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Sodium Chloride Is a Competitive Inhibitor of Polyphenol Oxidase

Michael Amin, Giovanna Sternberg, Lucca Vallejo, Randall Maxey,
Wan Hei Wong, Zollie Daily
Life Sciences
Santa Monica College
1900 Pico Blvd,
Santa Monica, California 90405 USA

Faculty Advisor: Dr. Olga Vagin

Abstract

Food browning resulting from the activity of polyphenol oxidase (PPO) is a contributing factor for food waste as browning causes poor appearance of products. To address this problem, the inhibitory effect of sodium chloride (NaCl) on the enzymatic activity of potato PPO was studied using catechol as a substrate. Enzymatic activity of PPO present in the potato extract was measured using the continuous spectrophotometric method to detect the gold melanin product of catechol oxidation. The initial rate of enzymatic reaction was determined from a slope of the time course of product accumulation during a 4-minute period after adding the PPO-containing potato extract to the reaction mixture containing a particular concentration of catechol. To calculate specific PPO activity per mg of protein of the potato extract, protein concentration was measured by the BCA method using bovine serum albumin as a standard. The increasing concentration of NaCl at the same concentration of catechol and PPO resulted in a gradual decrease in enzymatic activity, indicating that NaCl is an effective inhibitor of PPO. To determine the type of inhibition by NaCl, the substrate dependence of PPO was measured in the absence of NaCl and at 0.2 M NaCl. The Lineweaver-Burk plot was generated by using inverse values of catechol concentration and specific activity to calculate the maximal velocity (V_{max}) and Michaelis constant (K_m) in the presence and in the absence of NaCl. The results demonstrated that NaCl significantly increased the K_m and did not alter the V_{max} of PPO-catalyzed catechol oxidation. These results suggest that NaCl is a competitive inhibitor of PPO. The results might help develop methods to decrease food browning.

Keywords: Competitive Inhibition, Polyphenol Oxidase, Food Browning

1. Introduction

The foods produced by the agriculture industry do not last forever. Once food begins to brown, most individuals decide to throw away their food, believing it is no longer safe to eat. About one-third of the food produced in the world for human consumption every year gets wasted[1]. This means that 1.3 billion tons of food that were intended to be consumed are thrown away every year. Since 2015, the number of undernourished people in the world has been increasing, reaching about 821 million in 2018[2]. With an increasing number of individuals without access to food since 2015, it is crucial to support research on ways to decrease the amount of food being wasted. By investigating what causes food browning, knowledge can be applied to inhibit this biochemical process on an industrial level.

Food browning occurs due to the interaction between the substrate catechol and the enzyme polyphenol oxidase (PPO) during catechol oxidation[3]. The oxidation of catechol has as a product of pink/gold melanin, which spontaneously turns into brown/back melanin over time. When PPO catalyzes the reaction of catechol oxidation, the color change from pink/gold melanin to brown/black melanin occurs more rapidly. By introducing an inhibitor, the rate of browning could decrease or potentially stop. NaCl, or table salt, is a widely known inhibitor that is commonly used by individuals to prevent browning of fruits and vegetables like avocados and potatoes. By knowing more about its mechanism, an industrial solution to browning could be crafted.

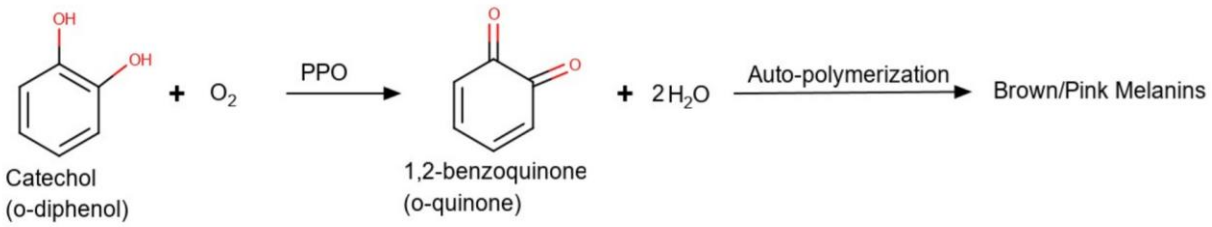

Figure 1. The reaction between PPO and catechol, simplified.

PPO has genetic variability between plant, animal, and fungal species. However, all PPOs have two copper ions in the active site, where each copper ion is connected to histidine residues[4]. Upon exposure to oxygen, PPO catalyzes the oxidation of an o-diphenolic substrate to its corresponding o-quinone. These resulting o-quinones auto-polymerize to form brown melanins[5]. Previous research proposes that one of the oxygen atoms of the substrate binds to one of the copper atoms on the PPO active site prior to being transformed into quinones[5].

Previous research suggests that in low concentrations, cysteine and ascorbic acid are effective competitive inhibitors in a PPO-catechol system[6]. Sulfites are a common preservative that inhibit browning by preventing quinones from polymerizing and forming melanins[7]. In previous research, NaCl has been shown to exhibit browning inhibition in PPO-catechol systems[8]. However, the mechanism of the inhibiting effect of NaCl on PPO is unclear. The current study focuses on whether NaCl acts as a competitive or non-competitive inhibitor by comparing the Michaelis-Menten kinetics of PPO in the absence and in the presence of NaCl.

This research aims to contribute with more data on this mechanism of inhibition as it is not fully understood. Different concentrations of NaCl were added to the reaction between catechol and PPO. Graphical data showed that the presence of NaCl had no significant effect on the V_{max} value but did increase the K_m value. This relationship suggests that NaCl acts as a competitive inhibitor of PPO.

2. Methods and Materials

All the glassware and materials associated with the making of potato enzyme was placed into a 4°C refrigerator 30 minutes prior to making a buffer solution. At the same time, the centrifuge machine was set to be cooled to 5°C, so that it reaches the optimum temperature 30 minutes later. In preparing the potato enzyme, a third of a potato was peeled, cut into chunks, and cold blended. 400 mL of cold 0.2M potassium phosphate monobasic buffer solution (in pH 6) was added into the blend and mixed with the blend for about 1 minute. A cold 600 mL beaker was placed onto ice in an ice bucket. The blended homogenate was filtered into the beaker with several layers of cheesecloth and a cold glass funnel. After this, the same filtering steps to transfer the homogenate into a cold 500 mL Erlenmeyer flask was repeated. The filtered homogenate was poured into 4 cold centrifuge bottles, which each holds around 100 mL. The centrifuge bottles held a maximum of ¾ their capacity. Each pair of bottles was balanced on a pan balance before being loaded into the centrifuge with transfer pipets. Balanced bottles pairs were placed in a cold centrifuge across from each other and were centrifuged at about 2500 rpm for 5 minutes. Once the centrifuge finished, the centrifuge bottles were taken out and carefully handled such as to not mix the supernatant and the precipitates. Another cold 500 mL Erlenmeyer flask was placed in an ice bucket. Layers of cheesecloth and a cold glass funnel was used to slowly filter the supernatant into a flask. Finally, the flask containing the supernatant was placed over ice and covered with parafilm. The Erlenmeyer flask was labeled. Protein concentration in the potato extract was determined by the BCA method[9].

The reaction mixture for measuring activity of PPO in a total volume of 5 ml contained indicated concentrations of catechol, NaCl, and 1 mL of enzyme solution, which was added to each sample as the final step to start the reaction. The absorbance at 475 nm, which reflects the amount of pink/gold melanin, in each sample was recorded every 30 seconds for 10 minutes using a spectrophotometer.

3. Results

To find whether NaCl acts as an inhibitor of polyphenol oxidase and to discover what kind of inhibitor it is, absorbance spectrophotometry was used to measure the rate of formation of pink/gold melanin from the enzymatic reaction. Absorbance of light is assumed to be directly proportional to the concentration of melanin under the Beer-Lambert Law[10]. An inhibition curve was created to show the potency of NaCl as an inhibitor, and a Michaelis- Menten plot was used to compare the results from the control samples to the samples containing NaCl. To discover the type of enzyme inhibition that occurs between polyphenol oxidase and NaCl, a Lineweaver-Burk plot was used to compare the control samples to the samples containing the inhibitor.

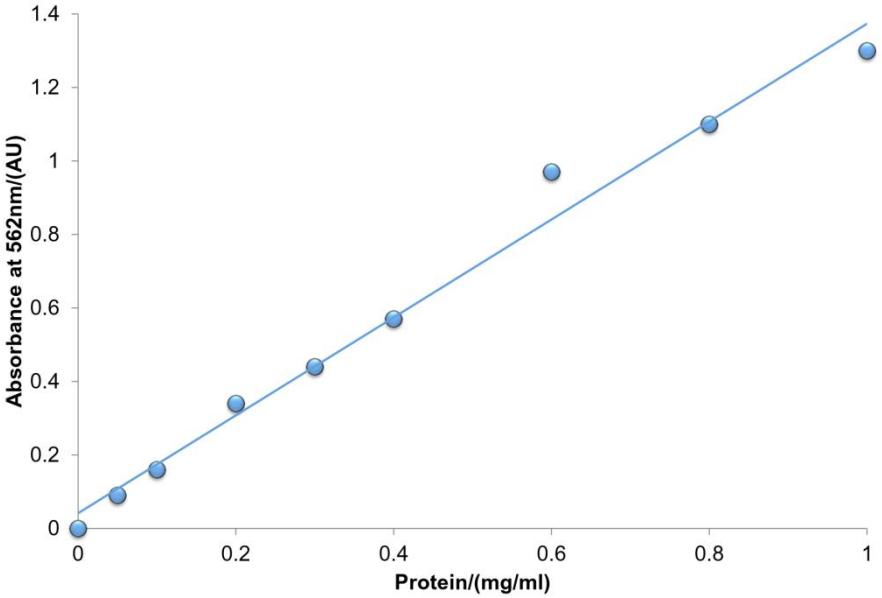

Figure 2. A standard curve to determine protein concentration in the potato extract. Albumin solutions with known concentration were used as standards.

First, it was necessary to determine the amount of protein in the potato extract that was used as an enzyme solution in all experiments to measure PPO activity. The BCA method was applied to generate a standard curve to correlate protein concentrations to the absorbance. A 1 mg/ml solution of Bovine Serum Albumin was diluted to specific ratios to generate standard solutions with different protein concentrations (Figure 1). The absorbance at 562 nm of protein/BCA was measured both in standards (Figure 1) and in different dilutions of the potato extract. The concentration of the protein in the potato extract was calculated as a mean from 3 parallel experiments and was determined to be 1.16 mg/ml.

Since all samples used to determine enzymatic activity of PPO had 1 ml of the potato extract, the approximate total amount of protein in each reaction mixture was 1.16 mg. This value was used later to calculate the specific enzymatic activity. First, the total activity was determined for each condition as described below (Figure 3), and this total activity was divided by the total amount of protein in the reaction mixture.

Total protein in the potato extract includes PPO and other water-soluble proteins extracted from the potatoes. The amount of total protein varied between 0.9 and 1.3 mg/ml in different preparations and was approximately 1.16 mg/ml in the particular potato extract that was used in experiments presented in Figs. 2-5. The constant proportionality of the total activity of PPO to the amount of total protein in the enzyme preparation allowed us to account for the variability in PPO content in different preparations made on different days. PPO concentration is proportional to total protein concentration. Therefore, total protein concentration can be used as a useful representative, or alias, of PPO concentration in the comparison of activity of different samples.

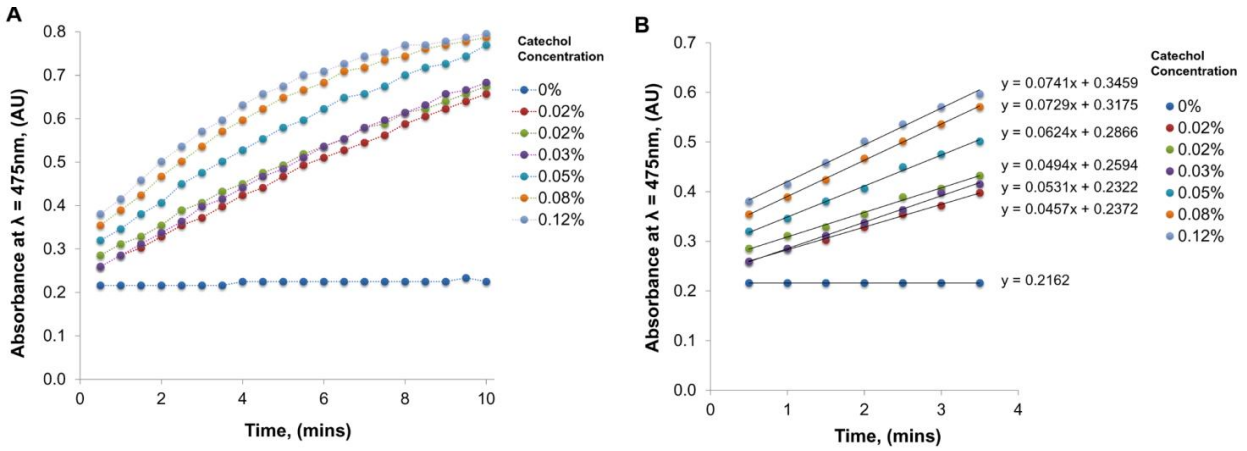

Figure 3. Time course of PPO-catalyzed reaction at different concentrations of catechol, %. A, full time course; B, initial period of the time course to find the initial rate of reactions.

The total activity of enzymes corresponds to the initial rate of the enzyme-catalyzed reaction. The initial rate was determined by finding the best time period for each time course (Figure 2A) where the change in absorbance with time was linear. From the linear approximation of the time course during this initial period, the initial rates of the reaction were determined by finding the slope of the time courses (Figure 2B). The specific enzymatic activity was determined by dividing the initial rates by the amount of protein in the reaction mixture.

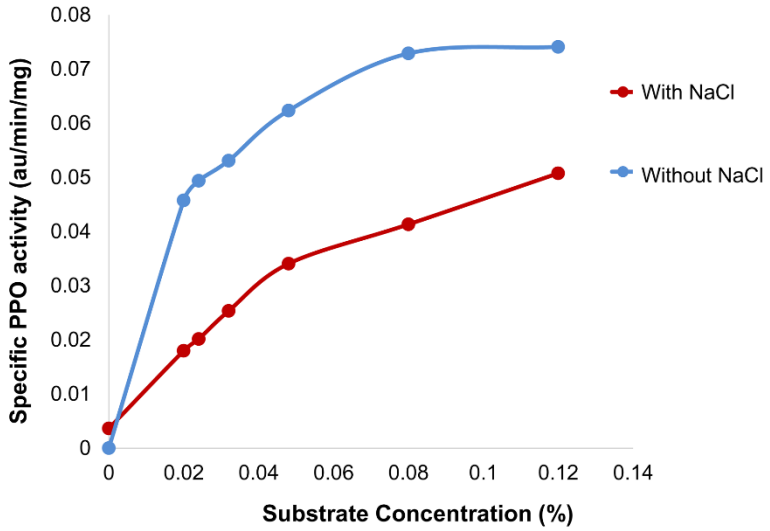

Figure 4. Effect of catechol concentration on PPO Activity in the presence and in the absence of NaCl (Michaelis-Menten plot).

The values of specific activities were plotted vs different catechol concentrations, producing the Michaelis–Menten plot (Figure 3, blue curve). A similar experiment to determine PPO specific activity at different catechol concentrations was performed in the presence of 0.2M of NaCl (Figure 3, red curve). Both in the absence and in the presence of NaCl, the increase in catechol concentration increased the specific activity. At lower catechol concentrations, the increase was linear, but at higher concentrations of catechol, the curve reached a plateau since the

enzyme became saturated by the substrate[11]. In the presence of NaCl, the saturation appeared to occur at higher catechol concentration, suggesting that NaCl increase the K_m for catechol.

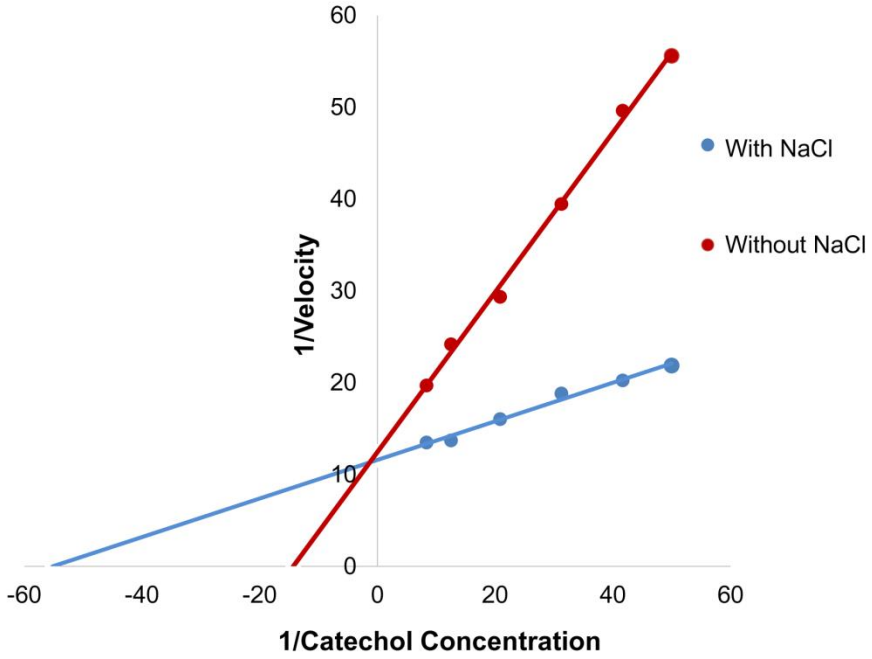

Figure 5. Lineweaver-Burk plot. Velocity is enzymatic activity, or initial slope.

Table 1. Mathematical definitions of main features of a Lineweaver-Burk plot.

Terms	**Corresponds to...**
X- intercept	$-\frac{1}{Km}$
Y- intercept	$\frac{1}{Vmax}$

Table 2. K_m and V_{max} values derived from Lineweaver-Burk plots. Average of four trials ± standard deviation is shown. *Statistical difference from the Km value in the absence of NaCl, p=0.0018, Student's t-test, n=4.

	Without NaCl	With NaCl
K_m *(catechol concentration, %)*	0.036 ± 0.014	0.087 ± 0.017*
V_{max} $(\frac{au}{min*mg})$	0.112 ± 0.027	0.091 ± 0.013

The experiment was conducted 4 times with NaCl and 4 times without NaCl. Figure 4 is the Lineweaver-Burk plot for one of these trials. The value of the x-intercept on this plot corresponds to $-1/K_m$, and the value of the y-intercept is $1/V_{max}$ (Table 1). The K_m and V_{max} values were calculated for each trial. The K_m and V_{max} values from the trials with

and without NaCl were used to find the averages and standard deviation of each respective group. The relationship between the control samples and the samples with NaCl can be seen by comparing the K_m and V_{max} values in Table 2. A Student's t-test yielded p values for both K_m and V_{max} that indicated a statistically significant difference in K_m values in the presence and in the absence of NaCl (p=0.0018). In contrast, the difference between V_{max} in the presence and in the absence of NaCl was shown to not be statistically significant (p=0.1002). These results suggest that NaCl functions as a competitive inhibitor of PPO.

Finally, the effect of different NaCl concentrations on PPO activity at different concentrations of catechol was studied. The specific activity of PPO at various concentrations of NaCl were determined the same way as described above for the experiments with varying catechol concentrations (Figures 1 and 2). Briefly, a time course of PPO-catalyzed reaction was created for each NaCl concentration, and the total activity of PPO was determined from the slope of the linear regression to the initial portion of this time course. Specific activity was determined by dividing the total activity by the amount of protein present in the reaction mixture.

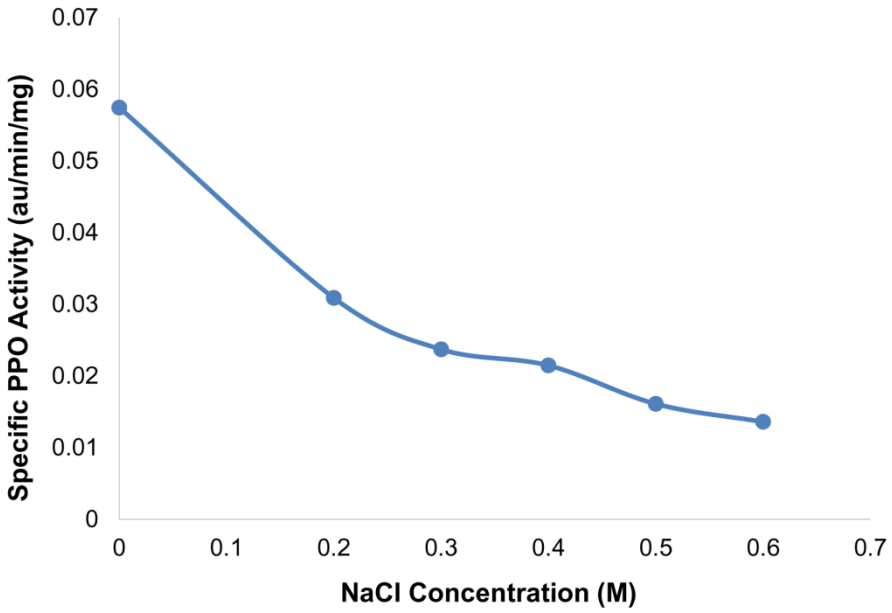

Figure 6. The effect of NaCl concentration on PPO specific activity.

The inhibition curve of the Polyphenol Oxidase reaction in the presence of NaCl was generated by plotting the specific enzymatic activity over the concentration of the inhibitor, NaCl (Figure 5). The specific PPO activity gradually decreased with higher concentrations of NaCl. Based on the inhibition curve, the K_i (the inhibition constant) of NaCl, which is defined as the concentration of NaCl causing 50% inhibition of the enzyme, is estimated to be around 0.2 M NaCl.

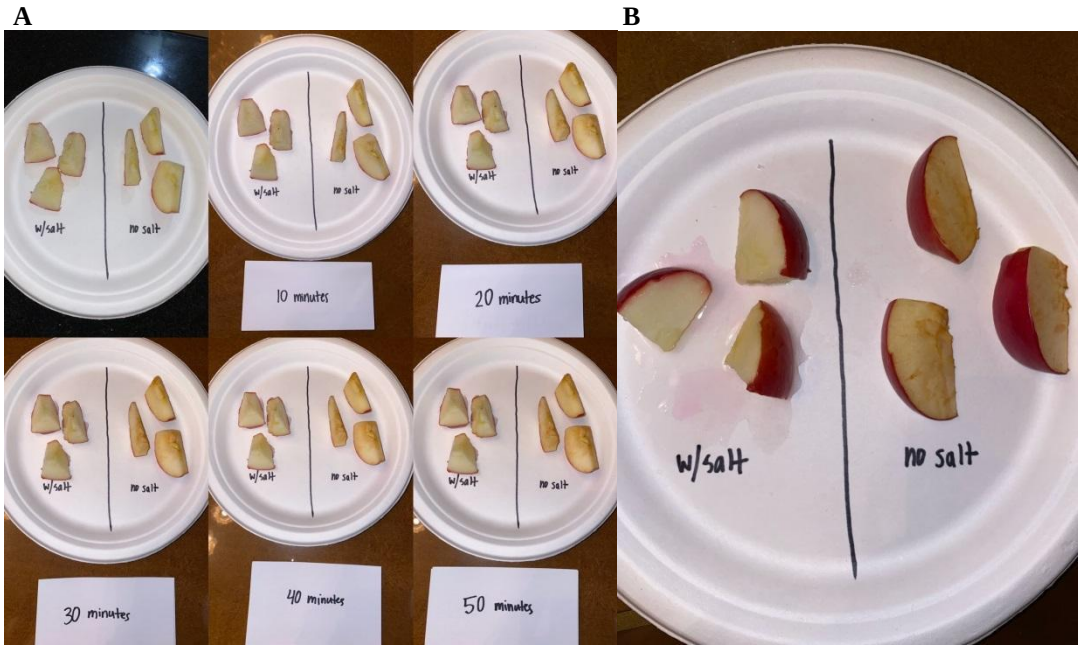

Figure 7. a) Basic kitchen experiment using NaCl to prevent the oxidation of apple slices. b) A close-up picture to provide a clearer view of the result.

To relate the results of this experiment to real life applications, a self-designed kitchen experiment was performed to demonstrate the effect NaCl has on PPO. One of the most common types of food containing this enzyme are apples. Therefore, two groups of apples were tested to show the effect of NaCl inhibition on the browning of damaged plant tissue (Figure 6A). After 50 minutes, the group of apple slices that did not have NaCl added was significantly browned, while the group with NaCl had little to no browning (Figure 6B).

4. Discussion

In Figure 3, it appears that as the concentration of substrate increases, so does the enzymatic activity of PPO for solutions with and without NaCl. This increase is not constant: as the slope decreases, the substrate concentration increases. As the proportion of enzymes to substrate decreases, the substrate molecules have a lower number of unoccupied enzymes to react with[11,12].

The solution containing NaCl has a lower rate of increase in comparison to the solution without NaCl. This suggests that NaCl is directly affecting the enzymatic activity of PPO. Competitive inhibition could be the mechanism bringing about this observation[13].

During competitive inhibition, inhibitors prevent reactions from taking place by binding to the active site of enzymes[14]. This type of inhibition can be overcome by increasing the concentration of substrate, thus increasing the likelihood that the substrate will come into contact with the enzyme[13,14,15]. The Michaelis-Menten model of enzyme kinetics indicates that the V_{max} value stays the same in the presence and absence of a competitive inhibitor. However, the Michaelis-Menten constant (K_m) increases in the presence of a competitive inhibitor[13,14,15]. V_{max} and K_m values are unique factors used to identify competitive inhibitors.

In Figure 4, the Lineweaver-Burk plot displays two trendlines that share a similar y-intercept. This indicates that the V_{max} value is approximately the same in the presence of NaCl and in the absence of NaCl. Since the presence of NaCl did not affect the V_{max} value and increased the K_m value, it appears that NaCl is a competitive inhibitor of PPO.

The data from this report supports the notion that NaCl can be used to slow down the browning of fruits and vegetables.

The question remains of the role of sodium and chloride ions in competitive inhibition. Sodium chlorite and sodium metabisulfite exhibit PPO inhibition[16, 17].This may make one conclude that the cation(Na+) plays the only role in PPO inhibition. However, other research elucidates that the “degree and type” of inhibition is anion dependent[18]. It appears that both the cation and anion play a major role, where the anion determines the mechanism and strength of inhibition.

Without a future control study where salts such as KCl and NaBr are used, however, one cannot fully understand the effect of each ion in NaCl.

A potential drawback of using NaCl to prevent browning is that it requires a higher concentration to reach the same level of inhibition as other known inhibitors[19]. Although NaCl is ubiquitous and affordable, it may affect the flavor of the food in an undesirable way.

A potentially beneficial future experiment is observing NaCl's inhibitory effect on other copper containing enzymes such as ascorbic acid oxidase. In doing so, one may be able to discover if NaCl is interacting with the dicopper center in PPO. Other methods for preventing melanin formation should also be explored for their commercial application. Other methods include deactivating PPO via denaturation or eliminating one of the naturally occurring substrates like polyphenols or molecular oxygen[20]. Because of the genetic variability of PPO, different methods may be more effective at browning inhibition in different plants.

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