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Insights into L-Ascorbate Oxidase Kinetics: A Green Zucchini Approach

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Abstract

L-Ascorbate oxidase (EC 1.10.3.3) is a vital enzyme found in various plant species, with cucurbits being a notable source, playing a crucial role in the oxidation of ascorbic acid to dehydroascorbic acid. The enzyme is of significant interest due to its essential role in producing ascorbic acid, a key product with wide-ranging applications in the agri-food and pharmaceutical industries. Given the importance of ascorbic acid in various domains, a deeper understanding of this enzyme is warranted. This study focuses on a thorough investigation of zucchini L-ascorbate oxidase, specifically from *Cucurbita pepo medullosa*. The methodology encompasses the extraction and purification of the enzyme, with a focus on maintaining structural integrity during these processes. The subsequent examination of kinetic parameters involved the determination of enzyme activity, substrate concentration, and reaction conditions. Our findings reveal a detailed characterization of L-ascorbate oxidase, with a particular emphasis on its structural composition. Spectroscopic properties of its copper atoms offer insights into the binding and reduction of substrates. The enzyme also contains carbohydrate residues, which contribute to its overall composition. These insights into the enzyme's structure provide a foundation for understanding its function. The research underscores the importance of L-ascorbate oxidase in the context of ascorbic acid metabolism and oxygen activation within biological systems. By exploring its structure, and kinetic properties, we contribute to a deeper comprehension of its functional significance. This understanding has implications for potential applications in diverse sectors, including agriculture, nutrition, and healthcare.

Key words: Ascorbate oxidase, ascorbic acid, metalloprotein, *Zucchini, Cucurbita pepo medullosa.*

Introduction

Plants represent a vast source of enzymes and molecules of interest that are widely used in various research sectors and biotechnologies. Most higher plants, particularly cucurbits, produce L-ascorbate oxidase (EC 1.10.3.3), a metalloenzyme capable of reversibly catalyzing the oxidation of ascorbic acid, a product extensively used in the agri-food and pharmaceutical industries, into dehydroascorbic acid, simultaneously reducing molecular oxygen to water. While L-ascorbate oxidase has been extensively characterized at the molecular level, its biological role is still poorly understood [1].

L-ascorbate oxidase is an enzyme belonging to the blue multi-copper oxidase group. It catalyzes the reduction of molecular oxygen to water by four electrons, with the simultaneous oxidation of the organic substrate by the loss of one electron (ascorbic acid to dehydroascorbic acid). It is isolated from most higher plants, with cucurbits being the most common source [2, 3].It is also produced by microorganisms[4].This enzyme extracted from *Cucurbita pepo Medullosa* is a glycoprotein consisting of two identical subunits of globular proteins with 552 amino acids and containing eight copper atoms[5, 6]. It contains 10% carbohydrates and amino acid composition analysis of the pure enzyme shows that it is an acidic protein, and has a high percentage of acidic aspartic and glutamic acid residues compared to histidine, lysine, and arginine residues [7].This work is a contribution to the study of the kinetic parameters of zucchini L-ascorbate oxidase (*Cucurbita pepo medullosa*), after its extraction and purification in the aim of exploring its functional significance and potential applications.

Materials and Methods

Purification of *Cucurbita pepo medullosa* L-ascorbate oxidase

Preparation of the crude extract of L-ascorbate oxidase

The method used to obtain the enzymatic preparation is a modification of a protocol conducted by Lee and Dawson (1973) [8]. Green zucchini, *Cucurbita pepo medullosa*, is used as the enzyme source for this study and is purchased commercially. The extraction of L-ascorbate oxidase involves fresh zucchinis, approximately 15 to 20 cm in length, are used. Their peels (approximately 142g), about 0.6 cm thick, are cut into small pieces and blended with a volume of 142 ml of phosphate/EDTA buffer (0.1M, pH 5.6). A small quantity of solid sodium borate

($Na_2B_4O_7 \cdot 10H_2O$) is periodically added to the mixture to adjust its pH to near neutrality (pH 6.8), as the pH of the raw juice is around 5.9. The resulting homogenate is filtered, and the filtrate is centrifuged for 10 minutes at 10,000 g (11,000 rpm) at 4°C. The supernatant, with a volume of 165 ml and a yellow-green color, constitutes the crude extract of L-ascorbate oxidase. It is stored at -10°C in sealed tubes for enzymatic activity determination and purification monitoring.

Ammonium sulfate precipitation

This method utilizes the differential solubility of proteins. Ammonium sulfate ($(NH_4)_2SO_4$) is preferably used due to its low cost, high precipitating power, high solubility, and minimal denaturing effect on proteins [9].The differential precipitation with ammonium sulfate is carried out as follows: The crude extract is brought to 65% saturation by the slow addition of solid ammonium sulfate (40.8g per 100ml of solution) at 4°C with stirring for 30 minutes. After a 72-hour contact time at 4°C, the green precipitate is collected on filter paper, and the clear filtrate is discarded. The green precipitate, with a volume of 2.6 ml, is stored at -10°C.

Fractionation with acetone

The addition of defined proportions of organic solvents, such as acetone, to a buffered aqueous solution containing a protein mixture changes the polarity of the medium and differentially alters the solubility of proteins. Some become insoluble and precipitate. This precipitation should be carried out at low temperatures (-20°C) to prevent denaturation [10]. Fractionation of L-ascorbate oxidase with acetone is performed where a suitable amount (1.96g) of the precipitate obtained after ammonium sulfate precipitation is suspended in approximately 6 ml of chilled distilled water to extract the soluble enzyme. After agitation for 30 minutes, suspension, and centrifugation for 10 minutes at 11,700 g (11,898 rpm) at 4°C to remove insoluble residues, 0.08g of NaCl is added to the supernatant. To this supernatant, placed on ice, slowly add pre-chilled acetone that has been stored at -15°C, and let it precipitate for 10 minutes. The volume of acetone added is 9 times the volume of the supernatant. Remove as much supernatant as possible and collect the precipitate on filter paper. Suspend this precipitate in approximately 14 ml of phosphate/EDTA buffer (0.1M, pH 5.6) and keep it cold. Transfer the obtained solution into a dialysis bag (Spectra/Por® 1.1; Biotech DispoDialyser®, MWCO: 8,000) and dialyze against 200 ml of phosphate/EDTA buffer (0.1M, pH 5.6) for about six hours, changing the dialysis buffer every two hours. The dialysate, with a volume of 10.9 ml, constitutes the purified L-ascorbate oxidase extract.

Enzymatic Activity Determination

A 10mM stock solution of ascorbic acid using HCl/EDTA solution (1mM, pH 2.2) as the solvent was prepared, then diluted to 10-fold with KH2PO4 solution (0.2M, EDTA 1mM, pH 4). In a series of test tubes, incubate 0.5 ml of substrate (1mM ascorbic acid) and 0.5 ml of Na2HPO4 solution (10mM, pH 7.8) at 30°C for 5 minutes, then add 0.1 ml of enzyme solution. Mix the tube contents well and reintubate in a water bath at 30°C for various reaction times. After each incubation period, stop the reaction by acidifying the reaction mixture, adding 3 ml of 0.2M HCl solution to the reaction medium. For the "0" time (blank reaction), first, add the reaction stop solution, then the enzyme solution. The remaining substrate (ascorbic acid) is then measured by spectrophotometry at 245 nm against the corresponding blank for time 0.

Results and Discussion

Regarding the kinetics of ascorbic acid oxidation, our experimental results were analyzed using a two-parameter kinetic model that provides the concentration of the substrate consumed as a function of time. This model is expressed as follows:

$[Sc] = A [1 - \exp(-Bt)]$

[Sc]: Concentration of consumed substrate (mol.l-1);

A: Constant (mol.l-1);

B: Constant (second-1);

t: Time (seconds).

This model allows for the evaluation of the initial reaction rate, which corresponds to the limit that the expression d[Sc]/dt approaches as time tends to zero [11]. By seeking the value of the derivative of equation "1" at the initial moment of the reaction, we obtain:

$V_{in} = [d(Sc)/dt]_0 = Ax B$

Where Vin (mol.l-1.s-1) is the initial rate of the ascorbic acid oxidation reaction by ascorbate oxidase.

The protein concentration in the ascorbate oxidase preparation was 7.5 ± 0.1 mg/ml. The specific activity of ascorbate oxidase was estimated to be 92.05 ± 18.57 IU/mg. (Measurement conditions: [Ascorbic acid] = 1mM; enzyme dilution 1/25 (V/V); solvent: phosphate/EDTA buffer 0.1M – pH 5.6; 30°C).

Comparison of the activities of ascorbate oxidase extracted from zucchini fruits at two different growth stages. The size of zucchini fruits can be related to their degree of maturity. To detect any

potential effect of maturity on ascorbate oxidase content, we compared the activities of ascorbate oxidase extracts from green zucchini fruits at two different growth stages. **Figure 1** represents the kinetics of ascorbic acid oxidation by the two enzymatic extracts obtained.

The enzymatic activities obtained for ripe and less ripe zucchini extracts were 685.78 IU/ml and 1138.42 IU/ml, respectively. These results are consistent with those of Arrigoni et al. (2003) [12] and Diallinas et al. (1997) [13]. It has been shown that a rapid decrease in the expression and/or activity of ascorbate oxidase is detected during fruit maturation. Conversely, in rapidly growing tissues and the "young" epidermis of cucurbitaceous fruits, an increase in ascorbate oxidase activity is observed. These observations, among others, have led to the hypothesis that ascorbate oxidase is involved in the relaxation and reorganization of the cell wall during the rapid growth phase of cucurbitaceous fruits.

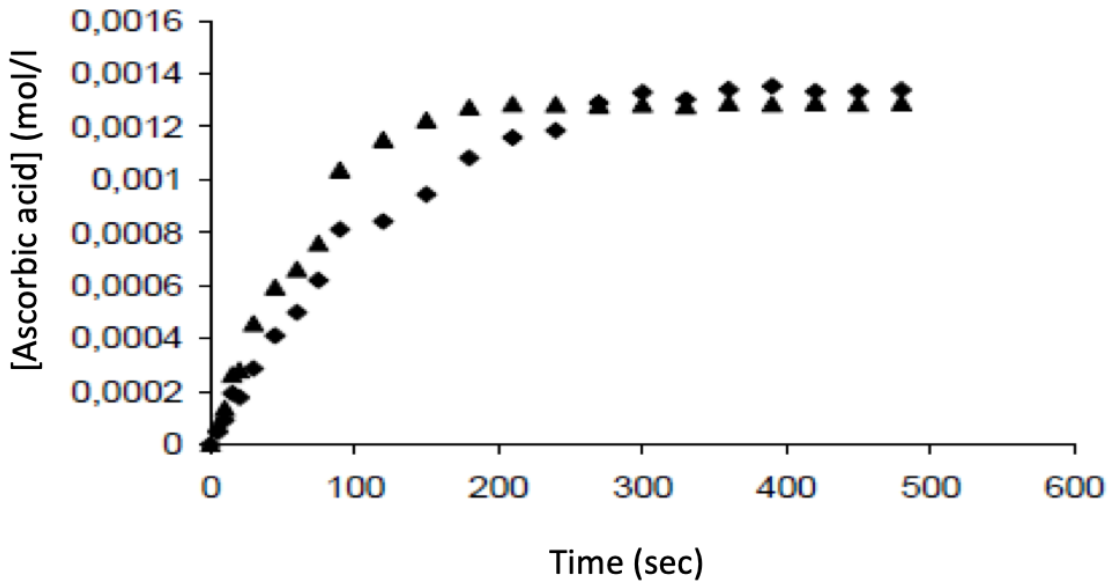

Figure 1: Kinetics of ascorbic acid oxidation by ascorbate oxidase extracted from ripe zucchini (♦) and zucchini harvested at an early stage of maturation (▲) with an average length of 17.5 cm and 10.5 cm and an average diameter of 3.5 cm and 2 cm, respectively. ([Ascorbic acid] = 1mM; enzyme dilution 1/25 (V/V); phosphate/EDTA buffer 0.1M – pH 5.6; 30°C).

1.1. Purification of *Cucurbita pepo medullosa* ascorbate oxidase

The purification of ascorbate oxidase from green zucchini involves, after extraction, precipitation with ammonium sulfate and fractionation with acetone. These two precipitation methods have seen their relative importance decrease with the advent of more sophisticated techniques.

However, they still offer numerous advantages, mainly as concentration methods rather than true purification methods. In general, the initial stages of purification are cost-effective and high-capacity techniques. All experimental results obtained for the different purification steps are summarized in Table 1. All manipulations were performed at a low temperature (4°C), and the determination of volumetric catalytic activities in the different fractions was done using a kinetic method.

Table 1: Purification table of *Cucurbita pepo medullosa* ascorbate oxidase.

Purification steps (142g of zucchini peels)	Total volume (ml)	Total activity (unit)	Total proteins (mg)	Specific activity (unit/mg)	Purification rate	Yield (%)
Raw extract	165	115265	1243	92.68	1	100
Precipitation by ammonium sulfate	2.6	57036	406.8	140.2	1.51	49.48
Acetone fractionation and dialysis	10.9	249.42	89.21	279.6	3.01	21.63

The ascorbate oxidase recovered after precipitation with ammonium sulfate and fractionation with acetone (followed by dialysis) is purified approximately three times. A total loss of enzymatic activity was observed in the fraction obtained after precipitation with ammonium sulfate. After precipitation with ammonium sulfate and fractionation with acetone of ascorbate oxidase from *Cucurbita pepo medullosa*.

2. Effect of pH

Enzymatic activity is highly dependent on pH. Generally, an enzyme has an optimal pH range where its catalytic activity is maximal. Beyond this range, the enzyme is gradually inactivated [14]. According to a paper [15]. the optimum pH of ascorbate oxidase is 5.7. A range of ascorbic acid solutions with pH values ranging from 5.4 to 8 is prepared. The enzyme preparation is diluted 25 times in phosphate/EDTA buffer (0.1M) with the same pH as the substrate solution of ascorbic acid. The effect of pH on the enzymatic activity of ascorbate oxidase is shown in **Figure 2.**

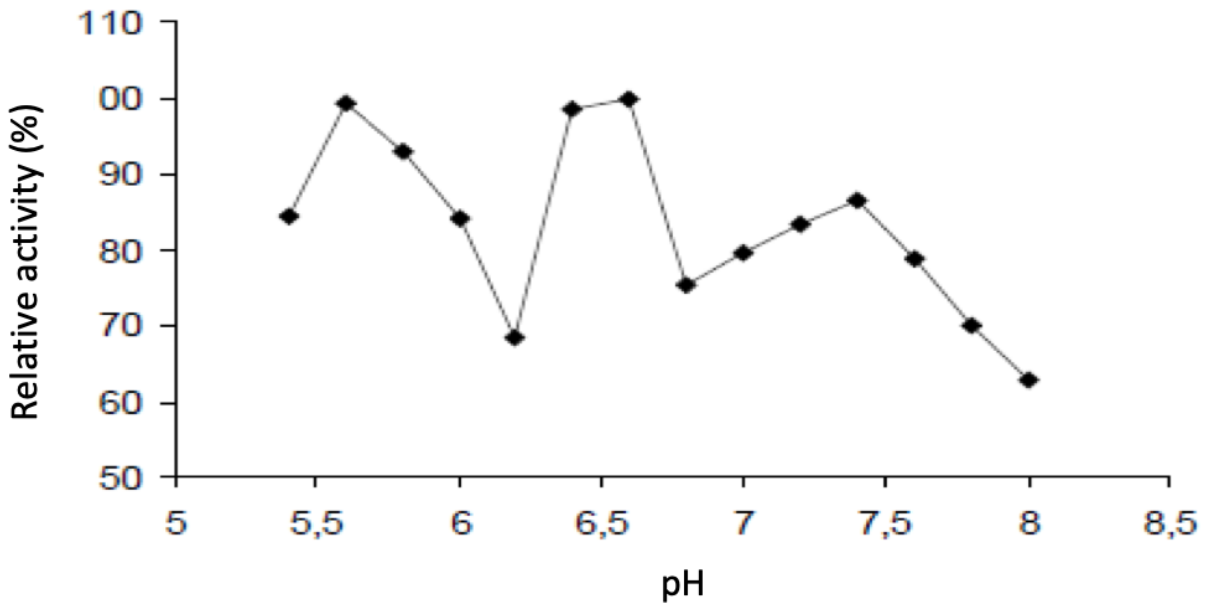

Figure 2: Effect of pH on ascorbate oxidase activity. ([Ascorbic acid] = 1mM; enzyme dilution 1/25 (V/V); phosphate/EDTA buffer 0.1M – pH 5.4 to 8; reaction time 1 minute; 30°C).

The results obtained show two peaks characterized by comparable enzymatic activities. The corresponding pH values are 5.6 (203.11 IU/ml) and 6.6 (204.49 IU/ml). According to an article [16] the enzymatic oxidation of ascorbic acid is maximal at a pH close to 5.6. Thus, another one [4] indicate that ascorbic acid is easily auto-oxidized under alkaline pH, so enzymatic oxidation can only be observed distinctly at acidic pH.

Effect of Temperature

The graphical representation of ascorbate oxidase enzymatic activity as a function of temperature (**Figure 3**) reveals an asymmetrical shape with two distinct phases corresponding to two different phenomena. In the ascending part of this curve (from 20°C to 45°C), activity increases with temperature. The increase in activity with temperature can be explained by an increase in the concentration of the activated complex when more thermal energy is provided to the reaction system. The enzymatic activity is 74.61 IU/ml at 20°C and 109.61 IU/ml at 45°C. This part of the curve corresponds to the activation curve [17-19].

In the descending part of the curve (beyond 45°C), enzymatic activity decreases. This is due to thermal denaturation. Thermal denaturation results from molecular agitation that disrupts the weak stabilizing bonds of the enzyme's spatial structure. There is a transition from a highly ordered state to a less ordered state, indicating an increase in entropy corresponding to the

disorganization of the enzyme's structure. The temperature from which this inactivation effect becomes visible is called the critical temperature. In the chosen measurement conditions, the temperature of 45°C, which corresponds to the maximum enzymatic activity (109.61 IU/ml), is the optimal temperature for ascorbate oxidase. This temperature represents the balance point between activation and thermal denaturation [14]. Kim et al. (1996) [4]measured an optimal temperature of 45°C for ascorbate oxidase. For a given enzyme, the measured optimal temperature depends on the experimental conditions, especially the pH and ionic strength of the medium, and the reaction duration. The shorter the duration, the higher the optimal temperature may be [10, 19]. In fact, determining the optimal temperature only provides an indication of the enzyme's thermal stability.

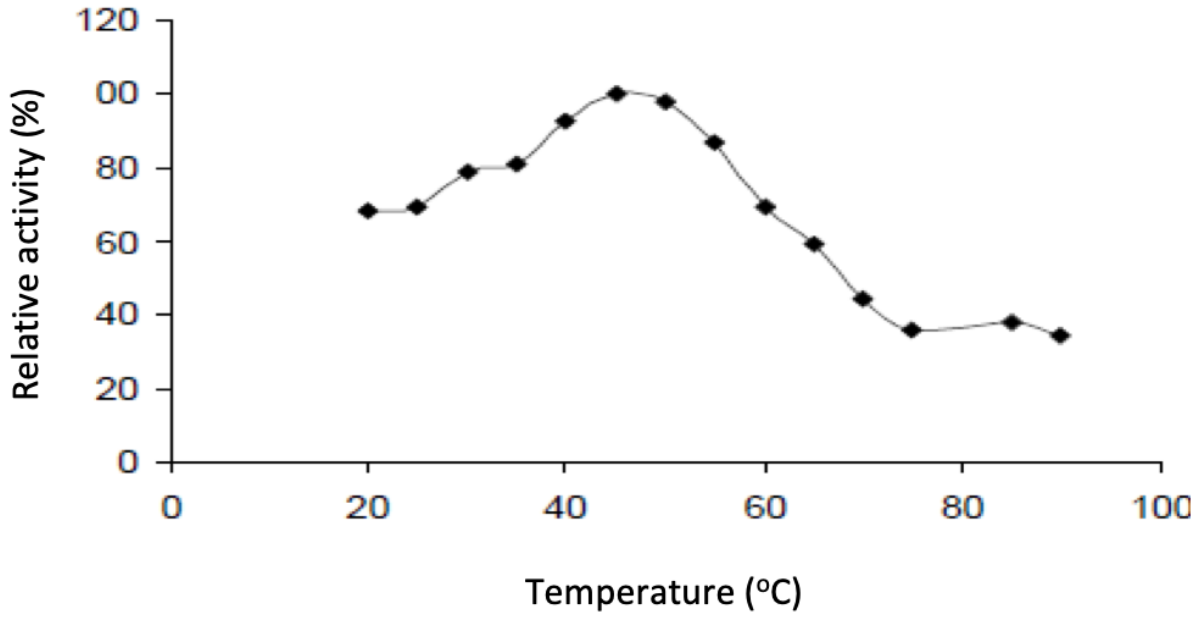

Figure 3: Effect of temperature on ascorbate oxidase activity. ([Ascorbic acid] = 1mM; enzyme dilution 1/25 (V/V); phosphate/EDTA buffer 0.1M - pH 5.6; reaction duration: 1 minute).

The ascending part of the relative activity curve as a function of the temperature of the reaction medium (**Figure 3**) allows the estimation of the activation energy for the oxidation of ascorbic acid by ascorbate oxidase. Thermal activation energy sometimes provides interesting insights into the chemical mechanism of the enzyme-catalyzed reaction [20]. The determined thermal activation energy is 12.41 kJ/mol. This result is close to that obtained by other researchers [21]

who determined an activation energy of 15.67 kJ/mol for ascorbate oxidase prepared from *Cucurbita pepo medullosa.*

Determination of Kinetic Parameters of Ascorbate Oxidase:

To determine the kinetic parameters Km and Vmax of ascorbate oxidase, the concentration of the substrate subjected to the enzyme was varied from 0.25 to 2mM. The reaction rate of ascorbic acid oxidation by ascorbate oxidase was measured at different substrate concentrations. The representation in inverse coordinates (known as Lineweaver-Burk plot) for unsaturated substrate concentrations is the most commonly used (**Figure 4**) [10, 14, 22, 23].

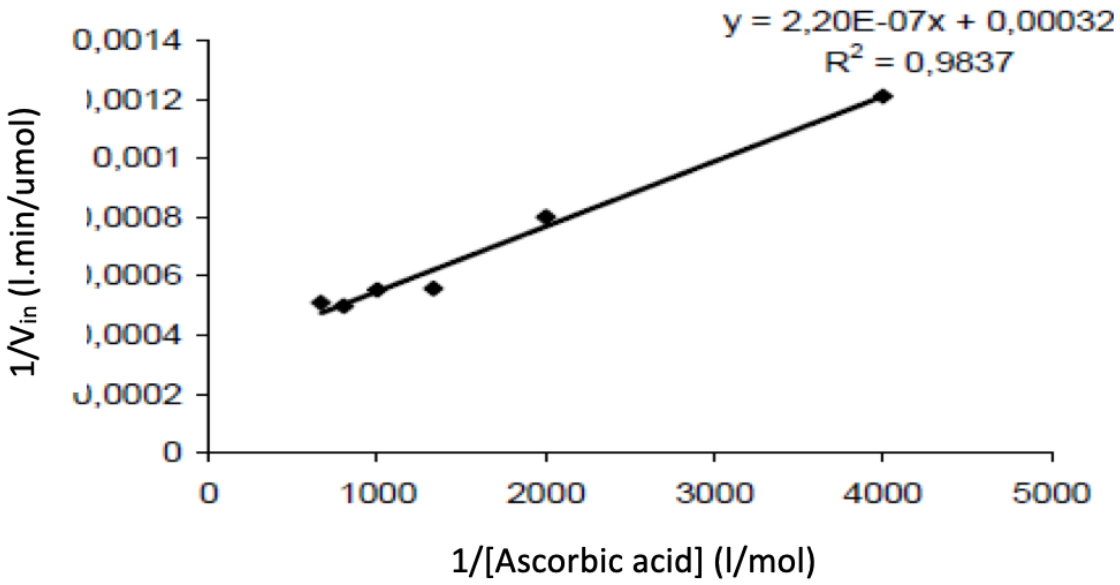

Figure 4: Lineweaver-Burk plot in inverse coordinates. (30°C; phosphate/EDTA buffer 0.1M – pH 5.6; enzyme dilution 1/25 V/V).

From this representation, the following kinetic parameters were obtained (at 30°C and pH 5.6):

Vmax = 3044.9 mol.l-1.min-1.

Km = 6.7 x10-4 mol.l-1.

R2 = 0.9837

There is heterogeneity in the values of the Michaelis constant (Km) of ascorbate oxidase reported in the literature. For example, according to Kim et al. (1996) [4] Km = 0.48 mmol.l-1; according to Matsumoto et al. (1981) [15], Km = 1.4 mmol.l-1; and according to a technical data sheet from the supplier “Roche”, Km = 0.3 mmol.l-1. Thus, the values of kinetic constants depend greatly on the source, enzyme purity, and operating conditions.

Conclusion

The first phase of our study involved extracting and partially purifying ascorbate oxidase from a natural source which is green zucchini (*Cucurbita pepo medullosa*). That ascorbate oxidase is a metalloenzyme that catalyzes the oxidation of ascorbic acid to dehydroascorbic acid in the presence of molecular oxygen. We studied the kinetic parameters of this enzyme, and the study of the influence of pH and temperature on ascorbate oxidase activity allowed us to estimate an optimum pH of 5.6, an optimal temperature of 45°C, and n of substrate concentration allowed us to obtain the following kinetic parameters (at 30°C and pH 5.6): Vmax = 3044.9 mol.l-1.min-1, Km = 6.7 x10-4 mol.l-1, r2 = 0.9837. This understanding hopefully would have implications for potential applications in diverse sectors, including agriculture, nutrition, and healthcare.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Authors contributions

All authors contributed equally to this work.

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References:

1. Messerschmidt A, Luecke H, Huber R. X-ray structures and Mechanistic implications of three functional derivatives of ascorbate Oxidase from zucchini reduced, peroxide and azide forms. J. Mol. Biol., 1993; 230: 997-1014.

2. Ohkawa J, Okada N, Shinmyo A, Takano M. Primary structure of cucumber (*cucumis sativus*) ascorbate oxidase deduced from cdna sequence: homology with blue copper proteins and tissue specific expression. Proc. Natl. Acad. Sci. 1989; 86: 1239-1243.

3. Farver O, Wherland S, Pecht I. Intramolecular electron transfer in ascorbate oxidase is enhanced in the presence of oxygen. J. Biol. Chem. 1994; 269(37): 22933-22936.

4. Kim Y, Yu S, Lee S, Hwang Y, Kang S. A heme-containing Ascorbate oxidase from *pleurotusostreatus*. J. Biol. Chem. 1996; 271(6): 3105-3111.

5. Farver O, Pecht I. Low activation barriers characterize Intramolecular electron transfer in ascorbate oxidase. Proc. Natl. Acad. Sci. 1992; 89: 8283-8287.

6. Santucci R, Ferri T, Morpurgo L, Savini I, Avigliano L. Unmediated heterogeneous electron transfer reaction of ascorbate Oxidase and laccase at a gold electrod. Biochem. J. 1998; 332: 611-615.

7. Marchesini A, Kroneck P. Ascorbate oxidase from cucurbita Pepo medullosa: new method of purification and reinvestigation of Properties. Eur. J. Biochem. 1979; 101: 65-76.

8. Lee M, Dawson C. Ascorbate oxidase: further studies on the Purification of the enzyme. J. Biol. Chem. 1973; 248(19): 6590-6602.

9. Durand G, Monsan P. Enzymes: production and industrial use. Paris: Bordas, Gauthier–Villars. 1982; 47-62, 121-124, 201-231, 260-268.

10. Loncle D. Enzyme engineering. Paris: doin. 1992; 3-21, 131-146, 351-377, 339-350.

11. Wallach J, Schwartz-cavrilovic C, Maginot J, Cavrilovic M. Biochemical analysis manipulations. 3rd edition. Doin.1996; 224-228, 353-354.

12. Arrigoni O, Chinni E, Ciraci S, De tullio M. In vivo elicitation of ascorbate oxidase activity by dioxygen and its possible role Photosynthesizing leaves. Botanica. 2003; 14: 127-134.

13. Diallinas G, Pateraki I, Sanmartin M, Scossa A, Stilianou E, Panopoulos N, Kanellis A. Melon ascorbate oxidase: cloning of multigene family, induction during fruit development and repression by wounding. Plant mol. biol. 1997; 34: 759-770.

14. Kamoun P. Apparatus and methods in biochemistry. Paris: Flammarion, Medicine Sciences. 1997; 199-212, 223-230, 291-298.

15. Matsumoto K, Yamada K, Osajima Y. Ascorbate electrode for determination of l-ascorbic acid in food. Anal. Chem. 1981; 53(13): 1974-1979.

16. Marchesini A, Capelletti P, Canonica L, Danieli B, Tollari S. Evidence about the catecholoxidase activity of the enzyme Ascorbate oxidase extracted from *cucurbita pepo medullosa.* Biochimica et biophysica acta. 1977; 484: 290-300.

17. Murray R, Granner D, Mayes P, Rodwell V. Harper biochemistry. 25th edition. Belgium: de Boeck. 2003; 48-136, 627-641.

18. Weil H.J. General biochemistry. 8th edition. Paris: Masson. 1997; 19-87.

19. Coutouly G. Enzyme engineering an introduction. Paris: Masson and Doin. 1991; 21-38, 99-141, 188-203.

20. Pelmont J. Enzymes. Algiers: university publications office. 1993; 167-203, 217-240.

21. Carvalho L, Lima C, Medeiros P. Ascorbate oxidase from *Cucurbita maxima*. Phytochemistry. 1981; 20(10): 2423-2424.

22. Burnstein C. Enzymatic biotechnology: instructions for use: Food industry – environment – medical. Paris: economica. 2000; 63 -103.

23. Palmer T. Understanding enzymes. 2nd edition. Ellis Horwood Limited. 1985; 162 -163.