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Review Article

UV - Spectroscopy: as a tool to determine enzyme activity

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Abstract

Enzymes are Biological catalysts called enzymes that quicken metabolic reactions in living things. They play essential roles in metabolism, energy storage, and other biological processes. Enzymes can be isolated and used in commercial operations due to their catalytic properties. These enzymes act in low quantities without being consumed, making them highly efficient. UV-Vis spectroscopy is commonly employed to study enzyme kinetics and interactions, allowing real-time monitoring of enzymatic activity by measuring changes in absorbance at specific wavelengths. This technique is crucial for observing concentration changes of substrates and products during enzymatic reactions. The OPA spectrophotometric assay is a widely used method for measuring enzyme activity, particularly for proteinases. The assay involves reacting O-phthalaldehyde (OPA) with primary amines, forming a fluorescent adduct that can be measured at 340 nm to quantify proteolysis. The assay is efficient, rapid, and highly sensitive, making it suitable for various research and clinical applications. The NADH-linked enzyme assay utilizes the UV absorbance of NADH at 340 nm to track enzymatic reactions, especially those involving dehydrogenases like lactate or alcohol dehydrogenases. Changes in absorbance over time are used to determine reaction rates and enzyme activity. In addition, curve fitting is an essential technique in UV-Vis spectroscopy that helps interpret complex data. By fitting mathematical models to the experimental spectra, researchers can resolve overlapping peaks, perform quantitative analysis, and study reaction kinetics. Both the OPA and NADH-linked assays, alongside curve fitting, provide powerful tools for studying enzyme mechanisms and activities in diverse scientific fields.

Keywords: Enzymes, Enzyme kinetics, UV-Vis spectroscopy, OPA spectrophotometric assay, NADH-linked enzyme assay, Curve fitting.

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1. Introduction[1-6]

Enzymes are biological catalysts, sometimes referred to as biocatalysts that accelerate biochemical reactions in living things these processes serve several tasks in nature, such as metabolism and energy storage within cells. They can be isolated from cells and utilized as catalysts for a variety of significant commercial operations. Enzymes operate as catalysts, accelerating processes without being consumed themselves in the process and only being needed in extremely low quantities.1-6

Enzymatic activity: The study of how enzymes interact with substrates, or the molecules they work upon, and how rapidly these interactions happen is known as enzyme kinetics. Using UV-visible spectroscopy, one may effectively study the kinetics of enzymes. (**Figure 1**)

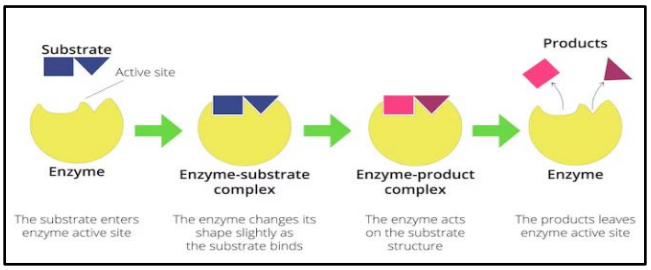

Figure 1: Mechanism of enzymatic activity

We can track concentration changes over time by measuring a sample's absorbance of UV or visible light.

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Since many enzymes and substrates have distinctive absorption spectra in this wavelength range, this is very helpful for researching enzymatic reactions.

Enzymes: Oxidoreductase, Transferase, Hydrolyses, Lyases, Isomerases, Ligases. UV-Visible spectroscopy is a strong method for studying enzyme kinetics and interactions. By measuring the absorbance of UV or visible light, changes in substrate and product concentrations, providing real-time insights into enzymatic activity. Many enzymes and substrates absorb light in unique patterns within this range, which is useful for studying their reactions. Using a spectrophotometer (a device that measures light absorption), we can track these changes by applying Beer's law. As the enzyme reacts with the substrate, the amount of product increases or the substrate decreases. This technique's sensitivity and versatility make it indispensable for monitoring enzyme function in diverse scientific fields, from biochemistry to industrial biotechnology.

2. Materials and Methods

2.1. Opa Spectrophotometric Assay[7-10]

Spectrophotometry assays are analytical techniques that utilize the amount of light absorbed by the sample at a particular wavelength to ascertain the drug concentration in the specimen. Both the OPA commonly used approaches, such as ninhydrin and TNBS (2,4,6-trinitrobenzene sulfonic acid) assays, are equally sensitive. On the other hand, it is quicker and more convenient, which makes it a better option in many situations. To identify and measure proteinases, which are enzymes that break down proteins, O-phthalaldehyde (OPA) is a reagent that is frequently employed in these tests.

In a standard OPA test for proteinases, the proteinase and OPA reagent mix to produce a luminous (colour) result. A spectrophotometer is then used to measure the fluorescence intensity in order to determine the proteinase content in the sample. This OPA is frequently used to measure the amount of proteins present in a sample, identify and measure free amino acids in biological materials, and support research in peptide bond and sequence analysis. It is frequently employed in clinical diagnostics, biochemistry, and molecular biology research to examine the connections and activities of proteins used in clinical labs to measure serum protein levels and do other diagnostic procedures

2.2. OPA Spectrophotometric assay

The OPA (o-Phthaldialdehyde) spectrophotometric assay is a sensitive method used to measure the concentration of amino groups released during protein hydrolysis. It involves reacting the OPA reagent with primary amines, forming a colour adduct that can be quantified by measuring absorbance at 340 nm. This assay is advantageous for its simplicity, rapid execution, and ability to accurately quantify proteolysis in various samples, making it valuable in protein chemistry and dairy science research.

2.3. Mechanism

When a thiol (2 -mercaptoethanol) is present, OPA reacts with the primary amines and lysine side chains of proteins in an basic environment. After being excited between 330 and 390 nm, the process generates a fluorescence signal between 436 and 475 nm.

2.4. How to perform the assay

A small aliquot of sample is added and OPA reagent (Take 2 mL methanol dissolve 80 mg of OPA, 0.2 mL of 2-mercaptoethanol or ethanethiol, 50 mL of 0.1 M sodium borate, and 2% (w/v) sodium dodecyl sulfate (NaDodSO4). Makeup up to 100 mL with water. Prepare fresh daily. In a quartz cuvette, mixed, and incubated. The absorbance is then measured at 340 nm. The increase in absorbance (ΔA340) indicates the release of amino groups, reflecting the extent of proteolysis.

Incubate the protein substrate with the proteinase enzyme in a suitable buffer solution. Add 20-50 µL of the reaction mixture to 1 mL of the OPA reagent. Mix properly and incubate for 120 second at RT. (25°C).

The suggested approach states that the study's foundation is the reaction between o-phthaldialdehyde (OPA) and β-mercaptoethanol and primary amines, which results in a product.

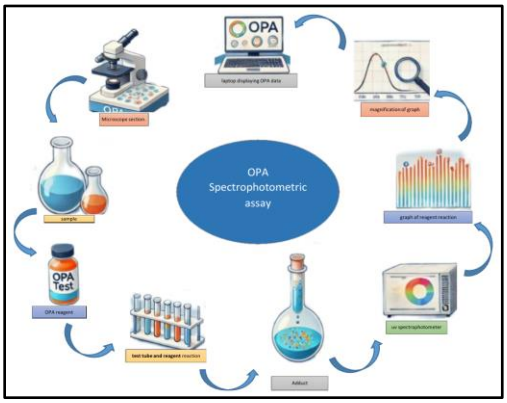

Figure 2: OPA based enzymatic activity

2.5. Investigations

The study investigates the molar absorptivity of o-phthaldialdehyde (OPA) adducts formed with various amino acids and peptides. The key findings indicate that the side-chain characteristics of amino acids do not significantly influence the molar absorptivity of the resulting OPA adducts.

1. **OPA Adduct Absorptivities:** The assay begins with the determination of average absorptivity and linearity of absorbance concerning sample concentration.
2. **Proteolytic Hydrolysis**: The study explored the hydrolysis of milk proteins using trypsin and chymotrypsin. Enzyme activity was measured by adding protein substrates to the enzymes in a buffered solution and tracking the release of amino groups over time.
3. **Kinetic Analysis**: The formation of the OPA-amine adduct is rapid, with half-times of about 10-15 seconds. Maximum absorbance is reached within 1-2 minutes and then decreases exponentially with half-times of 36-58 minutes**.** The results indicated that the rate of reaction followed a hyperbolic pattern, allowing for the determination of Michael's - Menten kinetics (K_M = 1.68 mg/ml).
4. **Proteolytic Activity in Milk:** The assay was further tested on the proteolytic activity of different strains of Streptococcus lactic in milk
5. **Molar Absorptivity**: The study tabulated the molar absorptivity's of various amino acids and peptides when reacted with OPA, finding that the side chains of amino acids did not significantly influence their absorptivity. The mean absorptivity across tested compounds was found to be approximately 6000 $M^{-1}cm^{-1}$. (Table 1)

Table 1: Molar absorptivities of OPA adduct of various amino acids at 340nm

Amino acid or peptide	Absorbance at 340nm
Gly	5880
Ala	6090
Ser	6000
Leu	6020
Phe	5830
Glu	6120
Gly-Leu	6050

3. Nadh Linked Enzyme Assay[11-12]

Changes in the UV-visible absorption spectra of nicotinamide adenine dinucleotide coenzyme (NADH/NAD1) and its derivatives (NADPH/NADP1) were used to examine their chemical stability. When the cofactor's (reduced form) spectra were observed at 340 nm in various buffers, the phosphate buffer demonstrated a quicker rate of breakdown. The adduct formation between phosphate and NADH (pyridine ring) was attributed. With no discernible interacting effects, the primary effects of the NADH oxidation were pH, temperature, and buffer, in ascending order.

Enzymes are employed as physiologically active components in biosensors and in a wide range of analytical processes to increase selectivity in complex sample analysis in clinical, environmental, food, and pharmaceutical tests. Numerous studies on NADH monitoring are mentioned because many enzymes rely on the nicotinamide adenine dinucleotide (NADH) cofactor to interact with the substrate. Under certain storage conditions, NADH is known to be an unstable reagent.

3.1. Principle

NADH (Nicotinamide Adenine Dinucleotide, reduced form) absorbs UV light at a wavelength of approximately 340 nm. This property is exploited in enzyme-linked assays to detect the presence of NADH.

3.2. How to perform the assay

1. **Enzyme Reaction**: The assay involves an enzyme that catalyzes a reaction producing NADH. For example, glucose dehydrogenase can be used to oxidize glucose, producing NADH in the process.
2. **Substrate addition:** The addition of the substrate, such as glucose, to the reaction mixture is done in conjunction with the enzyme and NAD+, which is the oxidized form of NADH.

The mixture is exposed to UV-Vis spectroscopy once the reaction is complete. The sample's absorbance is measured using the spectrophotometer at 340 nm

The absorbance of tryptophan (Trp), tyrosine (Tyr), and a trace quantity of cystine causes proteins to have absorption maxima between 275 and 280 nm. These absorbance's are slightly red shifted when moved from polar to nonpolar settings and are dependent on the microenvironment of their chromophores. As a result, native proteins have residues that are buried and exposed to solvent, which aids in absorption.

Quantification: The amount of NADH produced is directly proportional to the absorbance measured at 340 nm. By comparing the absorbance to a standard curve generated with known concentrations of NADH, the concentration of NADH in the sample can be determined by this. (**Figure 2**)

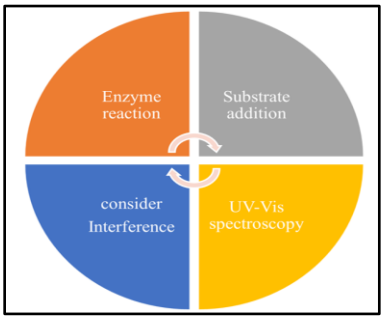

Figure 3: NADH linked enzymatic activity

4. Investigation

4.1. Concentrations of nucleic acids

Nucleic acids strong absorbance at 260 nm, which is frequently expressed as "A260 units," is used to calculate their quantities is equal to 50 mg, 33 mg, and 40 mg of RNA for double-stranded DNA. Because proteins absorb poorly, contamination has little effect on their quantities. Proteins account for just around 2% of the absorbance at 260 nm in a 1:1 mixture of proteins and nucleic acids. Therefore, the concentration of nucleic acids is determined by measuring their absorbance at 260 nm.

5. Absorbance of Proteins

Protein absorbance is result of the peptide bonds in the protein backbone, which absorb light in the far-ultraviolet spectrum (180-230 nm), as well as by the aromatic side chains of phenylalanine, tryptophan, and tyrosine. Heme groups, reduced nicotinamide adenine dinucleotide (NADH), reduced flavin adenine dinucleotide (FADH2), and cofactors containing copper are among the protein cofactors that absorb light in the visible and ultraviolet spectrum.

5.1. Protein unfolding

Heat or denaturants like urea or guanidinium chloride (GdmCl) can unfold native proteins.

The absorbance changes at 287–292nm can be measured based on temperature or denaturant concentration. The refractive index decreases slightly with temperature, protein concentration changes due to thermal expansion, and dissociable group ionization can change. In the absence of structural transitions, these factors have a negligible impact on protein absorbance, which results in a modest temperature dependence. Even in the absence of structural changes, the absorbance of tyrosine and tryptophan at 287 nm and 291 nm somewhat rise upon the additio of denaturants.

6. Curve-Fitting Routine Method[13-18]

Curve fitting in UV-Vis spectroscopy is a powerful method used to analyze and interpret spectral data Curve fitting is a mathematical method used to deconvolute these overlapping signals and extract meaningful information from the spectra. By fitting a chosen model (such as Gaussian, Lorentzian, or polynomial functions) to the experimental data, curve fitting allows for the precise determination of peak positions, intensities, and widths. This technique is particularly useful for quantitative analysis, peak deconvolution, kinetic studies, etc. Overall, curve fitting enhances the interpretative power of UV-Vis spectroscopy, enabling more accurate and detailed analysis of complex samples.

6.1. Principle

Curve fitting involves fitting a mathematical function to the experimental data points obtained from a UV-Vis spectrum. This helps in resolving overlapping peaks, estimating peak positions, and determining concentrations of analytes.

6.2. How to perform

6.2.1. Data collection

1. ***Measure Absorbance***: The first step is to measure the absorbance of the sample at various wavelengths using a UV-Vis spectrophotometer.
2. ***Plot Spectrum***: The absorbance data is plotted against wavelength to create a spectrum.

6.2.2. Choosing a Model

1. Select Mathematical Functions: Common functions used for curve fitting include Gaussian, Lorentzian, and polynomial functions. The choice of function depends on the shape of the peaks in the spectrum2.
2. Initial Parameters: Initial guesses for the parameters of the chosen function (e.g., peak positions, heights, widths) are required.

6.2.3. Fitting the model

1. **Optimization Algorithms:** To best fit the experimental data, the model's parameters are modified using algorithms like genetic algorithms, least squares fitting, and other optimizationapproach
2. **Minimize Error:** Often expressed as the sum of squared errors (SSE), the objective is to reduce the discrepancy between the experimental data and the fitted mode.

7. Analyzing Results

1. **Peak Parameters**: Once the best fit is achieved, the parameters of the fitted peaks (e.g., position, height, width) can be extracted and analyzed.
2. **Quantitative Analysis**: The fitted model can be used to determine concentrations of analytes by comparing the fitted absorbance values to a calibration curve. (**Figure 3**)

Figure 4: Curve-fitting method

7.1. Investigations

This technique is particularly useful for: An investigation using curve fitting in UV-Vis spectroscopy might involve determining the concentration of a substance in a mixture, identifying the number of components in a sample, or studying the kinetics of a reaction.

1. **Quantitative Analysis**: Determining the concentration of analytes by comparing the fitted curve to calibration standards.
2. **Peak Deconvolution**: Resolving closely spaced or overlapping peaks, which is essential for complex mixtures.
3. **Kinetic Studies**: Monitoring the time-dependent changes in absorbance to study reaction kinetics. Curve fitting is an essential method in UV-Vis spectroscopy that enhances the analysis and interpretation of spectral data. By applying mathematical models to experimental data, it allows for the accurate determination of peak positions, intensities, and widths. This method is invaluable for deconvoluting overlapping peaks, conducting quantitative analyses, and studying kinetic processes. It can be applied to a wide range of samples and conditions, making it a versatile tool in both research and industrial application.

8. Discussion

The OPA spectrophotometric method is a valuable tool in biochemical analysis for detecting and quantifying proteolysis. The assay involves adding one reagent solution to the sample, making it simple and user-friendly. Its high sensitivity, rapid reaction time, versatility, and practicality make it an essential technique for various scientific and industrial applications. Its suitable for various protein substrates and can be adapted for different experimental setups. A chemical reaction that turns chemicals into products is called an enzyme. One can determine the rate of the reaction by measuring the increase in product concentration or the decrease in substrate concentration as a function of reaction time. When both the substrate and product absorbance varies, the change in absorbance over time can be used to directly track the progress of an enzyme process. Changes in concentration have a linear relationship with changes in absorbance, and when the reacting species' absorption coefficients are known absorbance data can be used directly to calculate reaction rates.

Table 2: Overview of methods used for estimation of enzyme activity by UV spectroscopy

Method	Principle	Application
OPA Method	Reaction of O-phthaldehyde (OPA) with target enzyme in order to produce luminous (colored) compound which will absorb UV- Vis light, so measurement can be done by UV Spectrophotometer.	It is used to perform assay of amines, peptides, proteins and amino acids. OPA can be used to determine protein content in various sample including biological tissue and vaccine
NADH-Linked Enzyme Assay	The NADH-Linked enzyme assay is based on the oxidation-reduction reaction between NADH and a substrate. In this reaction, the enzyme catalyzes the conversion of substrate resulting in the oxidation of NADH to NAD^+	It is used to measure the activity of enzymes like dehydrogenases, reductases, oxidases.
Curve-Fitting Routine Method	Curve fitting involves finding a mathematical function the best describes a set of data points.the goal is to create a smooth curve that minimizes the difference between the observed data points and the predicted values	It helps in data analysis and identify trends and pattern in data. It help in developing model for classification and regression tasks.

7.1. For example

NADH-linked enzyme reactions, such as those mediated by lactate, malate, or alcohol dehydrogenases, are excellent examples of absorbance-based enzyme assays.

Maximum absorption is seen in NADH's reduced nicotinamide ring. The reduced nicotinamide ring in NADH loses its maximum absorbance, which is around 340 nm, when it is oxidized to NAD1. The activities of these

dehydrogenases can be directly measured by monitoring the decrease in A340 over time.

A "silent" enzyme reaction that yields a colourless product can occasionally be combined with another enzyme reaction that uses the result of the first enzymatic reaction. for a conversion that results in a change in absorbance. A high concentration of the indicator enzyme can be used to accomplish this.

9. Conclusion

UV-Vis spectroscopy, including the OPA spectrophotometric and NADH-linked enzyme assays, offers high sensitivity, rapid reaction times, and versatility for various scientific and industrial applications. OPA Method Offers a quick and convenient alternative to traditional methods like ninhydrin and TNBS assays. NADH Monitoring with advancements in buffer conditions and storage stability enhancing its accuracy. Compared to other UV spectroscopy techniques, these methods provide unique advantages in terms of simplicity, speed, and practical applications. Advancements in curve fitting further enhance the analytical capabilities of UV-Vis spectroscopy, making it an indispensable tool in biochemical analysis and beyond.

10. Source of Funding

None

11. Conflict of Interest

None.

12. Acknowledgement

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