detailed various histochemical and biochemical techniques employing the substrate α-naphthyl acetate with different types of diazonium salts suitable for one assay but not for the others [(3)].

Nachlas and Seligman were the first to describe a post-coupling azo dye method for the demonstration of nonspecific esterases using β-naphthyl acetate as a substrate [(4)]. Later works used α-naphthyl acetate as results were found to be more sensitive [(5)]. These researchers developed methods for biochemical measurement of esterases activity, using a simultaneous-coupling azo dye reaction with two different diazonium salts, *p*-Nitrobenzenediazonium tetrafluoroborate (*p*-NBDTFB) and Fast Violet B. Production of the azo

dye then could be measured with a spectrophotometer at the wavelength of maximum absorbance (λ_{max}) of the azo dye. This was found to be a sensitive assay allowing direct comparison between biochemical and histochemical data with one of the most widely used histochemical substrates for esterases, α-naphthyl acetate. Yet, the problem with the use of such diazonium salts was that they require optimum pH levels different from those present in vivo (pH 5.0 for *p*-NBDTFB, pH 8.0 for Fast Violet B) [5].

On the contrary, hexazotized pararoseaniline (HP), another diazonium salt widely used in histochemistry for staining of fresh frozen sections, was proved to couple effectively with α-naphthol, the product of α-naphthyl acetate hydrolysis, to produce the colored final reaction product (FRP) at physiological pH of 7.4 [3].

Generally, there are few quantitative methods utilizing substrates entirely satisfactory for histochemical work with α-naphthyl acetate. Conventional methods to determine esterase activity in tissue homogenate make the enzyme hydrolyzes α-naphthyl acetate and then a Fast Blue B dye complex is formed with α-naphthol [6]. Other spectrophotometric assays, which are limited to α-naphthyl ester substrates, continuously record esterase activity at 510 nm by monitoring absorbance changes due to the formation of an azo dye complex with Fast Blue RR salt [7]. Thus, most of the quantitative histochemical techniques used for the detection of α-naphthyl acetate esterases (ANAE) activity are either limited to the spectrophotometric assay performed on tissue sections [8], or they have used different types of chromogens other than the HP [9-11].

In addition, some trials for the use of ultraviolet (UV) absorbance in the determination of α-naphthol release in tissue homogenate have reported insensitivity because of the high background UV absorbance. These methods depend on the direct measurement of the product of enzymatic reaction rather than the quantification of the amount of the colored FRP. Thus, they are liable for the overlap in absorption spectra of α-naphthyl acetate and α-naphthol [5].

This work tries to establish a standard biochemical method for the detection and quantification of ANAE activity in solution, using HP as a coupling agent at physiological pH, so that enzyme activity can be accurately measured in tissue homogenates.

Methods

Reagents were obtained from ADWIC, basic fuchsin (C.I. 42510) from SD Fine-Chem, sodium nitrite from United Company for Chemicals, α-naphthol from Nice Chemicals, and hog liver esterase (EC 3.1.1.1.) from Sigma.

Buffers

Phosphate buffer 0.2 M (pH 7.4) was prepared by dissolving 11.4 g of Na_2HPO_4 and 2.7 g of KH_2PO_4 in distilled water. Volume was completed to 500 ml and the pH was adjusted with 1 N HCl [12].

Bovine serum albumin

Stock solution of bovine serum albumin (BSA) (10 mg/ml) was prepared and stored in refrigerator (+4 ^{o}C). A final concentration of 0.67 mg/ml of BSA in the assay medium was adequate for maintaining the azo dye in solution [5,13].

Diazonium salt

Preparation of the diazonium salt was based on the method of demonstration of α-naphthyl acetate esterases [4,12]. Pararoseaniline was made by dissolving 1 g of basic fuchsin in distilled water. Then, 5 ml of concentrated HCl was added and the solution was warmed slowly, cooled to room temperature, filtered and stored at 4 ^{o}C. Hexazotization of the uncoupled diazonium was done by adding 1 ml of pararoseaniline to 1 ml of freshly prepared 4% sodium nitrite. The mixture was stirred and put in ice-cooled bath.

The absorption spectra of the pararoseaniline and the uncoupled HP were measured as follows: a mixture of 4 ml phosphate buffer, 0.1

ml acetone, 0.32 ml pararoseaniline or HP, and 0.293 ml BSA was prepared. 3 ml of the mixture was pipetted in a quartz cuvette and the absorbance was recorded with Centra 5 UV-Vis Double-Beam Spectrometer at 350-650 nm wavelengths with 5 nm intervals. Control solution in which distilled water substituted the pararoseaniline or the HP was used. Measurements were done within 5 minutes of making the mixture.

Determination of λ_{max} of the azo dye

A standard solution of α-naphthol was made by dissolving 144 mg in 25 ml 99% acetone to give a final concentration of 40 mM. Then, 0.1 ml of this solution was added to a 3 ml quartz cuvette containing 2.2 ml buffer, 0.2 ml BSA and 0.5 ml HP. The wavelength absorbance was adjusted to zero immediately before addition of α-naphthol and the solution was then read at 350-650 nm. An absorption spectrum is thus obtained and the λ_{max} of the coupled HP is determined automatically.

Relationship between α-naphthol concentration and optical density of FRP

To make a standard curve, a series of various concentrations of α-naphthol were prepared (5-80 mM) and the absorbance was read immediately (that is, within 5 minutes of addition of the diazonium salt) as it is likely that if the azo dye is kept over extended periods of time the spectral characteristics would be altered [5]. Regression analysis is performed using Microsoft Excel 2010 tool in order to define a regression equation and the line of best-fit.

Effect of enzyme concentration on α-naphthol release

The detection of the enzymatically released α-naphthol was done by mixing 2 ml buffer, 0.2 ml BSA (10 mg/ml), 0.5 ml HP and 0.2 ml enzyme solution to fill in the 3 ml cuvette. Commercial hog liver esterase of original package at a concentration of 10 mg/ml was used after 1:1000 dilution in distilled water and kept on ice until use. Volumes between zero and 100 µl (0-1.0 µg/ml) were assayed with HP. The solution was incubated at 25°C and the reaction was initiated by the addition of 0.1 ml α-naphthyl acetate (8 mg/ml). The change in absorbance at λ_{max} was recorded continuously over a period of 1 minute [5] and the regression analysis was done accordingly.

Results

The measurements of the optical density of the pararoseaniline stock solution and the uncoupled HP are shown in figure1; the absorption spectra show higher optical density values of the non-hexazotized pararoseaniline in comparison to the uncoupled HP at wavelengths shorter than 425 nm, at which point both spectra intercept each other.

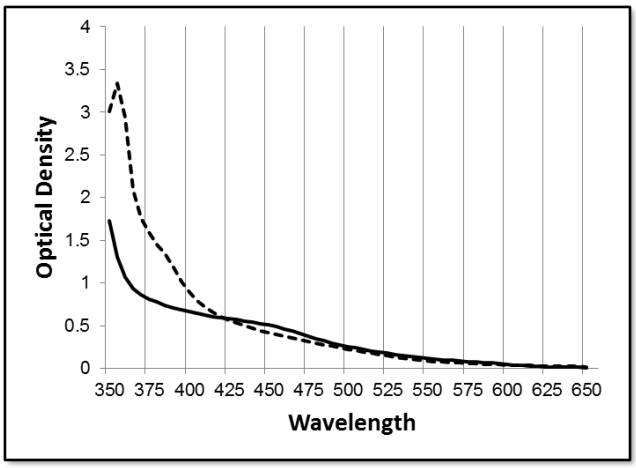


Figure 1. Absorption spectra of non-hexazotized pararoseaniline solution (_ _ _ _) and uncoupled HP (____) mixture. Both spectra slope down at higher wavelengths with an interception at 425 nm.

Coupling with 40 mM α-naphthol is illustrated in figure 2. The absorption spectrum of azo dye formed upon coupling of HP to α-naphthol revealed peak absorption (λ_{max}) at 425 nm.

A standard curve with best-fit correlation and regression equation of various concentrations (5-80 mM) of α-naphthol in relation to the optical density of the coupled azo dye at 425 nm is seen in figure 3. There was a linear correlation

between α-naphthol concentration and the optical density of the FRP.

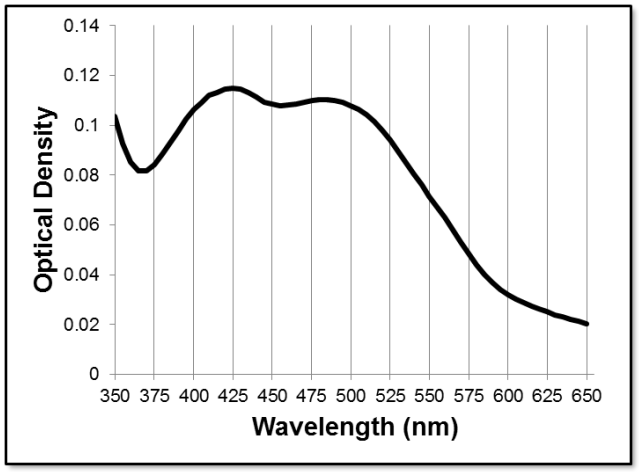


Figure 2. Absorption spectrum of azo dye formed upon coupling of HP to 40 mM α-naphthol, demonstrating peak absorption (λ_{max}) at 425 nm.

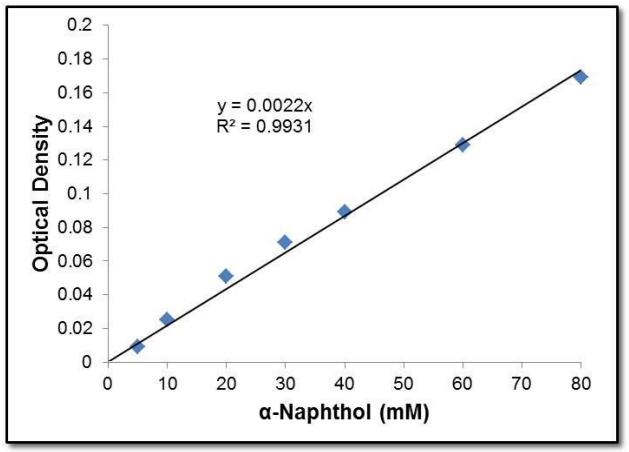


Figure 3. Standard curve and regression equation of various concentrations (5-80 mM) of α-naphthol in relation to the optical density of the coupled HP at 425 nm

The effect of enzyme concentration on the hydrolysis of α-naphthyl acetate to α-naphthol, measured by the optical density of the FRP produced upon coupling with the HP, is demonstrated in figure 4; the reaction showed linear relationship with enzyme concentration.

Discussion

The simultaneous coupling azo dye methods are well documented to be more sensitive especially at lower enzyme activity; the histochemical information obtained from esteratic staining of tissue sections depend mainly on the use of HP, because of the high efficiency of its coupling reaction with α-naphthol [3].

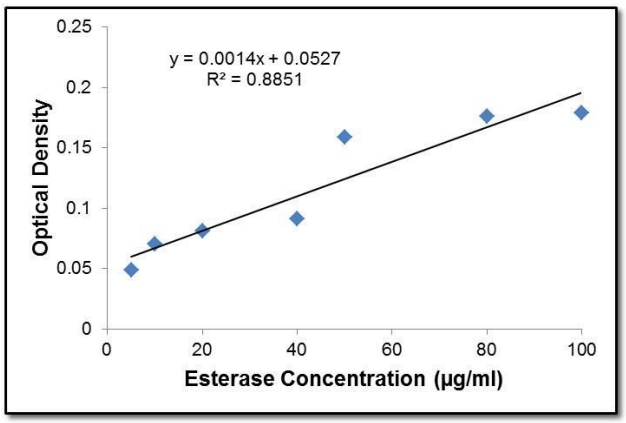
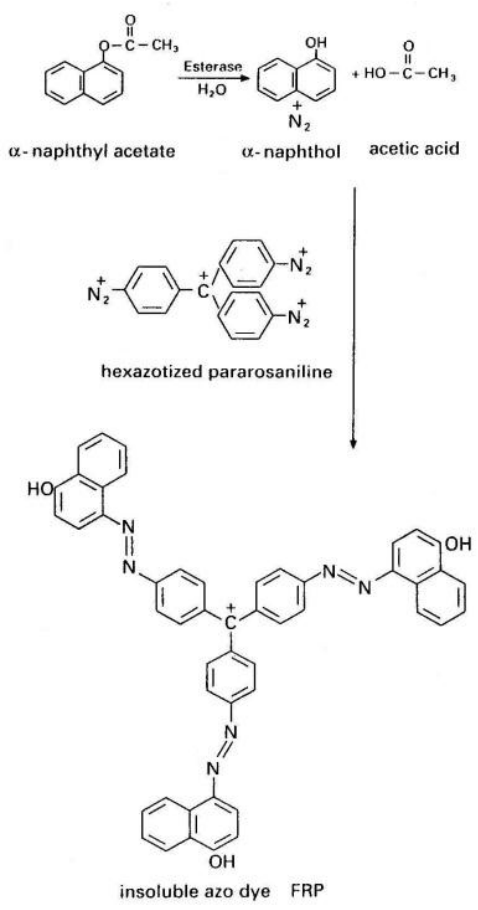


Figure 4. Optical density of the FRP as a function of esterase concentration. Measurements were done within 1 minute upon coupling with HP at its λ_{max} (425 nm). There is linear relationship within the range of enzyme concentration under study. The *y* intercept represents the amount of auto-hydrolysis

In figure 1, the absorption spectra show higher optical density of the non-hexazotized pararoseaniline in comparison to the uncoupled HP at wavelengths shorter than 425 nm, at which point both spectra intercept each other. The lower optical density readings of the uncoupled HP might be due to change in the molecular configuration after hexazotization of pararoseaniline according to the following chemical formula [12] showing below.

The absorption spectrum of azo dye formed upon coupling of HP to α-naphthol (figure 2) revealed peak absorption (λ_{max}) at 425 nm. This is in concordance with the documented λ_{max} for azo dye staining method in tissue sections [8]. In

other words, the use of BSA for maintaining the azo dye in solution did not alter the wavelength absorption characteristics of the incubation medium. This is also evident from the linearity of the plot of α-naphthol concentrations versus optical density measurements (figure 3), and from the linear relationship between the enzyme concentration and the optical density of the FRP (figure 4); both plots (figures 3 and 4) were measured at the assigned λ_{max} for azo dye (425 nm). Besides, this linearity reveals that the inhibitory effect of diazonium salts on enzymes did not present an overwhelming problem with the use of HP in biochemical assays, as it adheres to the Beer-Lambert Law at the concentrations measured, similar to what have been noticed in previous works with other azo dyes [14].



Upon comparing the benefit of the use of HP in the detection of ANAE activity, this azo dye works optimally at the physiological pH of 7.4, which is an important advantage in this study against other diazonium salts such as *p*-NBDTFB and Fast Violet B that work best at alkaline or acidic pH, respectively [5]. In other words, HP staining method in solution or tissue homogenate does not affect the physiological environment needed for the optimum activity of the enzyme.

In contrast to the UV assay methods, tissue homogenates with components that possess high UV absorbance can be assayed using HP without the complicating factor of a distorting background because wavelength absorption falls within the range of the visible spectrum (350-650 nm). In addition, the simultaneous coupling in tissue homogenate solutions is a continuous assay, enabling initial reaction velocities to be measured over very short periods of time. Thus, problems arising from instability of diazonium salts and azo dyes are minimized [5].

In this study, esterase-catalyzed α-naphthol hydrolysis demonstrated straight lines of the reaction progress, indicating that a steady-state was already reached at the beginning of the measurements. An intercept with the *y*-axis above zero may point to a relief from the steady-state attributed to auto-hydrolysis of the substrate (figure 4). The *y* intercept is, therefore, a measure of the amount of auto-hydrolysis.

This auto-hydrolysis may raise a question of non-enzymatic catalyzed staining of tissue sections, that is, there might be some degree of azo dye staining not due to the presence of the enzyme, rather, due to the auto-hydrolysis of the substrate α-naphthyl acetate. Therefore, the biochemical method used in this work for measuring ANAE activity in solution or tissue homogenate could eliminate an artifact inevitably present when determining enzyme activity in tissue sections.

Despite the finding that the λ_{max} of coupled HP in solution is similar to what has been recorded for tissue sections [8], some authors believe that living cell or tissue imaging, in which cells and tissues are kept intact during analysis, is the best approach to generating data on the activity of an enzyme [1]. Justification depends on the reflection of the in vivo situation, because activity measurements of a purified enzyme both in diluted solutions and in tissue or cell homogenates probably do not imitate

the activity of that enzyme in the crowded compartmentalized cell [15].

According to above mentioned perspective, it would be advisable to consider the segregation of cellular compartments by differential fragmentation and centrifugation in order to isolate the desired cellular fraction that contains the enzyme under study.

A debate concerning the work on biochemical assays may arise when considering enzyme histochemistry as the oldest histochemical modus operandi, with an approach that has minimized its impact in the '80s and '90s of the last century with the growing immunohistochemistry and in situ hybridization techniques. Recently, genes and gene expression have received almost all the attention. Studying enzyme activity was considered old-fashioned until scientists realized that gene expression tells us little about function; in the first decade of this century, enzyme activity localization and imaging had a strong revival because they focus on function. Imaging of living cells and tissues, in combination with localization of enzyme activity, became a strong match as metabolic mapping [1].

Yet, studying enzyme activity in solution or whole tissue homogenate is still a field of interest and the future of such work looks bright as continuous attention is paid to the innovation of novel methods for the detection of enzyme activity instead of the older procedures [16-18]. Therefore, it was reasonable to develop a simultaneous coupling method that utilizes HP for the detection of esterases activity in tissue homogenates solutions. This work adds to the methods used for such detection utilizing one of the widely used azo dyes, the HP, with technical parameters comparable to those applied in studying enzyme activity in tissue sections. Further studies comparing data obtained from tissue sections and tissue homogenates are recommended.

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