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Influence of Temperature and Glycine/Cysteine Inhibition on Cresolase Activity in Immature and Mature *Solanum lycopersicum* Extracts

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A B S T R A C T

Objective: Polyphenol oxidase is an enzyme involved in phenolic substrate hydroxylation (cresolase) and oxidation (catecholase), processes critical for enzymatic browning in stored fruits and vegetables. Its activity depends on the type of substrate. PPO also generates reactive oxygen species and quinones, which can diminish the nutritional quality of proteins

Objective: The research focuses on analyzing cresolase activity of extracted from *Solanum lycopersicum* at immature and mature stages. The study evaluates optimal conditions such as pH, temperature, and substrate concentration, as well as glycine and cysteine as inhibitor.

Methods: Tomatoes from Kurdistan, at immature and mature stages, were processed to obtain extracts. Cresolase was evaluated across pH levels. Protein concentrations were quantified via the Bradford method, and kinetic parameters were calculated using varying p-cresol concentrations. Inhibitory effects of glycine and cysteine were assessed, and catecholase thermal stability under immature conditions was tested at different temperatures. Nonlinear regression models, supported by SAS and GraphPad Prism software, were applied to analyze and optimize enzymatic activity trends.

Results: The study identified pH 6.7 as optimal in both immature and mature samples. At 0.16 mM p-cresol concentration, Vmax was 0.15 units/mg protein, Km was 0.11 mM, and catalytic efficiency reached 1.37 units/mg protein/mM. Glycine exhibited stronger inhibition than cysteine, with IC50 values of 1.7 mM in mature and 0.4 mM in immature samples. Enzyme activity peaked at 45 minutes with inhibitors and 40 minutes without inhibitors. Glycine-treated samples exhibited slower declines in activity than cysteine-treated ones. Optimal performance was at 45°C without inhibitors and 40°C with inhibitors, while higher temperatures caused activity loss due to structural stress.

Conclusion: Cresolase activity varies depending on pH, temperature, and inhibitors. Studies on kinetics and mathematical models can contribute to the development of strategies for controlling browning in food products and designing protective mechanisms for plants.

Keywords: Kinetics, Glycine, Cysteine, Cresolase, *Solanum lycopersicum*

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Introduction

Polyphenol oxidase (PPO) plays a crucial protective role in plants, fungi, and animals, catalyzing two key biochemical reactions: cresolase activity (monophenol oxidation) and catecholase activity (diphenol oxidation). Catecholase facilitates post-harvest enzymatic browning by converting catechols into quinones, forming pigments in damaged fruits and vegetables. Cresolase, however, initiates melanin biosynthesis and aids in plant defense by converting monophenols (e.g., L-tyrosine) into diphenols, which are further processed by catecholase. Together, these activities highlight PPO's functional diversity, influencing pigmentation, cellular regulation, and stress responses in biological systems [1]. These

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enzymatic activities position PPO as a key player in the defense mechanisms of plants. The substrates for cresolase and catecholase activities, p-cresol and catechol respectively, are plant-derived precursors. In plants, cresolase activity is generally slower compared to catecholase reactions [2]. PPO plays a crucial role in plant defense, particularly in the Solanaceae family, where it helps form melanin-based protective barriers against herbivores and microbial infections [3]. These barriers act as both physical and chemical shields, preventing invaders. Additionally, PPO regulates leaf curling responses in tomatoes and potatoes, aiding in stress adaptation. In fungi, such as Agaricus bisporus, PPO generates reactive quinones, reinforcing structural resistance against biotic threats. Its diverse protective functions underscore its significance in biotic and abiotic stress responses across species. Furthermore, PPO is implicated in regulatory functions, such as tissue-specific upregulation in apples [1], the induction of curling responses in potatoes and the activation of leaf curling in tomatoes [4]. PPO generates reactive oxygen species (ROS) and quinones, which interact with proteins and nucleic acids of invaders, neutralizing threats and enhancing plant defense mechanisms. Research from the 1990s showed that tomato PPOs are induced by systemin and jasmonate, signaling responses against herbivores. Studies with transgenic plants further confirmed PPO's role in resistance to insect herbivores and pathogens, highlighting its importance in plant protection [5]. Cresolase activity in polyphenol oxidase (PPO) is strongly influenced by substrate type, temperature, and pH, with monophenolic substrates exhibiting enhanced oxidation efficiency. PPO reaches peak activity at 40–45°C, but excessive heat can cause structural damage and enzymatic loss. While PPO poses challenges in the food industry, such as enzymatic browning, its role in bioprotection and pest resistance highlights its biological significance. A deeper understanding of PPO regulation can lead to improved plant defense mechanisms and industrial applications. The cresolase activity rates depend on the specific monophenolic substrate and are ranked as follows: para-hydroxyphenylacetic acid < tyramine < phenol < L-tyrosine. This order correlates with the oxidation efficiency of monophenols, which is enhanced by electron-donating side chains on the benzene ring [6]. Notably, key precursors such as tyrosine and para-aminophenol in mushrooms are synthesized via the shikimate pathway [7]. The shikimate pathway is a fundamental biosynthetic route in plants, fungi, and microorganisms, producing phenolic compounds and aromatic amino acids like phenylalanine and tyrosine, which serve as precursors for flavonoids, alkaloids, and lignin. In polyphenol oxidase (PPO) metabolism, tyrosine undergoes cresolase activity, leading to the formation of melanin and reactive quinones, strengthening plant defense mechanisms against herbivores, pathogens, and environmental stressors. This pathway is essential for structural integrity, pigmentation, and biochemical resilience in diverse biological systems [8]. PPO activity can also generate reactive oxygen species (ROS) [2] and reactive o-quinones that form covalent bonds with free amino and thiol groups. These reactions reduce the nutritional value of dietary proteins [9,10]. Polyphenol oxidase causes browning in agricultural products, but high hydrostatic pressure, ultrasound, and high-pressure CO_2 help reduce its activity and maintain food quality [11]. Polyphenol oxidase plays a crucial role in plant

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defense by producing reactive oxygen species (ROS) and quinones, reinforcing protective barriers against herbivores and pathogens. However, its involvement in post-harvest enzymatic browning affects food quality and nutrition. Advancing research on PPO regulation and non-thermal mitigation strategies is key to promoting agricultural sustainability and overcoming industrial challenges.

This study aimed to evaluate the cresolase activity of polyphenol oxidase (PPO) in Solanum lycopersicum under immature and mature conditions. It focused on determining optimal pH, temperature, and substrate concentration while assessing the inhibitory effects of glycine and cysteine. Additionally, the research analyzed kinetic profiles, catalytic efficiency, and sensitivity under standard and inhibitory conditions, offering valuable insights into PPO stability and functionality in biochemical and environmental applications.

Materials and Methods

This study was conducted in the research laboratory of Payame Noor University, Saqqez. Locally grown garden tomatoes (Solanum Lycopersicum) from Kurdistan Province, harvested in both immature and mature stages, were purchased and processed. After washing and removing excess parts, tomato tissues were disinfected in a 2% benomyl solution to prevent fungal activity. Extracts were prepared by homogenizing tomato tissues in 0.1 M phosphate buffer (pH 7) containing 0.02% phenylmethylsulfonyl fluoride (a protease inhibitor). Homogenates were centrifuged twice (10 min at 3000 × g, followed by 30 min at 15000 × g), and the supernatant was used as the crude extract for further enzymatic assays. To determine the optimal pH for cresolase activity, enzyme activity was measured across a pH range of 3 to 10 using p-cresol (0.14 mM) as the substrate. The initial reaction rates were recorded while maintaining constant substrate and extract concentrations, varying only the buffer pH. Protein concentrations were quantified using the Bradford method with Coomassie Brilliant Blue G250, measuring absorbance at 595 nm and referencing a bovine serum albumin standard curve. All assays were conducted at laboratory temperature, 27°C. During the homogenization process, the procedure was carried out at the same laboratory temperature. The homogenates were then stored at −20°C to ensure their stability for further analysis. To calculate Km and Vmax, initial reaction rates were assessed at the optimal pH of 6.7 using p-cresol concentrations ranging from 0 to 0.14 mM. Experiments included enzyme assays with varying concentrations of glycine (0–2.4 mM) and cysteine (0–2.4 mM) under both immature and mature conditions. Absorbance was recorded at 400 nm to quantify cresolase activity, reflecting the transformation of p-cresol into 4-methylbenzoquinone, which exhibits an extinction coefficient of 1433 $M^{-1}cm^{-1}$ (Santos et al., 2024). The enzymatic activity was calculated by measuring the increase in absorbance of quinone products at their respective wavelengths. The reaction rate was determined from the slope of the tangent to the curve of absorbance increase over time, focusing only on initial rates. Using the extinction coefficients of each substrate used in the reaction, the substrate consumption by the enzyme per unit time was determined. One unit of enzymatic activity is defined as the amount of enzyme that produces one micromole of quinone products per minute.

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(µmol/min/mg protein = unit/mg protein). In the experiment investigating the kinetics of polyphenol oxidase (PPO) and the effects of glycine and cysteine on its activity, one assay was conducted in the absence of cysteine and glycine, while the remaining assays included these compounds for comparison. The cuvettes of the spectrophotometer, containing the substrate p-cresol and the inhibitors cysteine and glycine, exhibited no increase in absorbance at 400 nm under their respective conditions, which was considered the negative control. Upon adding the homogenate containing active polyphenol oxidase with cresolase activity in the absence of cysteine and glycine, cresolase activity was observed, indicated by an increase in absorbance at 400 nm, serving as the positive control. All other assays with varying concentrations of cysteine and glycine were evaluated relative to these controls.

To evaluate the effect of temperature on the stability of catecholase activity of Solanum lycopersicum extract under immature conditions, enzymatic activity was measured after treatment at various temperatures in both the absence and presence of glycine and cysteine. Measurements were carried out using a constant concentration of P-cresol as the substrate. For each assay, 100 µL of extract was incubated at the target temperature for 10 minutes, followed by a 5-minute incubation on ice. Enzymatic activity was subsequently measured, and initial reaction rates were plotted against temperature changes, expressed as units per milligram of protein relative to the control. To ensure the accuracy and reliability of the study, each assay was conducted in triplicate. This methodological approach enhances the robustness of the data and provides greater confidence in the computational analyses performed.

Analytical Methods: This study utilized nonlinear regression analysis to identify the optimal response levels of PPO activity to various treatments. The regression models employed included

Piecewise Model : This model is characterized by two slopes (slope1 and slope2) on either side of the breakpoint (x_0), which represents the maximum numeric value of y. Parameters include the intercept (a), slope1 (positive), and slope2 (negative). The function is given as:

if $x < x_0$

$y = a + slope_1 x_0$

if $x > x_0$ then $y = a + slope_1 x_0 + slope_2 (x - x_0)$

2. Single-Phase Exponential Decay Model: This model describes scenarios where enzyme activity decreases with time or increased levels of a stimulant. Changes in y begin rapidly but slow down as y approaches a minimum value (NS). The function is given as:

$$Y = (Y_0 - NS) \times \exp(-k \times X) + NS$$

Here, Y_0 represents the maximum value of y, NS is the minimum value, and k is the decay slope [12].

3. Plateau Exponential-Decay Model: Similar to single-phase decay, this model accounts for rapid initial decreases followed by stabilization near a minimum (plateau). The function is:

Y= IF(X<X0, Y0)

$$Y = Pleatue + (Y_{max} - Pleatue) \times \exp(-k \times (X - X_0)))$$

Parameters include Plateau (minimum value), Ymax (maximum value), k (decay slope), and X_0 (breakpoint).

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4. Logistic Model: This model represents linear reductions in enzyme responses to varying treatment levels. It is defined as:

$$Y = Y_m \times {Y_o}/{((Y_m - Y_o) \times \exp(-k \times x) + Y_o)}$$

Here, Y_0 is the minimum value, Ym is the maximum value, k is the growth rate (inverse of X), and X_0 is the breakpoint from increasing to decreasing response rates [12].

5. Beta-Growth Model: A flexible sigmoidal function used to measure enzyme growth or response levels, defined as:

$$Y = Y_m \times (1 + ({T_e - X}/{(T_e - T_m)})) \times ({X}/{T_e})^{(\frac{T_e}{T_e - T_m})}$$

Parameters include Ym (maximum response), Te (time or value of X at maximum), and Tm (X value at the model's breakpoint. Nonlinear regression fitting was performed using SAS software (Proc nlin) and iterative optimization methods. GraphPad Prism software [12] was employed for parameter estimation, adjusting initial values until the best fit was achieved. These models provide precise insights into enzyme behavior under varying conditions.

Results

Optimal pH Determination of Cresolase Activity in Immature and Mature Conditions

To determine the optimal pH for cresolase activity, enzyme activity was assessed across a pH range of 2 to 10 under both immature and mature conditions, using p-cresol as the substrate. The findings revealed that the enzyme exhibited cresolase activity within the acidic pH range of 2 to 5, but lacked activity at high alkaline pH levels in both immature and mature states. As exhibited in Figure 1, the activity profile demonstrated three distinct peaks: pH 4, 6.7, and 8 for the immature state, and pH 4, 6.7, and 7.5 for the mature state. The optimal pH for cresolase activity was consistent at pH 6.7 for both conditions, with minor differences observed between the first and third peaks in immature and mature states. Subsequent enzymatic activity assays were conducted using this optimal pH of 6.7.

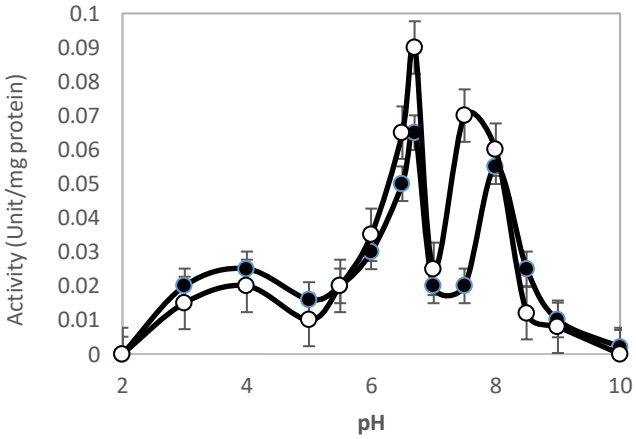


Figure 1: Determination of optimal pH for cresolase activity in Solanum lycopersicum under immature and mature conditions using p-cresol as the substrate.

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Kinetic Parameters for Cresolase Activity (C.I.S) Under Immature Conditions

Figure 2 illustrates the Michaelis-Menten curve for C.I.S at different p-cresol concentrations. The data exhibited a gradual increase in the initial reaction rate as p-cresol concentrations from 0 to 0.2 mM. At 0.16 mM p-cresol, the enzyme reached its maximum efficiency in catalyzing cresolase reactions. Vmax was determined to be 0.15 units/mg protein, while the Km was calculated as 0.11 mM. The catalytic efficiency was estimated at 1.37 units/mg protein/mM. Similarly, kinetic parameters was determined in the presence of glycine (C.I.S-G). The activity profile exhibited sigmoidal kinetics; initial increases in enzyme activity were slow and gradual between 0.05 and 0.1 mM p-cresol, followed by a sharp rise between 0.1 and 0.14 mM (table 1). Additional kinetic parameters for cresolase activity were calculated under various conditions, including C.I.S-C (presence of cysteine) as well as Cr.MS, Cr.MS-G, and Cr.MS-C (mature). The results are presented in Table 1.

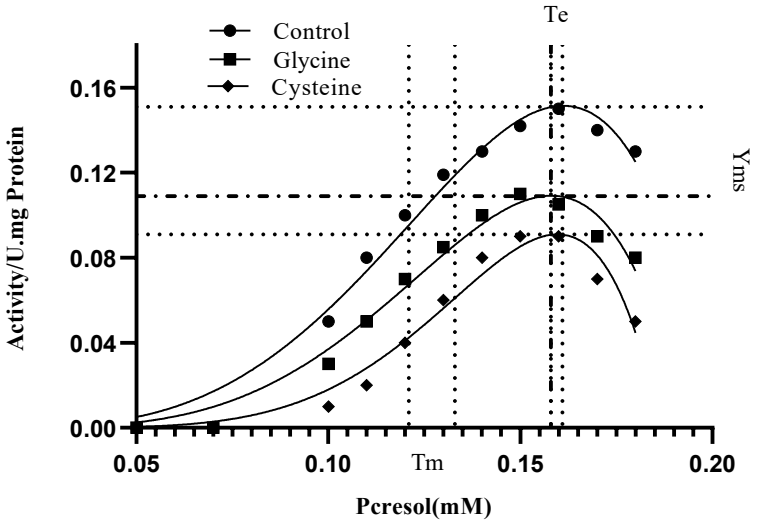


Figure 2: Determination of kinetic parameters for cresolase activity under immature conditions. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

Quantitative analysis showed that cresolase activity followed a beta-growth model, with higher maximum activity in both immature and mature extracts without inhibitors. Sensitivity to p-cresol was reduced in the presence of glycine and cysteine, with glycine exhibiting a stronger effect than cysteine. The Tm breakpoint occurred at higher p-cresol concentrations under control conditions, while enzyme response accelerated with glycine and cysteine, leading to faster inhibition as substrate concentrations increased. (Table 1).

Table 1 summarizes the coefficients of the beta-growth model applied to cresolase activity at varying p-cresol levels with or without cysteine and glycine, in both immature and mature.

	Tm	Ym	R2	Treatment
Premature				
0.151	0.161	0.121	98.2	Control

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0.109	0.158	0.123	97.7	Glycine
0.091	0.158	0.133	96.7	Cysteine
Mature				
0.160	0.129	0.192	99	Control
0.155	0.114	0.129	97.2	Glycine
0.147	0.105	0.086	94.4	Cysteine

Kinetic Parameters for Cresolase Activity (C.I.S-G and C.M.S-G) Under Immature and Mature Conditions
To assess C.I.S-G and C.M.S-G behavior, enzymatic activity were measured in the presence of varying glycine concentrations. A gradual decline in cresolase activity was observed in presence of Gly. This reduction began with increasing glycine concentrations, where cresolase activity in mature conditions reached a plateau at 1.6–2 mM glycine, while under immature conditions, the plateau was reached at 1.1–2 mM glycine (Figure 3).

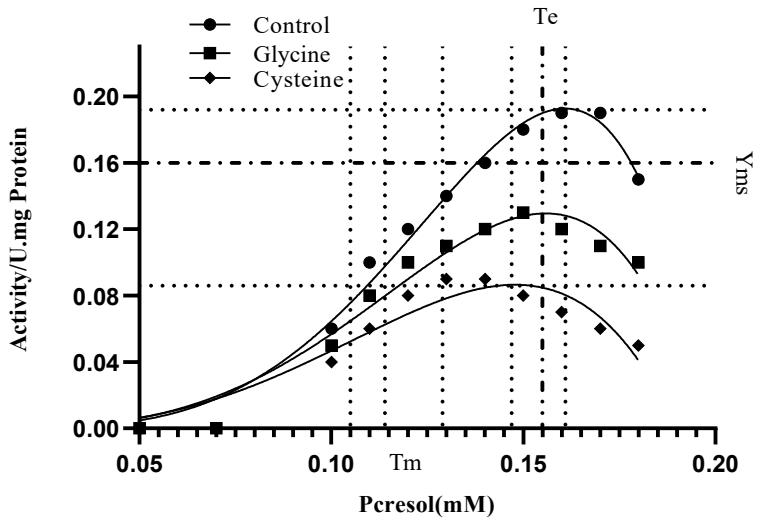


Figure 3: Determination of kinetic parameters for cresolase activity under mature conditions. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

Beyond these concentrations, no significant changes in activity were detected for either extract. At 1.7 mM glycine, cresolase activity in mature extracts was reduced to 50% of its initial level, while under immature conditions, a 50% reduction occurred at 0.4 mM glycine. The results indicated that cresolase activity in immature and mature extracts followed a single-phase exponential decay model (Table 2) as glycine and cysteine concentrations increased.

Table 2: Summary of kinetic parameters for cresolase activity in the presence of p-cresol substrates in immature and mature Solanum lycopersicum extracts.

Mature Solanum.L			Prematur Solanum L.			Presence	
Catalytic Efficincy	Km (mM)	Vmax U/mg. p	Catalytic Efficincy	Km (mM)	Vmax U/mg. p		

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U/mg.P. mM-1			U/mg.P. mM-1				
1.7	0.11	0.19	1.37	0.11	0.15	----	
1.2	0.11	0.13	1	0.11	0.11	Gly	P-cresol
0.9	0.11	0.09	0.9	0.11	0.09	Cys	

The rate of activity decline was significantly steeper in immature extracts than in mature ones. The shorter enzyme half-life (T1/2) in immature extracts supported this observation, indicating greater cresolase sensitivity to cysteine in immature extracts compared to mature ones. Cresolase activity varied more extensively in mature extracts, requiring higher cysteine concentrations to reach minimal levels, whereas immature extracts exhibited greater sensitivity at lower concentrations. Glycine inhibition was more pronounced in immature extracts, with faster activity decline. Enzyme half-life analysis confirmed these trends, and the single-phase exponential decay model, supported by high R^2 values, effectively described cresolase activity dynamics under varying inhibitor concentrations. (Table 3,4,5).

Table 3: Coefficients of the single-phase exponential decay model applied to changes in cresolase activity at varying glycine and cysteine levels in immature and mature s.

treatment	Yo	NS	K	T1/2	R2
		Cysteine			
Premature	99.01	24.5	3.41	0.203	99.8
Ripe	99.52	37.71	0.84	0.826	99.3
		Glycine			
Premature	99.92	6.36	5.23	0.132	99.9
Ripe	98.03	41.92	2.42	0.31	98.4

Table 4: Summary of kinetic parameters for IC50 determination of cresolase activity in the presence of p-cresol substrate in immature and mature.

IC50(mM)	Presence	Activity	Solanum.L
1.7	Gly	Cresolase	Mature
1.1	Cys		
0.4	Gly		Premature
0.15	Cys		

Table 5: Summary of kinetic parameters for IC50 determination of cresolase activity in the presence of p-cresol substrate in immature and mature.

Mature			Premature			Inhibitor	Activity	Substrate
Inhibition	Km	Vmax	Inhibition	Km	Vmax			
Non Competetive	Fixed	↘	Non Competetive	Fixed	↘	Gly	Cresolase	P-cresol
Non Competetive	Fixed	↘	Non Competetive	Fixed	↘	Cys		

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Effect of Temperature on the Stability and Cresolase Activity

The findings show that cresolase activity in immature Solanum lycopersicum extracts peaked at 45°C, while in the presence of cysteine and glycine, maximum activity was observed at 40°C. Under 40–45°C with 10-minute incubation, activity reached 0.24 units/mg protein without inhibitors, 0.16 units/mg with glycine, and 0.13 units/mg with cysteine, highlighting the regulatory effects of these inhibitors on enzymatic function (Figure 4,5).

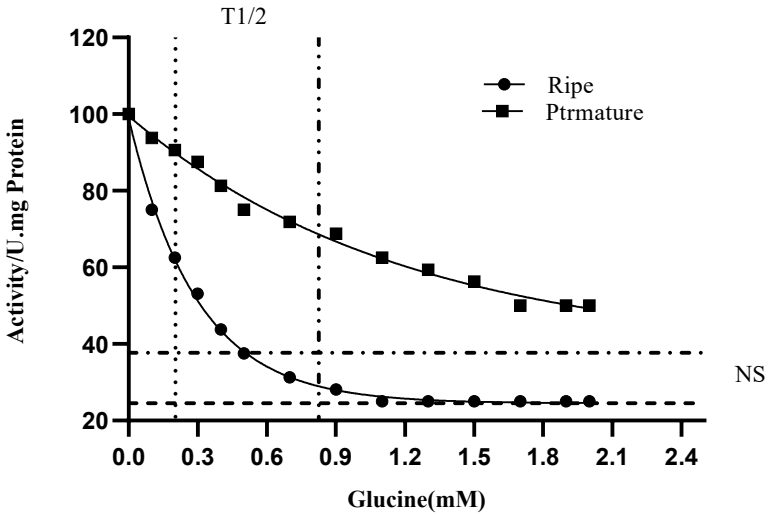


Figure 4: Determination of kinetic parameters for cresolase activity under immature (●) and mature (■) conditions, with fixed p-cresol concentration and varying glycine levels.

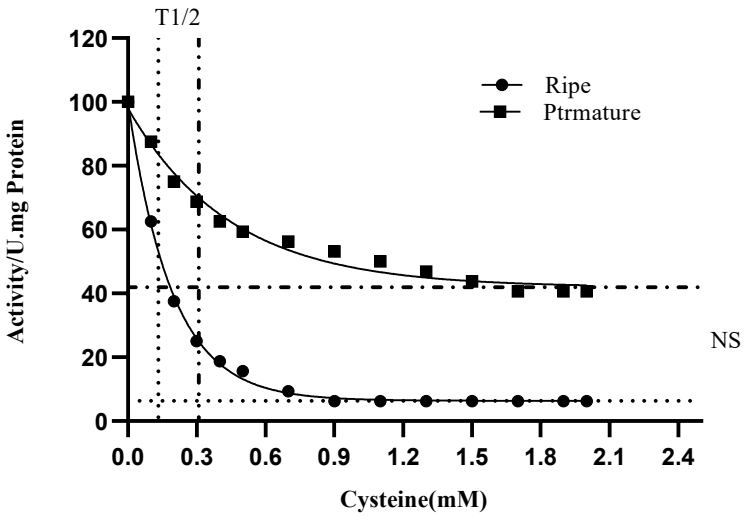


Figure 5: Determination of kinetic parameters for cresolase activity under immature (●) and mature (■) conditions, with fixed p-cresol concentration and varying cysteine levels.

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This demonstrates a 90% increase in cresolase activity at 45°C in the absence of inhibitors compared to room temperature, confirming the activating effect of 45°C on cresolase activity. In the presence of glycine and cysteine, cresolase activity at 40°C increased by 30% and 40%, respectively, confirming the activating effect of temperatures ranging from 40–45°C on cresolase activity (Figure 6, 7).

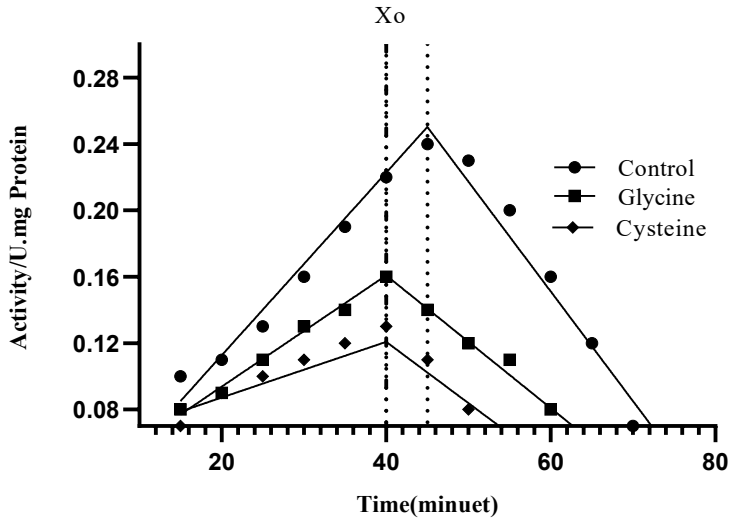


Figure 6: The effect of temperature on cresolase activity under immature conditions. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

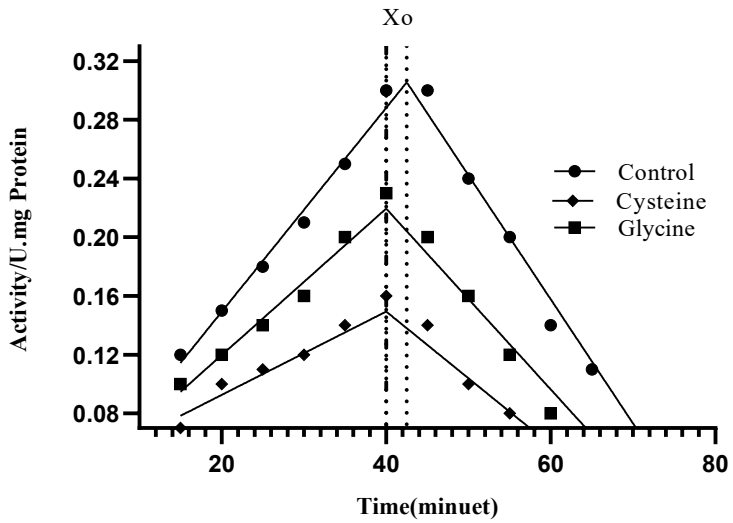


Figure 7: The effect of temperature on cresolase activity under mature conditions of p-cresol. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

Elevating the temperature beyond 40°C resulted in reduced enzyme activity, with significant declines observed at 70–80°C. Incubation at these higher temperatures led to structural alterations in the enzyme, reducing activity by 70–100%. Thus, the optimal temperature for cresolase activity under immature conditions was 45°C without inhibitors, while in the presence of cysteine and glycine, it was 40°C (Table 3). Nonlinear regression analysis revealed that cresolase activity followed a

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piecewise model in both immature and mature Solanum lycopersicum extracts, regardless of cysteine or glycine presence. In immature extracts, activity steadily increased for 40 minutes, showing greater sensitivity to glycine than cysteine. In mature extracts, peak activity occurred at 40 minutes with inhibitors, slightly delayed to 42.5 minutes in control conditions. Post-breakpoint decline was more pronounced without inhibitors, highlighting their role in stabilizing enzyme activity over time. (Table 6).

Table 6: Coefficients of the piecewise model applied to changes in cresolase activity at varying p-cresol levels with or without cysteine and glycine in immature and mature

treatment	intercept	slope1	slope2	X0	R2
			Premature		
Control	0.0024	0.0055	-0.0066	45	98
Glycine	0.0266	0.0035	-0.0039	40	99.5
Cysteine	0.053	0.0016	-0.0036	40	93
			Ripe		
Control	0.0083	0.007	-0.0084	42.32	98.7
Glycine	0.0198	0.0049	-0.0061	40	97.6
Cysteine	0.0358	0.0028	-0.0045	40	93

Evaluation of Time Effects on Stability and Cresolase Activity Under Mature and Immature Conditions

According to Figures 8 and 9, the C.I.S reached its peak after 45 minutes of enzyme incubation at 40°C, exhibiting a 381% increase in the absence of cysteine and glycine, and increases of 293% and 223% in the presence of glycine and cysteine, respectively. Cresolase activity reached its maximum at 40°C following 45 minutes of incubation under immature conditions. Further treatment beyond 40 to 60 minutes did not result in additional significant increases in catecholase activity. Quantitative analysis of the effect of time on activity and stability of C.I.S and C.M.S exhibited that enzyme responses followed a logistic growth model in all three states (absence or presence of glycine and cysteine) (Figures 8 and 9). Under immature conditions, cresolase activity shifted from linear to exponential growth earlier without glycine or cysteine, whereas their presence delayed this transition. Following this phase, enzyme activity declined after peaking at 40 minutes, dropping significantly afterward. In the presence of glycine and cysteine, peak activity occurred at 45 minutes. The logistic model confirmed that maximum activity was higher with glycine than cysteine in both mature and immature extracts, indicating great er enzyme sensitivity to glycine. (Table 7).

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Table 7: Coefficients of the logistic model applied to changes in cresolase activity over time in the presence and absence of cysteine and glycine under mature and immature conditions

Treatment	Ym	Yo	K	Xint	R2
		Premature			
Control	417.7	104.1	0.0716	13.96	99.3
Glycine	337.3	90.95	0.054	18.23	98.4
Cysteine	271.9	92.15	0.043	22.89	98.3
		Ripe			
Control	343.5	101.7	0.076	13.1	99.7
Glycine	226.5	98.4	0.042	23.53	99.4
Cysteine	186.1	104.6	0.033	30.18	99

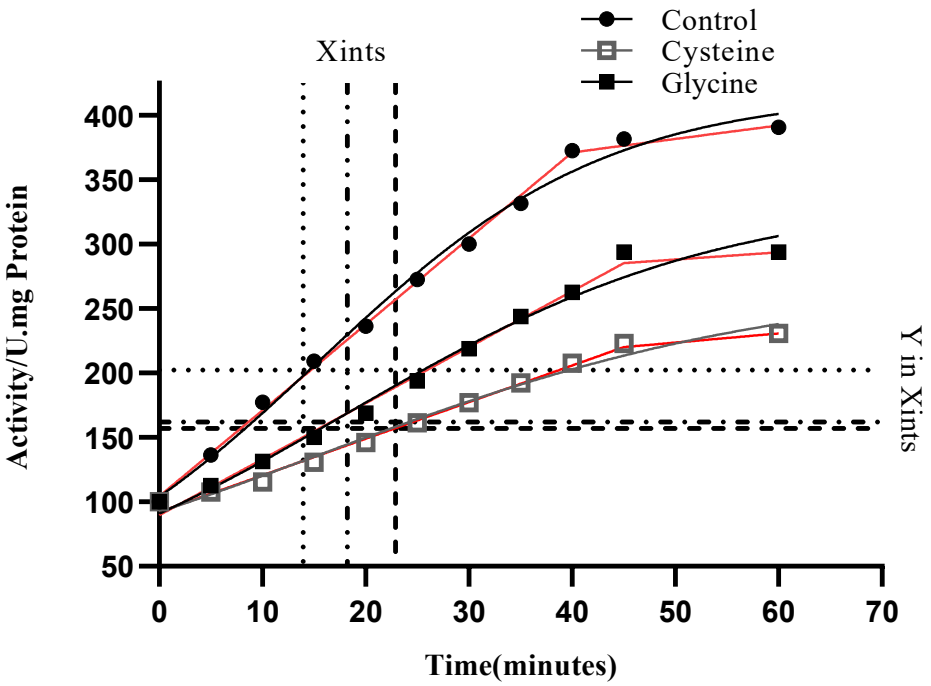


Figure 8: Effect of time on cresolase activity under immature conditions at the optimal temperature. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

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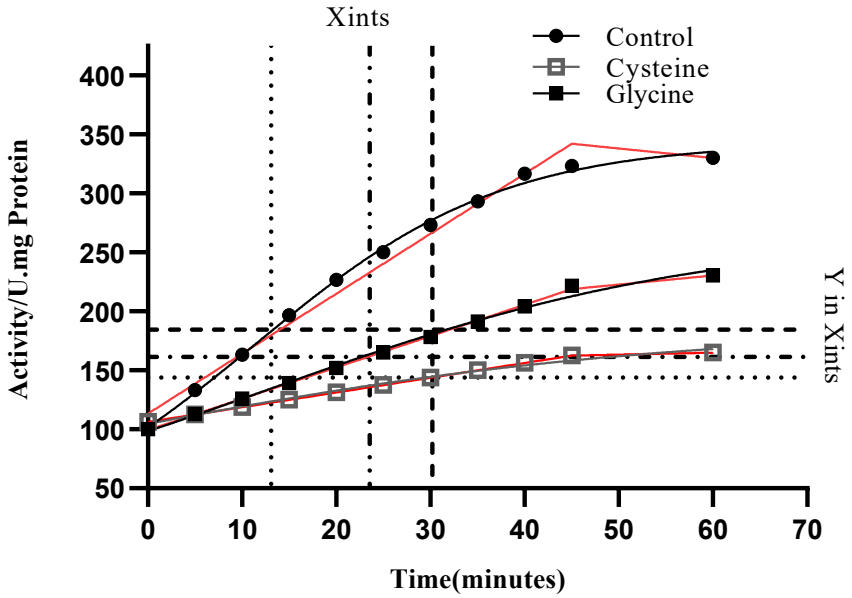


Figure 9: Effect of time on and cresolase activity under mature conditions at the optimal temperature. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

Evaluation of Time Effects on Stability and Cresolase Activity at 50°C

Figure 10 and 11 illustrates C.I.S extract after time-based incubation at 50°C in the presence of p-cresol. The results exhibited that C.I.S peaked after 25 minutes of enzyme treatment at 50°C, with a 172% increase in the absence of inhibitors. Activity in the presence of glycine and cysteine peaked after 20 minutes of enzyme treatment at 50°C, exhibiting increases of 68% and 30%, respectively.

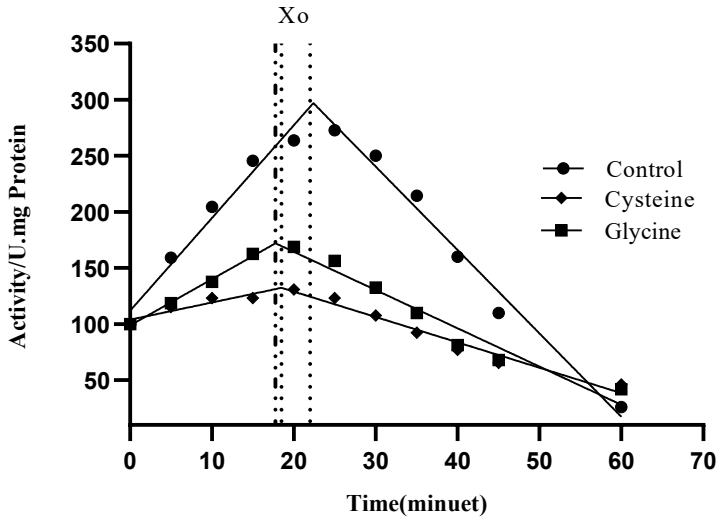


Figure 10: Effect of time on cresolase activity under immature conditions at the optimal temperature. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

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Extending the treatment time beyond 20–25 minutes to 60 minutes resulted in a decline in enzyme activity. At 50°C, enzyme activity varied significantly, with 10-minute treatments exhibiting cresolase activities of 104%, 37%, and 23% for control conditions, glycine presence, and cysteine presence, respectively. However, treatments exceeding 45 minutes led to gradual reductions in cresolase activity compared to control conditions. After 60 minutes at 50°C, cresolase activity decreased by 50% and 55% in the presence of glycine and cysteine, respectively, although enzymatic activity was retained. Nonlinear regression analysis of the effect of time on C.I.S demonstrated that enzyme responses followed a piecewise model in all three states (absence of cysteine and glycine, and presence of each inhibitor) (Figure 10).

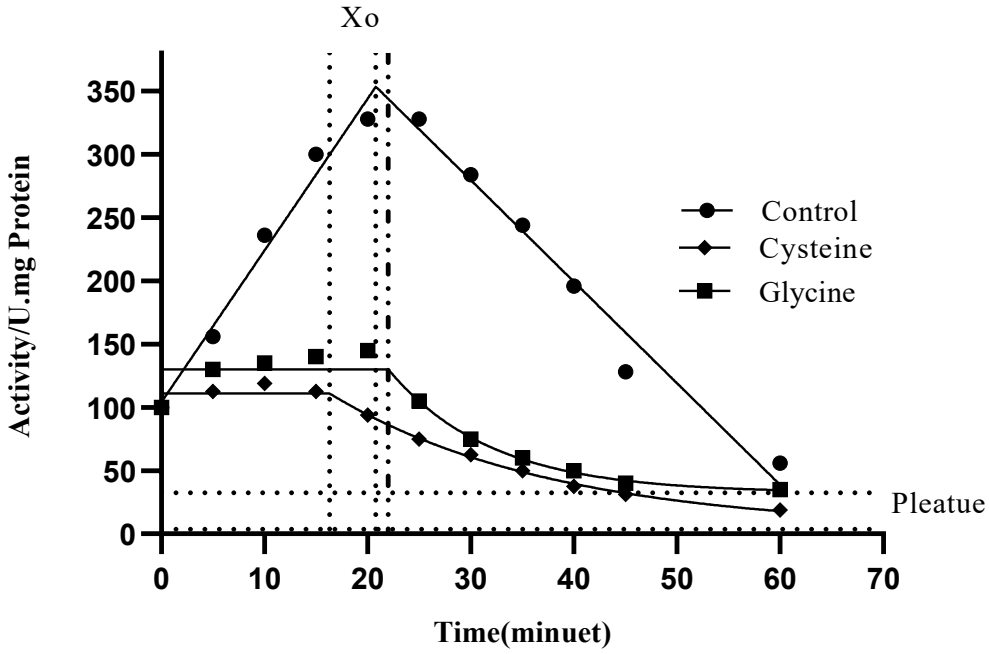


Figure 11: Effect of time on cresolase activity under mature conditions at the optimal temperature. Absence of Cys and Gly (●), presence of Gly (■), presence of Cys (▲).

Under immature conditions, cresolase activity increased steadily until reaching its peak at 22.01 minutes, with a slope of 8.27 units/mg protein per time unit increase, before transitioning into a decline. Enzyme activity increased more rapidly in the presence of glycine than cysteine, highlighting the enzyme's greater sensitivity to glycine in the environment (Table 8,9). Peak cresolase activity was interpolated at 17.77 minutes with glycine and 18.5 minutes with cysteine. In mature Solanum lycopersicum extracts, enzyme activity followed a piecewise model under control conditions but adhered to a plateau exponential decay model with inhibitors. Maximum activity in control extracts occurred at 20.79 minutes, while enzyme stability persisted until 22 minutes with glycine and 16.31 minutes with cysteine. Post-breakpoint activity decline followed slopes of 0.1 mg protein per unit time for glycine and 0.046 mg protein per unit time for cysteine, reaching minimum values of 32.33 and 3.62 units/mg protein, respectively (Table 9).

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Table 8: Coefficients of the piecewise model applied to changes in cresolase activity over time in the presence and absence of cysteine and glycine under mature and immature conditions

Treatment	intercept	Xo	Slope1	Slope1	R2
		Premature			
Control	104.3	40	6.6	1.4	99.3
Glycine	89.66	45	4.34	0.56	98.4
Cysteine	92.17	45	2.84	0.70	98.3
		Ripe			
Control	113.2	45	5.08	-0.80	99.8
Glycine	99.37	45	2.65	0.76	99.4
Cysteine	106.3	45	1.2	0.16	99.4

Table 9: Coefficients of the piecewise and plateau exponential decay models applied to changes in cresolase activity at varying p-cresol levels in the presence and absence of cysteine and glycine under immature and mature conditions

Treatment	intercept	slope1		slope2	X0	R2	
Premature							
Control	111.8	8.27		-7.51	22.01	96.7	
Glycine	98.75	4.12		-3.42	17.77	96	
Cysteine	103.8	1.53		-2.26	18.5	97	
Ripe							
Control	104	12		-8.03	20.79	97.6	
	X0	Yo	Pleatue	halftime	k	Span	R2
Glycine	22	130	32.33	6.93	0.1	97.67	98
Cysteine	16.31	110.9	3.62	15.01	0.046	107.3	98.3

Discussion

In Solanum lycopersicum, cresolase activity is detectable in both immature and mature extracts, reflecting similarities to PPOs in other organisms [21]. The presence of p-cresol facilitates measurement, with optimal substrate pH differing between maturity stages. Activity profiles revealed three peaks under both conditions: at pH 4, 6.7, and 8 for immature, and pH 4, 6.7, and 7.5 for mature extracts. The optimal pH in both cases was 6.7, though minor variations were observed in the first and third peaks. Various studies confirm the findings of this research data in such a way that the optimum pH of PPO was 7.0 for African bush mango (Irvingia gabonensis) fruit peel [22], tomato (Solanum lycopersicum) [23], sweet potato (Ipomoea batatas L. Lam) [24], and Areca nut (Areca catechu L.) kernel [25], when catechol was served as substrate [13]. The optimum pHs of PPO in fennel (*Foeniculum vulgare* Mill.) seeds were 6.0, 5.0, 5.0, and 7.0 when catechol, 4-methylcatechol, 4-tertbutylcatechol, and pyrogallol were served as substrates, respectively [26], Similarly, different PPOs within the same tissue, like tea leaves, exhibit varying optimal pHs, such as 5.5

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and 6.0 [27]. While most PPOs exhibited a neutral optimum pH, some are acidic [28]. Factors like extraction methods and environmental conditions can also influence the optimal pH. This variability highlights the diversity and adaptability of PPOs across species and conditions [13]. The maximum cresolase velocity (Vmax) under immature conditions followed this trend: Vmax without glycine and cysteine > Vmax with glycine > Vmax with cysteine.

Values of Vmax were determined as 0.15 units/mg protein in the absence of inhibitors, 0.11 units/mg with glycine, and 0.09 units/mg with cysteine. In all cases, the Michaelis constant (Km) was approximately 0.11 mM. A similar trend was observed for mature extracts, with Vmax values of 0.19, 0.13, and 0.09 units/mg protein in the absence of inhibitors, in the presence of glycine, and the presence of cysteine, respectively. The catalytic efficiency of cresolase activity under immature conditions was calculated as 1.37, 1, and 0.9 units/mg protein/mM for no inhibitors, glycine, and cysteine, respectively. Under mature conditions, these values were 1.7, 1.2, and 0.9 units/mg protein/mM. This demonstrates that both glycine and cysteine reduce not only cresolase activity but also the enzyme's catalytic efficiency. The results presented in the study conducted by Everet et al. (2007) support the findings of the present research, as they indicate that these amino acids interfere with the enzyme's ability to catalyze reactions. This likely occurs through alterations in the enzyme's structure or its interaction with substrates, aligning with the conclusions of the current study. Such mechanisms can have a direct impact on enzyme activity, which may lead to significant implications in understanding their biochemical function [29]. These results align with findings for PPO extracted from Thermomicrobium roseum, which exhibited fourfold higher activity with pyrogallol compared to catechol [14]. A similar study conducted by Saeidian in 2013 also demonstrated that PPO in tomatoes exhibited significantly higher activity with pyrogallol compared to catechol [15]. pH modifications and SDS treatment enhanced PPO activity, supporting the study's findings. P-cresol activated latent PPO by inducing conformational changes, transitioning the enzyme to an active state with high catalytic efficiency. Cresolase activity followed a hyperbolic pattern, aligning with Michaelis-Menten kinetics, indicating strong substrate affinity during quinone production. Interestingly, cresolase kinetics in Solanum lycopersicum are consistent with the sigmoidal kinetics reported in saffron and lettuce [16], confirming that the kinetics observed in saffron and lettuce support the type of kinetics found in Solanum lycopersicum [17,18]. Sigmoidal kinetics in enzymatic activities indicate allosteric interactions, where substrate molecules or other factors bind to non-active (allosteric) sites of the enzyme, inducing structural changes [30]. These structural modifications can affect enzyme activity, with the binding of one substrate molecule potentially facilitating (positive cooperativity) or hindering (negative cooperativity) the binding of subsequent molecules. Such behavior manifests as a sigmoidal curve on the reaction rate versus substrate concentration graph, differing from the simple hyperbolic kinetics of Michaelis-Menten [30]. Sigmoidal kinetics are typically observed in multi-subunit enzymes, where each subunit interacts with the substrate, creating allosteric effects [31]. Since the pH profile under immature and mature conditions exhibited three peaks at three different pH values, it

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confirms that polyphenol oxidase in Solanum lycopersicum likely contains at least three distinct isoenzymes. Thus, sigmoidal kinetics can indicate the complexity of enzyme regulation in response to varying substrate concentrations, often crucial in metabolic pathways for precise biological control [31]. Meanwhile, studies on PPO in fruits like olives (Olea europaea) have exhibited hyperbolic kinetics during ripening, accompanied by notable increases in Vmax, Km, and catalytic efficiency [32]. These findings contradict the data obtained for Solanum lycopersicum, suggesting that polyphenol oxidase in olives is likely composed of a single chain [19]. Substrate inhibition of PPO activity was observed with p-cresol, supporting previous findings of irreversible enzyme deactivation due to the oxidation of phenolic substrates into quinone products [20]. Excessive quinone accumulation competes with substrates for active site binding, inhibiting the enzyme. Glycine and cysteine acted as noncompetitive inhibitors of cresolase activity in both immature and mature extracts, as Km values remained constant, while Vmax decreased. The IC50 values (inhibitor concentration reducing enzyme activity by 50%) differed significantly. For the p-cresol substrate, IC50 in the presence of glycine was 1.7 mM (mature) and 0.4 mM (immature), while IC50 with cysteine was 1.1 mM (mature) and 0.15 mM (immature). This ranked the sensitivity as follows:

Cre-Gly-mature < Cre-Cys-mature < Cre-Gly-immature < Cre-Cys-immature.

Temperature significantly influences cresolase activity, with optimal activity observed within the 40–45°C range depending on extract type and the presence of inhibitors. Under inhibitor-free conditions, maximum activity was noted at 45°C, while the addition of glycine and cysteine reduced the optimal temperature to 40°C. This trend is consistent with findings in other organisms where inhibitors often modify the structural dynamics of enzymatic active sites, leading to adjustments in activity thresholds [33]. At optimal temperatures, structural changes enhance substrate binding to the active site, promoting catalytic efficiency. However, excessive temperatures cause conformational damage to the enzyme, diminishing activity until complete deactivation occurs [34]. Temperature-dependent PPO activity is consistent across species, with fungi and bacteria showing structural susceptibility at higher temperatures. P-cresol is a reliable substrate, and further studies on amino acids, isoenzyme identification, and 3D structural analysis could enhance PPO kinetic understanding. In vivo research on pH and temperature effects would provide valuable insights into natural enzymatic responses.

Conclusion

Polyphenol oxidase (PPO) in *Solanum lycopersicum* exhibits cresolase activity, with optimal performance at pH 6.7 and 40–45°C. P-cresol activates the enzyme, while glycine and cysteine inhibit its activity, with cysteine being more effective. The enzyme's behavior under various conditions suggests its role in controlling enzymatic browning in food products and developing protective mechanisms in plants against environmental stress. Its kinetics, analyzed through Michaelis-Menten and other models, provide insights into intracellular biochemical reactions.

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Conflicts of interest: There are no conflicts of interest to declare regarding this study.

Ethical considerations: This study adhered to strict ethical guidelines, ensuring integrity, transparency, and responsibility in all phases. Experimental protocols were carefully designed to guarantee responsible resource use, and results were honestly analyzed and reported, aligning with scientific ethical standards.

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