Assessing the Influence of pH on Tyrosinase Activity Using Spectrophotometry

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Abstract

Tyrosinase is an enzyme, which is a class of proteins that function as biological catalysts which lowers the activation energy of a particular reaction without being consumed, therefore accelerating it. Enzymatic function, and therefore activity, is determined by its shape which can be affected by environmental conditions such as pH. Tyrosinase is one particular enzyme that is associated with the melanin production in animals as well as fruits and vegetables. Because of this, the regulation of its activity is particularly significant to cosmetic and agricultural industries, with our experiments particularly focusing on the pH's effect. In order to assess tyrosinase activity we took advantage of the tyrosinase catalyzed production of DOPAchrome, which is pigmented. We then measured the absorbance of a mushroom homogenate at 475 nm using buffered solutions of pH 4, 7, and 9, as the DOPAchrome production leads to higher absorbance values over time. T-Tests comparing the mean activity, defined as net absorbance at 475 nm * volume uL, found that the mean activity of 0 at pH 4 was significantly lower than the pH 7 average of 2.5 with a P-value of $1.01*10^{-8}<0.05$. Contrary to our expectations, we found that the mean activity of 3.3 at pH 9 was significantly higher compared to pH 7, with a P-value of $4.48*10^{-4}<0.05$. This suggests that the tyrosinase enzyme denatures at acidic pH 4, but retains its optimal function at pH 7 and pH 9. Knowing the optimal range for tyrosinase function may help to make advances in biomedical, agricultural, and cosmetic industries in which tyrosinase catalyzed production of melanin causes issues of pigmentation.

Introduction

Enzymes are an important class of proteins which help to increase the speed at which biological processes occur without being used up itself. By acting as catalysts, enzymes are able to lower the activation energy that a reaction requires to proceed, therefore increasing the rate of reaction. In order to do this, enzymes have an active site to which substrates can bind to. Tyrosinase is one example of an enzyme and is associated with the production of melanin in both plants and animals. It does this by catalyzing several reactions, starting with the oxidation of tyrosine into dihydroxyphenylalanine, which we will be calling DOPA. From there, it catalyzes the multi-step oxidation of DOPA to DOPAchrome, which eventually becomes Melanin through an autocatalytic reaction[3]. The 3-dimensional shape of tyrosinase's active site, and the enzyme in general, is important for maintaining this function as a catalyst. However the pH of its environment, as well as other conditions such as temperature, can affect the site by denaturing it, and thus reducing its activity and effectiveness. Tyrosinase is involved with conditions such as the unappealing discoloration of fruits and vegetables, as well as hyperpigmentation and melanoma in human skin[6]. Understanding how tyrosinase is affected by environmental conditions such as pH will help to provide insight and assistance to medical, cosmetic, and industrial contexts that involve melanin production, as tyrosinase activity is correlated with this process. Previous studies have shown that tyrosinase activity is decreased to 50% at basic and acidic pH's[1], and we aim to use alternative methods to support these conclusions. If the optimal pH of the tyrosinase enzyme is 7 then the buffed solution at pH 7 would have a statistically significant higher total activity, measured by its absorbance at 475 nm over time, than the solutions at pH 4 and 9, which we predict to have an activity of 0. To address this hypothesis, we will assess tyrosinase activity through the measurement of absorbance of the solution over time using a spectrophotometer, as the DOPAchrome produced in tyrosinase catalyzed melanin production absorbs light at 475 nm[3].

Methodology

The activity of tyrosinase was determined using a spectrophotometer to measure the absorbance of 3 different pH's, being 4, 7, and 9. A homogenate was prepared by the TA using Button Mushrooms blended with 200mL 20mM potassium phosphate buffer, then filtered through cheesecloth. To measure absorbance a spectrophotometer was used, which was set to a wavelength of 475 nm. As for the tubes, the pH was set using various buffers. To all of the solutions, apart from the blanks used for spectrophotometer calibration, 0.4 mL of DOPA was added. For the blank acidic pH 4 tubes, a 3 mL Mcilvain Buffer containing 100 mM Citrate/100mM Na_2PO_4 and 0.35mM SDS was used, and 2.6 mL of the buffer was used for the experimental tubes. For the blank neutral pH 7 tubes, 3mL of buffer with 100mM KPO4 with 0.35mM SDS was used, with 2.6mL of this buffer for the experimental tubes. Finally, for the blank basic pH 9 tubes, 3mL a solution of 100 mM Tris-HCl buffer with 0.35mM SDS was used, with 2.6 mL of the solution going to the experimental tubes. After these solutions were made, 0.2mL of the mushroom homogenate in a 20 mM KPO_4 buffer was added to each solution, which started the reaction. For each condition, the blank tube was used to blank the spectrophotometer, then took measurements of absorbance for all 3 pH's for 16 minutes in 2 minute intervals after the start of reaction and plotted to determine absorbance over time, which we used as our net activity value. This was then repeated 4 times for each pH condition in order to use an average activity. Once the data was gathered, 2, Two-Tailed and Two sample T-Tests with equal variance were performed. For the first one, the mean total activity of pH 4 and pH 7 was compared. For the second test, the mean total activity of pH 7 and pH 9 was compared. A T-Test was chosen in order to determine if the differences in mean total activity between the aforementioned conditions were due to chance (not different) or if they were statistically significant, especially because of our smaller sample size. If the T-Tests resulted in p values below 0.05 then the activities are significantly different.

Results

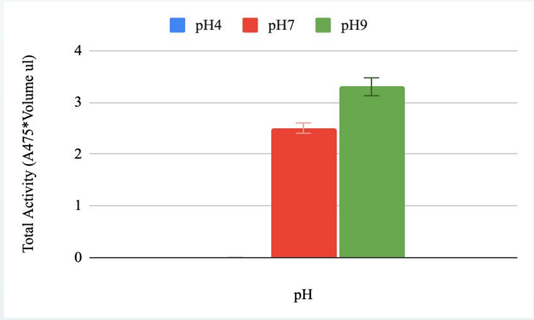

Figure 1: Bar Graph showing the effect of pH on total tyrosinase activity. Total activity was calculated by average change of Absorbance at 475 nm (of DOPAchrome) multiplied by volume in uL. Error bars represent 1 standard deviation. Absorbance measurements at 475 nm were taken over a 16 minute period in 2 minute intervals to determine A475 over time.

	pH 4	pH 7	pH 9
Mean Total Activity	0	2.5	3.3
Standard deviation	0	0.1	0.1732050808
Variance	0	0.01	0.03
Count	4	4	4
T-Test P Value (Comparing Mean Total Activity)	$1.01*10^{-8}$		$4.48*10^{-4}$

Table 1: Table of mean total activity, standard deviation, variance, count, and p-values of T-Tests of the activity of tyrosinase between the 4 trials of 3 different pH's. Total activity was calculated by average change of absorbance at 475 nm (of DOPAchrome) multiplied by volume in uL. Absorbance measurements at 475 nm were taken over a 16 minute period in 2 minute intervals to determine A475 over time.

Our experiment found that the mean total enzymatic activity, as defined by net absorbance at 475 nm * volume uL, was 0 at pH 4, 2.5 at pH 7, and 3.3 at pH 9. The standard deviation was 0 for pH 4, 0.1 for pH 7, and 0.173 for pH 4. All pH conditions had sample sizes for 4. 8 Absorbance at 475 nm measurements were taken in 2 minutes intervals for 16 minutes to determine net absorbance over time.

Two tailed and two sample T-Tests were performed comparing activity at pH 4 and 9 to the neutral pH of 7. The activity at pH 4 was significantly less compared to pH 7 (T-Test $p=1.01*10^{-8} > 0.05$). The higher activity at pH 9 was significantly greater compared to pH 9 (T-Test $p= 4.48*10^{-4} > 0.05$)

$$t = \frac{\overline{x_1} - \overline{x_2}}{\sqrt{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)}}$$

Discussion & Conclusion

Figure 1 showed significantly different activity between the three pH's studied, which is consistent with other studies that found that "pH has a large effect on tyrosinase catalysis"[2]. More specifically, the activity in acidic conditions of pH 4 was significantly lower compared to the activity at pH 7, indicating a reduced production of DOPAchrome. The lack of activity in the pH 4 tubes could be due to denaturing of the enzyme in acidic conditions, which reduces its functionality and effectiveness as a catalyst for biological reactions. Unexpectedly though, the activity in basic conditions of pH 9 were significantly higher compared to the activity at pH 7. We believe that this could have been caused by methodology error, such as cross contamination of the buffer solutions, causing the enzyme to be closer to a neutral pH rather than a basic one. The unexpected result of activity at pH 9 was found in other studies where a "minor stability optimum at pH 9" was observed, although they asserted that there was no known explanation for this[1]. If the activity of tyrosinase can be reduced through acidic conditions, this could be used as an alternative inhibitor of tyrosinase for cosmetic and industrial purposes. Traditionally, molecular tyrosinase inhibitors are used for the reduction of browning of vegetables and treatment of hyperpigmentation in humans[5]. However the data of tyrosinase activity in this experiment derives from a mushroom homogenate, whose structure is different from the tyrosinase found in humans[4]. Therefore, it may be inappropriate to apply these findings to tyrosinase found in humans.

Initially, we hypothesized that the tyrosinase activity at both basic and acidic conditions would be lower compared to the activity at pH 7. However, figure 1 only displayed this pattern for an acidic pH of 4. Interestingly enough, the activity at a basic pH of 9 was higher compared to pH 7. T-Tests comparing the activities of pH 7 to pH 4 and pH 9 found P-Values of less than 0.01. Because of this, we can determine that these differences were statistically significant and not likely due to chance, meaning that there were true differences between the values for mean total activity, allowing us to use these differences in assessing the accuracy of our hypothesis. From this, we found that our hypothesis that both the basic and acidic buffered solution would have lower activity compared to our neutral solution was only sustained for an acidic pH. In the future, we would like to repeat this experiment using a wider range of basic and acidic pH's, such as pH 8, 8.5, and 10 to better understand the activity of tyrosinase in different environmental conditions, as well as to ensure that the higher activity at pH 9 was not due to human experimental errors by increasing replication.

References & Acknowledgements

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