Evaluation of Urease Activity Assessed on Urea Concentration Using Tannic Acid-Silver Nitrate Colorimetric Method

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Editor’s note: Urease, a nickel-dependent enzyme, hydrolyzes urea into ammonia and carbon dioxide. Imohiosen et al. explored how urea concentration affects urease activity using a colorimetric method with tannic acid and silver nitrate. Low concentrations (≤ 1.00 mM) limited enzyme activity, while an optimal concentration of 3.00 mM produced the highest absorbance (0.949), indicating maximum activity. Concentrations above 4.00 mM decreased absorbance, likely due to pH shifts. Low urea levels showed sharp SPR peaks at 414 nM, whereas higher levels produced broader peaks, suggesting substrate inhibition. These findings support the development of a sensitive urea biosensor.

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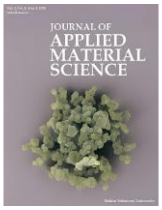

Original Research

Evaluation of Urease Activity Assessed on Urea Concentration Using Tannic Acid-Silver Nitrate Colorimetric Method

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Abstract

Urease, a nickel-dependent enzyme, hydrolyses urea into ammonia and carbon dioxide. The unhydrolyzed urea, in the presence of silver nitrate and tannic acid, forms a yellow-brown complex. This study examines the effect of urea concentration on urease activity using a colorimetric method that involves tannic acid and silver nitrate. The results revealed that low concentrations of urea, less than or equal to 1.00 mM, may limit substrate availability, leading to an underestimation of enzyme activity. An optimal urea concentration of 3.00 mM produced the highest absorbance, 0.949, indicating maximal urease activity (V_{max}). The high urea concentrations, greater than or equal to 4.00 mM, resulted in lower absorbance, possibly due to pH shifts or altered reaction kinetics. Low urea concentrations show narrow or sharp SPR peaks at around 414 nM, whereas higher concentrations display broader or wider peaks. The Lineweaver-Burk method shows that the enzyme kinetics of the urea hydrolysis efficiency were greatest at about 3 mM. At this concentration, catalytic efficiency and enzyme/substrate binding were very high (i.e., low Km and near-saturation V_{max}). Higher substrate concentrations led to decreased reaction rates. The changing wavelengths suggested substrate inhibition and possible sensor behavior related to nanoparticles. These results indicate that this approach is viable for a very sensitive urea biosensor.

Keywords: Urea; Tannic acid; Silver nitrate; Colorimetric method.

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1. Introduction

Metal nanoparticles like AgNPs have drawn significant attention owing to their unique physicochemical properties, which make them highly applicable in environmental remediation, medicine, agriculture, and more [1]. Silver nanoparticles green synthesis, which exploits biological agents such as plant extracts, microbes, and biomolecules, has arisen as an ecological and eco-friendly alternative to physical synthesis and conventional chemical methods. This approach not only decreases environmental impact but also ensures the production of biocompatible nanoparticles with enhanced biological activities [2].

Urease-based biosensors are usually used to sense urea in biological and environmental samples. This could consist of using urease to catalyze the breakdown of urea, with the products being determined to infer the concentration of urea. Silver nitrate ($AgNO_3$) could be used in these sensors to examine the ions present or to modify surfaces, making the sensor more sensitive. Urea can be hydrolyzed via a catalytic enzyme, urease, into ammonia and carbon dioxide. It is vital in several biological processes and is also used in diagnostic tests [3-5]. To maintain biochemical reactions of stable pH, a phosphate buffer (PB) is used. This is vital, since most enzyme activity, like that of urease, highly depends on pH [6]. Urea is a substrate for the urease enzymes, whose concentration is often measured in various applications. Tannic acid, a polyphenol, acts as a reducing agent. It interacts with enzymes such as urease, potentially inhibiting their activity, and is also used to produce silver nanoparticles [7]. Silver nitrate is a source of silver ions, and in the presence of tannic acid, a reducing agent, the silver ions are reduced to silver nanoparticles (AgNPs). Color change shows the presence or concentration of the target substances. Hence, these AgNPs can be used in colorimetric assays [8].

Even though colorimetric assays based on silver tannic acid complexation have proven extremely common for determining urease activity, little is known about the effects of substrate concentration on assay accuracy and signal linearity. Specifically, the impact of urea concentration on absorbance response, SPR peak behavior, and signal distortion that might have been a result of pH- or kinetics-induced changes has not been systematically considered. Such inefficiency can be the cause of under- or overestimating urease activity, which shows the necessity of well-defined operating conditions to perform an accurate enzymatic measurement.

Colorimetric urea sensing involves measuring urea content based on a visible color change, usually through an enzyme or chemical reaction. The enzyme urease, responsible for the hydrolysis of urea into ammonia and carbon dioxide, forms the basis for a highly sought-after colorimetric urea sensing method. The pH indicator can, as ammonia produced changes the pH of the solution, change color. Urease-mediated colorimetric sensing: In one investigation, it was demonstrated that urease was used to catalyze the hydrolysis of urea. The presence of urea was signaled by a yellow-to-red color change when a pH-sensitive dye, i.e., phenol red, was used. This method provides a rapid and effective method to detect urea in environmental samples and biofluids.

With the help of a pH indicator, the presence of urea can lead to a pH change when it reacts with urease and alters the color. Phenol red is a common pH indicator used in this way; its color shifts depending on the basicity or acidity of the solution. Phenol red-based colorimetric sensor: This system employs phenol red that changes from yellow (acidic) to red (basic) in the presence of released ammonia through urea hydrolysis. The intensity of the color change varies directly with the concentration of urea.

Surface Plasma Resonance (SPR) is based on the collective oscillation of conduction electrons at the surface of metallic nanoparticles (like silver) when excited by light. This SPR peak appears in the UV/Vis spectrum and is highly sensitive to the following factors: shape and particle size, interparticle spacing, and Dielectric environment (such as pH and solvent) [9].

Nanoparticles with a smaller size, SPR normally happens at blue-shift (shorter wavelength) due to stronger absorption and less scattering. For particles with a larger size, SPR shifts to longer wavelengths (red-shift) and also shows a broader SPR peak, due to greater scattering and multipolar modes. It may also display multiple resonance modes (e.g., dipolar, quadrupolar). The widening and broadening of the SPR peak are symbols of clustering or polydispersity in the size of nanoparticles, aggregation, which is due to pH-induced instability and complex shapes or large particle dimensions (e.g., rods, stars) [9]. This study aimed to examine the evaluation of colorimetric sensing of urea concentrations via the tannic acid-silver nitrate method.

2. Experimental

2.1. Materials

Analytical-grade reagents were used in this study. Urease (AR grade), urea (99% AR grade), phosphate buffer (AR grade pH 7.0), tannic acid AR (gallotannic acid), $AgNO_3$ (AR grade) were used as synthesis starting materials.

2.2. Measurements

Urease Incubation: Urease enzyme (50 µL, 5 µ/mL) in 0.5 mM of PB (phosphate buffer) solution is incubated with 50 µL of various urea concentrations (0.00, 1.00, 2.00, 3.00, 4.00, 5.00, and 10.00 mM) individually for 10 min at 37 ± 1 °C in a water bath/ incubator [10-12].

Sensing Solution Addition: Subsequently, the sensing solution consisting of 800 µL of tannic acid (0.025 mg/mL) and 100 µL of $AgNO_3$ (15 mM) was added to the aforementioned solution and incubated for another 20 mins [10-12].

Spectrophotometric Measurement: The intensity of the color change is measured spectrophotometrically in a wavelength range of 300 – 600 nM. UV-Vis Spectroscopy, this technique is used to confirm the formation of AgNPs by detecting the surface plasmon resonance (SPR) peak, typically in the range of 400-450 nm [10-12].

3. Results and discussion

In Table 1, as the concentration of urea increases from 1.00 mM, 2.00 mM, and 3.00 mM, the absorbances are 0.902, 0.877, and 0.949, respectively. Optimal urea concentration, 3.00 mM (typically saturating). 3.00 mM has the highest absorbance of 0.949, which is the optimal urea concentration (typically saturating). This means its maximum capacity, which the enzyme is working, hence allowing for an accurate measurement of its activity (V_{max}).

In Figure 1, the too low urea concentration, 1.00 mM, leads to substrate limitation where the measured activity reflects the availability of urea rather than the enzyme's intrinsic activity. It may also result in color development that is too faint to be accurately quantified.

In Figure 1, the high urea concentrations of 4.00 mM, 5.00 mM, and 10.00 mM prolonged reaction may lead to important product inhibition by ammonia, altering the reaction kinetics and potentially imparting the colorimetric signal. It may also cause large pH shifts that can move the enzyme out of its optimal activity range. A

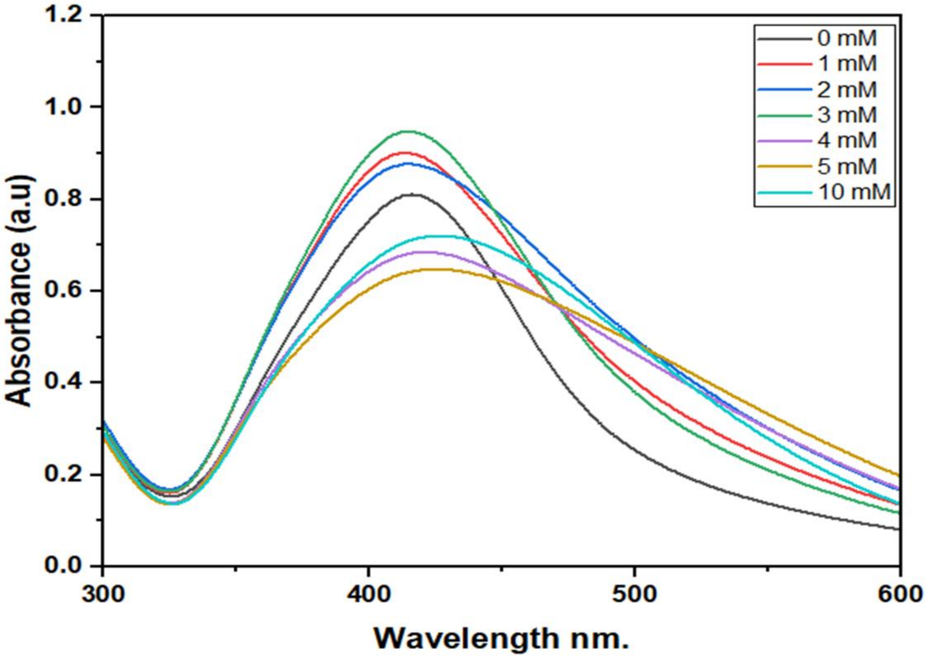

Figure 1. Graph of different urea concentrations wavelength (nM) against absorbance.

Table 1. UV/Vis results of urea concentration (mM), absorbance, and wavelength (nM)

Urea Conc. (mM)	Absorbance	Wavelength (nM)	Remark
0.00	0.811	415.0	
1.00	0.902	414.0	Too low
2.00	0.877	414.0	Low
3.00	0.949	414.0	Optimal (TS)
4.00	0.684	424.0	High
5.00	0.648	423.0	High
10.0	0.721	425.0	excessively high

Note: TS = typically saturating

higher urea concentration will result in more ammonia production, leading to more tannic acid reduction and a greater number of silver nanoparticles, thus causing a more pronounced color change.

According to [13, 14], moderate, high, and very high urea concentrations of 4 M, greater than 6 M, and greater than 8 M, respectively. 4 M show a minor red-shift, slightly broadening SPR peaks, and a gradual increase in pH. With a minor increase in particle size with moderate aggregation, urea starts to change its hydration shell and surface interactions. High urea concentration greater than 6 M shows broadening SPR peaks, red-shift, and a major increase in pH through hydrolysis. Apparent increase in particle size with high aggregation, urease-catalyzed hydrolysis produces NH_3, promoting aggregation and altering pH. Very high urea concentration, greater than 8 M, shows a non-linear shift, strong broadening of the SPR peaks, and alkaline pH. Clustering of particle size, very high aggregation, urea self-aggregation, and strength effects dominate; destabilization of colloids.

Curves for 1.00 mM, 2.00 mM, and 3.00 mM exhibit sharp absorption peaks, generally located at approximately 414 nM, which agrees with the work of [10, 11] that says the surface plasmon resonance (SPR) peak of AgNPs formation is detected in the range of 400-450 nm. According to [10, 11, 15], the SPR peak is characteristic of Surface Plasmon Resonance (SPR) for silver nanoparticles, which range between 400 nM and 430 nM. Hence, the urea concentrations of 4.00 mM, 5.00 mM, and 10.00 mM curves exhibit broad absorption peaks [12].

The colorimetric assay method based on urease may be utilized in daily situations when it is necessary to quickly test urea levels in farm soils and fertilizers to control the appropriate amount of nitrogen, and when screening blood or food items, to quickly determine the presence of disproportional urea concentrations by using straightforward color responses. Maximization of urea concentration guarantees precise, inexpensive monitoring without advanced instrumentation; it is applicable in the field or in the laboratory in regular usage [16, 17].

Table 2 and Figure 2, The Lineweaver–Burk diagram illustrates the reciprocal link between the concentration of the substrate (1/[S]) and velocity of the reaction (1/V), resulting in a linear graph with a bracket system where the kinetic values for an enzyme can be assessed. This linear equation approximating the line shown in the plot can be expressed as: 1/V = - 0.405 (1/[S]) + 1.444. The V_{max} (Maximum Reaction Velocity) is represented by the value of the y intercept: $1/V_{max} = 1.444$. Hence, Vmax = 0.693 a.u. The data imply a maximum catalysis of approximately 0.693 at saturation of enzyme active sites.

An enzyme's kinetic behavior is inversely proportional to concentration based on the slope of the Lineweaver-Burk Plot Curve, providing the distinguishing characteristic of enzyme kinetics. With a relatively low Vmax, the enzyme can likely operate at moderate catalytic efficiency.

Using the Lineweaver-Burk approach, the common approach of linearizing Michaelis-Menten kinetics was utilized, allowing us to determine important characteristics of the enzyme-catalyzed reaction. The behavior of the enzyme as it hydrolyses urea fits a normal saturation curve, where the amount of product produced is dependent on the amount of substrate available until all catalytic sites are filled with substrate [18]. The value of Vmax is an estimate of the maximum catalytic effectiveness of the enzyme, and since the Km

Table 2. Calculated reciprocal values for Lineweaver–Burk plot

[S] (mM)	V (Absorbance)	1/[S] (mM^{-1})	1/V
1.00	0.902	1.000	1.109
2.00	0.877	0.500	1.140
3.00	0.949	0.333	1.054
4.00	0.684	0.250	1.462
5.00	0.648	0.200	1.543
10.0	0.721	0.100	1.387

Note: V = Reaction velocity (represented by absorbance), [S] = Substrate concentration (urea, mM), Km = Michaelis constant, Vmax = Maximum reaction velocity. (The 0 mM concentration was excluded because reciprocal substrate concentration is undefined).

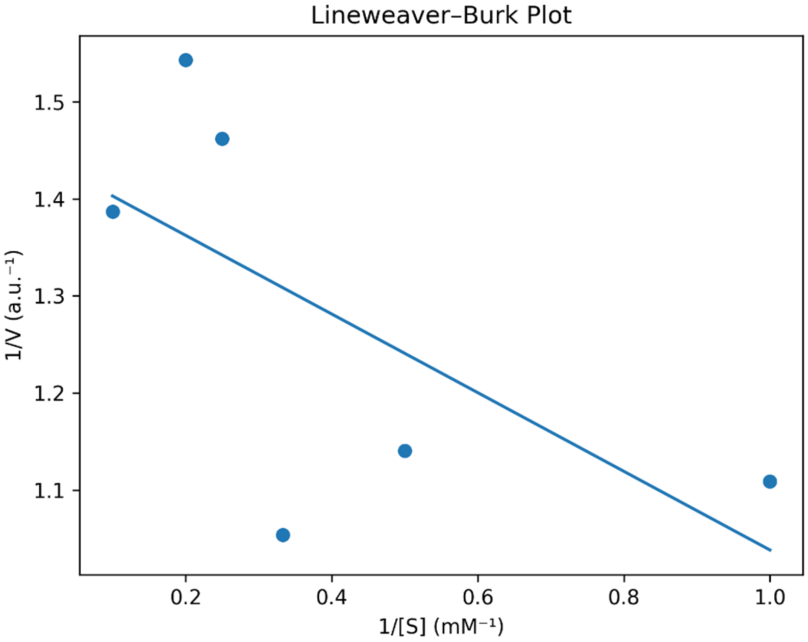

Figure 2. Lineweaver–Burk plot analysis for enzyme kinetics.

value (the amount of substrate needed for half-maximal activity at saturation) is low, it indicates a very high level of enzyme-substrate affinity and catalytic efficiency, making it a good candidate for use in analytical or biosensing applications. Additionally, reactions at higher levels of substrate are inhibited, inhibiting the rate of reaction at the very high levels (100 µM), indicating either inhibition by substrate or conformational changes of the enzyme. Moreover, upon addition of high substrate levels, a shift in the wavelength of the absorption spectrum is observed, suggesting that reactions may occur in an altered environment due to the use of the amplitude changes seen in the colorimetric sensing behavior of nanoparticle-based systems. Thus, the enzyme system exhibits good catalytic performance and high potential for the development of ultra-sensitive urea detection systems.

4. Conclusions

A yellow-brown complex is formed by the unhydrolyzed urea in the presence of silver nitrate and tannic acid. Urea concentrations of 1.00 mM, 2.00 mM, and 3.00 mM have absorbance values of 0.902, 0.877, and 0.949, respectively. Concentrations less than or equal to 1.00 mM may limit substrate availability, leading to underestimation of enzyme activity. The optimal concentration, 3.00 mM, has the highest absorbance, 0.949, indicating maximal urease activity (Vmax). High concentrations of 4.00 mM, 5.00 mM, and 10.00 mM have absorbance values of 0.684, 0.648, and 0.721, respectively. The concentrations, greater than or equal to 4.00 mM, resulted in lower absorbance, possibly due to pH shifts or altered reaction kinetics. Urea with low concentrations shows narrow or sharp SPR peaks around 414 nM, while higher concentrations display broader or wider peaks. The widening and broadening of the SPR peak are symbols of clustering or polydispersity in the size of nanoparticles, aggregation, which is due to pH-induced instability and complex shapes or large particle dimensions (e.g., rods, stars). A typically saturating (optimal) urea concentration is crucial for accurately assessing urease activity, which reflects its catalytic competence and falls within the linear range of the detection method. The Lineweaver-Burk graph was efficient for quantifying kinetic parameters. The enzyme had high substrate affinity (low Km) and a clear maximum reaction rate. This confirmed that the experimental assay was accurate for analytical enzyme activity studies and biosensing.

Conflict of Interest

The authors declare no conflict of interest.

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