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Original Research Article

Assessment of Kinetic Parameters of Peroxidase Isolated from Maturing *Solanum lycopersicum* Fruits for Analytical and Biotechnological Applications

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ABSTRACT

The wide application of peroxidase in biotechnology, food industries, environmental remediation and medical diagnosis has necessitated the interest for further research on the enzyme. This study investigated the kinetic parameters of maturing *Solanum lycopersicum* (tomato) fruit peroxidase with the prospect to ascertain its potentials and viability for analytical and biotechnological applications. Ammonium sulphate precipitation and gel filtration with sephadex G-100 were used to purify *Sonalum lycopersicum* peroxidase to homogeneity in two phases. Using o-dianisidine as a substrate, the optimal pH and temperature were found, while the Michaelis constant (Km) and maximum velocity (Vmax) were obtained using the Lineweaver–Burk graph. The purification factor and specific activity of the crude enzyme were 2.16 and 55.5 µ/mg respectively. Maturing *Solanum lycopersicum* fruit peroxidase was purified to homogeneity via a dual-step purification phases of gel filtration preceded by ammonium sulphate precipitation with specific activities of 34.11 µ/mg and 117.20 µ/mg in that order. The substrate used for the reaction was o-dianisidine. The enzyme adhered to Michaelis-Menten kinetics with Michaelis constant and maximum velocity of 5.23 mg/mL and 12.27 µmol/min, respectively. Maturing *Solanum lycopersicum* fruit peroxidase showed sensitivity under a range of pH (6-8) and temperature (40-90^{o}C) in its activity with 50°C and 5.9 as the temperature and pH optima, respectively. The result of this research has revealed that peroxidase from *Sonalum lycopersicum* exhibited physiochemical properties that are similar to what is obtainable *in vivo* which makes it suitable for analytical and biotechnological applications that in most cases mimics physiological conditions.

***Keywords*:** Peroxidase, *Solanum lycopersicum*, Purification, Kinetics, pH, Temperature.

Introduction

Peroxidases (EC1.11.1.7) are oxido-reductases that catalyze the reduction of peroxides such as hydrogen peroxide (H_2O_2). They are also involved in the oxidation of organic and inorganic compounds like xenobiotic and phenolic compounds.[1] In the presence of hydrogen peroxide, substrates such as ferricyanides and ascorbic acids receive electrons from peroxidase with the goal of rendering them into harmless constituents. Hydrogen peroxide, a by-product of oxygen metabolism in the body is degraded into water and oxygen by peroxidase.[2] Peroxidases are valuable tool industrially as they are useful in the production of detergents, foods and high fructose corn syrup.[3] Currently, peroxidases and particularly horseradish peroxidase are used for analytical and biosensor applications. They possess invaluable biochemical applications as they help in amplification of a weak signal with the aim of detecting a molecule or compound of interest.[4] The earliest but still useful analytical method is based on chemical kinetics where catalytic activity (usually determined by change in substrate concentration) of enzyme is taken into advantage. In that manner, enzyme is added to solution containing a suitable substrate and their reaction is monitored for a given time frame.

The applications of peroxidases beyond the shore of industries such as in the manufacturing of diagnostic kits and biosensors have made the enzyme to be more relevance. This has given rise to under-supply of the enzyme compared to its industrial demands. Currently, the only viable source of the enzyme is horseradish and this has necessitated the search for an alternative source of the enzyme of which plants remains a promising target. Maturing *Solanum lycopersicum* fruits could serve as option for commercial exploration of peroxidase as these enzymes are naturally found at high concentration in maturing *Solanum lycopersicum* fruits as it plays a cardinal role in fruit ripening.

The knowledge of kinetic parameters such as V_{max}, Km, optimum pH and temperature of a maturing *Solanum lycopersicum* fruits peroxidase would be a guide on its effective utilization for analytical and industrial purposes. It would also provide a fundamental insight on how to improve its efficiency such as high solubility, increased catalytic activity, thermostability and substrate affinity with the benefits of enhancing its biotechnological applications.

On the other hand, *Solanum lycopersicum* (tomato) is herbaceous plant that is part of the nightshade family with highly divided leaves that have long, slender hairs with distinctive scent and is commonly grown all over the world for its fruit; tomato.[5] Research evidence has shown

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that *Solanum lycopersicum* peroxidase resembles horseradish peroxidase in a standard peroxidase assay. In the same manner, maturing *Solanum lycopersicum* fruits peroxidase is reduced to ferroperoxidase and after which it is converted to oxyferroperoxidase (Compound III) in other to form peroxidase complexes with hydrogen peroxide (Compound I and II).[6] Currently, roots of horseradish serve as the only traditional source of peroxidase available on a commercial scale and as such, researches are tailored towards the discovery of new or alternative sources of peroxidases with characteristics that are exploitable and applicable in analytical, biomedical and industrial settings.[7] Hence this study evaluated the kinetic parameters of maturing tomato fruit peroxidase to determine its potentials and viability for analytical and biotechnological applications.

Materials and Methods

Peroxidase Extraction

Solanum lycopersicum fruits at half ripen stage were sourced from a garden in Agbo Oko in Oko Okpuje Ani in Okpuje Community, Nsukka Local Government Area of Enugu State, Nigeria with GPS coordinate (6.9028° N, 7.2923° E) on 12th December 2020. Identification and authentication of the sample took place at The Centre for Bioresources and Development Programme (CBDP), Nsukka by the Chief Taxonomist Mr. Alfred Ozioko. Crude peroxidase from *Sonalum lycopersicum* fruits was isolated following a procedure reported by Enachi *et al.*[8] with little modification as described by Benmrad *et al.*[10] The maturing fruits of *Solanum lycopersicum* were carefully washed under running water and 800 g was weighed and milled for homogenization with 1000 mL of ice-cold phosphate buffer of 0.1 M as medium. The mixture was vigorously shaken, and the solution filtered. At 4000 rpm and for a period of half an hour, the filtrate was centrifuged. The supernatant, which is the enzyme extract measured 2000 mL and was preserved in refrigerator.

Peroxidase purification

Ammonium sulphate precipitation was carried out at 80% saturation based on the result from pilot study. In 1.5 L of the crude extract, 654 g of the salt was carefully dissolved. The dissolution was done in a careful and gradual manner accompanied with gentle stirring to ensure total dissolution of salts. After centrifugation at 4000 rpm, there was re-dissolution of precipitate in 40 mL sodium phosphate buffering medium of 0.05 M concentration at pH 6.0. The solution was kept at temperature of 4°C awaiting further experiments. The column for Sephadex G-100 purification was prepared by; first, soaking 10 g of the gel overnight (12 h) to have the gel swell up. The soaking medium was sodium phosphate buffer solution of pH 6.0 at 0.05 M concentration. After pouring the gel carefully, it was allowed to settle so as to pack homogenously until the bed height (25 cm) was reached. The packed column was equilibrated with 0.05 M sodium phosphate buffer solution (pH 6.0) and 30 mL of the precipitated protein was introduced into the column. At a flow rate of 5 mL per minutes, 80 fractions were collected using 5 mL fraction tubes. The protein concentration of each fraction was monitored using a spectrophotometer.

Peroxidase assay and protein determination

The method of McLellan and Robinson guided the assay protocol.[9] Change in absorbance at 460 nm was recorded using Jenway 6405 Modeled UV/VIS spectrophotometer as a result of reduction of hydrogen peroxide to water in a medium containing hydrogen peroxide, o-dianisidine and enzyme extract at 30°C. The vial for assay protocol with a total volume of 3.0 mL is composed of 0.2 mL hydrogen peroxide, 0.3 mL 1% o-dianisidine, 2.4 mL sodium phosphate buffer and 0.1 M concentration of enzyme extract. The determination of the protein content of the enzyme followed the Lowry method.[11]

Determination of optimum pH and temperature

The percentage residual activity of peroxidase was obtained by incubating the enzyme in buffers with pH range of 4 -10 at interval of 10 to 60 min. The temperature stability for the peroxidase activity was determined via incubation of the enzyme solution at temperature range of 30-90°C (at intervals of 10°C) for 20 min using water bath. The assay protocol consisted of hydrogen peroxide (0.2 mL), 1% o-dianisidine (0.3 mL), sodium phosphate buffer (2.4 mL) and enzyme extract (0.1 M concentration).

Thermal inactivation

The rate of heat inactivation was investigated at different temperatures of 40-80°C. The % residual activity of the enzyme fraction was calculated using the formula below:

$$\%\ \text{Residual activity} = \text{At/Ao} \times 100$$

Where A_t and A_o are the activities of both heat treated and heat untreated samples respectively.

Determination of K_m and V_{max}

The substrate concentration effect on peroxidase activity enabled us to determine the kinetic parameters, K_m and V_{max}. This was achieved through the incubation of the enzyme in variable concentrations of the substrates using working buffer solution at 50°C. After thorough mixing, the spectrophotometric absorbance was carefully read at 480nm. The graphical plot (Lineweaver-Burk) of initial velocity against variable substrate concentration helped to determine the kinetic parameters of interest, V_{max} (velocity maximum) and K_m (Michaelis constant) of the enzyme.

Results and Discussion

Purification of peroxidase isolated from maturing Solanum lycopersicum fruits

In quest to have a purified enzyme, the crude enzyme obtained from extraction processes was subjected to ammonium sulphate precipitation at 80% ammonium sulphate saturation (Figure 1) which gave the maximum activity from pilot study. During precipitation the protein in solution was salted out based on its ionic strength according to the concentration of the salt. This may be linked to the kind of the amino acids present in the active site. In furthering the purification process, maturing *Solanum lycopersicum* fruits peroxidase was ran on sephadex G-100 gel filtration chromatographic column under ambient room temperature. The fractions between 7-18 that gave high peroxidase activity whose peaks (red) aligned with that of protein peaks (blue) were pooled together for characterization and kinetics analysis (Figure 2). After gel filtration, an enzyme with purification factor and specific activity of 2.2 and 119.61 µ/mg respectively were obtained. An increased purification factor when compared to ammonium sulphate could be attributed to desalting of the enzyme after gel filtration which was obvious with corresponding increase in activity when compared to the previous enzyme forms, that is, the crude and precipitated enzymes. This is an indication that it has high level of reduced protein contaminants as compared to the crude and the precipitates.

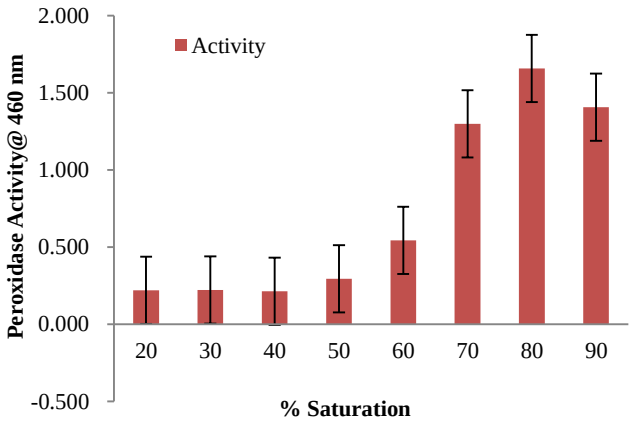

Figure 1: Ammonium sulphate precipitation profile

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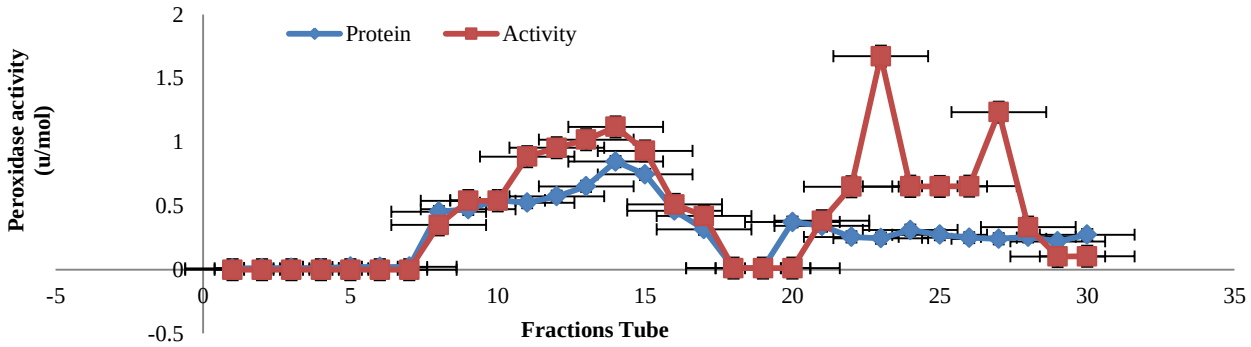

Figure 2: Gel filtration profile of peroxidase isolated from *Solanum lycopersicon*.

Table 1: Purification Table of *Sonalum lycopersicum* peroxidase

Fraction	Volume mL	Protein mg/mL	Total protein	Activity µmol/min	Total activity	Specific activity µ/mg	Purification fold	% recovery
Enzyme extract	500	1.774 ± 0.031	887.40	98.42 ± 0.004	4.9×10^3	0.56×10^2 ± 0.002	1.00	100
Ammonium sulphate	60	0.583 ± 0.011	34.10	30.10 ± 0.004	$1.3x10^3$	0.36×10^2 ± 0.009	0.65	25.5
Gel filtration	30	0.163 ± 0.021	4.89	19.50 ± 0.003	0.6×10^3	1.21×10^2 ± 0.001	3.33	46.5

A purification fold of 17.92 for peroxidase from lettuce stems on a three-step purification of ammonium sulphate precipitation, sephadexG-100 and concanavalin A affinity chromatography had earlier been reported.[12] Also, with a three-step purification process of ammonium sulphate precipitation, dialysis and a CM Sephadex ion exchange chromatography a purification fold of 9.7 was obtained for Turkish black radish peroxidase.[13] On a four-step purification process of Sephadex G-25, ammonium sulphate precipitation, DEAE Sepharose, and cancanavalin A Sepharose, peroxidase from horseradish were purified 2692 fold. The low purification fold achieved in this study could be attributed to only two phases of purification. Despite the low purification fold achieved when compared to the works from above cited literature, the purification method adopted in this study is inexpensive, fast, dependable and less inapt. The activity of the crude is the highest when compared to that of ammonium sulphate precipitate and gel filtration (Table 1). This could be as a result of other proteins that are present in the crude. The rise in specific activities for the enzyme after gel filtration could be attributed to the fact that more contaminated proteins are being removed after the purification.[14]

Kinetic of Maturing Solanum lycopersicum Fruits Peroxidase
Optimum pH

The optimum working pH for this enzyme was found to be 5.9 with activity of 0.7 (u/mol) as shown in Figure 3. Other pH values evaluated resulted to significantly lesser activities, with the lowest activity found within alkaline range. The result from this study is similar to what was obtained in peroxidases from other sources. For instance, in the cases of horseradish peroxidase and beans cell peroxidase, the pH optimum were 8.5 and 7.2 respectively.[15] Other studies have reported similar results where most peroxidases from different sources showed optimum activity in the pH range of 4.5–6.5 [16] The decrease in activity associated with low pH of 3.5 occurred as a result of enzyme inactivation due to acidic conditions of the reaction medium as low pH usually leads to protein denaturation, giving rise to loss of function. The pH optima of 5.9 is close to physiological or neutral pH of 7.0 needed for maximum performance of enzyme in analytical or biotechnological applications. Since such enzymes are usually immobilized, the target would be to use an immobilizer that could cause a shift of the enzyme pH towards neutrality**.** By adopting the gel/polymer entrapment method of immobilization, this enzyme could be conferred with this property.

Optimum temperature

The temperature profiles of peroxidase sourced from *Sonalum lycopersicum* and purified with sephadex-G100 is shown in figure 4.

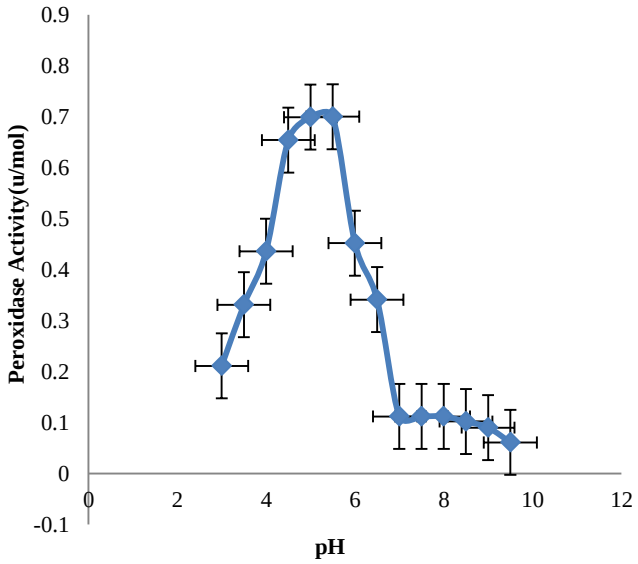

Figure 3: Effects of pH on peroxidase activity.

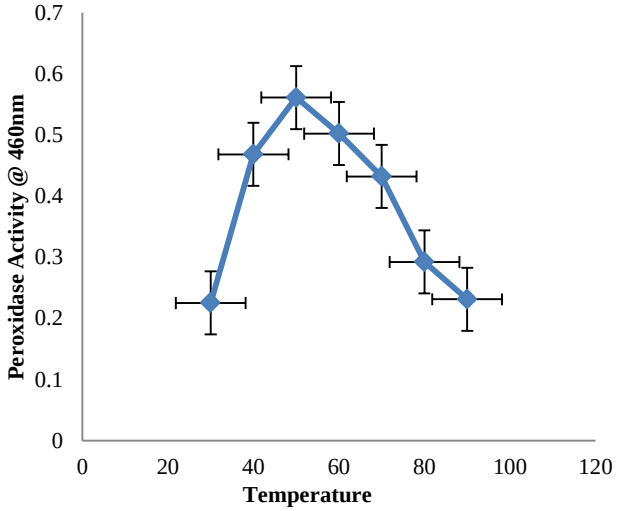

Figure 4: Effects of temperature on peroxidase activity

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A curve of a typical bell shape was obtained as depicted. The enzyme activity rose gradually up to the temperature of 50°C and then declined till a drop point of 90°C, a typical demonstration of enzyme inactivation at extreme temperature. Optimum temperature of 50°C is closely related to the findings of Sarika who reported maximum enzyme activity at 40°C in Concanavalin A agarose affinity chromatography purified horseradish peroxidase, an enzyme that symbolizes peroxidase of industrial and analytical relevance.[17] This finding is also in accordance with the earlier data reported by Ling *et al*.[18] A temperature of 50°C optima for peroxidase isolated from ripening *Solanum lycopersicum* fruit was also earlier reported from research findings.[19] The variation in temperature optima could be as a result of isoenzymes from different species of plants. Also, differences in optimum temperature may be as a result of different in the substrates used, assay time and enzyme concentration.[20] Most industrial applications of peroxidase use temperature ranges of 25 to 55°C and as such peroxidase from *Sonalum lycopersicum* with optimum temperature of 50°C can be applied in some of the processes.

Michaelis Constant K_m and Maximum Velocity V_{max}

K_m and V_{max} values of 5.44 mg/mL and 12.57 µmol/min respectively were graphically determined from double reciprocal plot (Figure 5) based on studies on effect of substrate concentration on the enzyme activity. A low K_m value suggests that the enzyme has a strong affinity for the substrate. The values are quite close to other peroxidases from plants and microbes.[21] However, the K_m and V_{max} values were shown to be 2.407 mg/mL and 21853 µmol/min for peroxidase from *Streptomyces sp.* MSC702.[22] Kinetic constants, K_m and V_{max} of 7.14 mM and 0.1 mole/min respectively were obtained for peroxidase isolated from *Brassica oleracea.*[23] The variation could be attributed to difference in the type of substrates used in the different studies.

Thermal Inactivation

Thermal inactivation at temperature of 50-80°C for peroxidase from maturing *Solanum lycopersicum* fruit resulted to biphasic curves as depicted in figure 6. The enzyme appear to be stable at high temperature. Even at extreme temperature of 70°C for a period of 80 minutes the conformational and structural integrity of the enzyme were still not affected with percentage residual activity calculated to be 66%. Thermal inactivation of Peroxidase isolated from precooked frozen *Brassica Species*,[24] sorghum,[25] and banana puree [26] all showed biphasic curves. Biphasic inactivation curves could have been the result of a multi-step process involving stable intermediates [27] or the presence of heat stable components.[28] It might also be attributable to activity restoration and regeneration,[27] or micro heterogeneity of covalently linked oligosaccharide residues at molecular levels,[29] or it may still be related to series-type inactivation kinetics.[28]

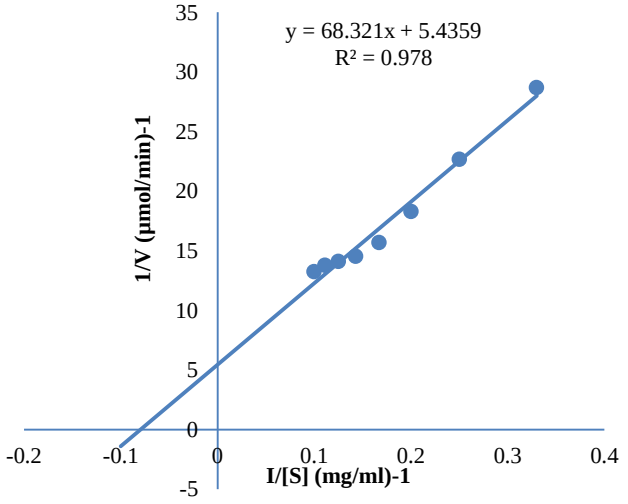

Figure 5: LineWeaver-Burk plot of peroxidase isolated from maturing *Sonalum lycopersicum* using different concentrations of O-dianisidine as substrate

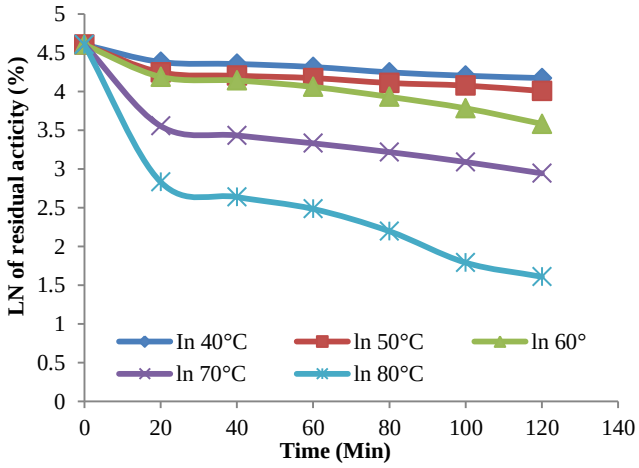

Figure 6: Percentage residual activities against time for peroxidase.

Conclusion

The present study concludes that maturing *Solanum lycopersicum* fruits produce substantial amount of peroxidase. The peroxidase that was partially purified showed maximum activity at optimum temperature of 50°C which is close to the temperature requirement for industrial processes. This shows the enzyme is highly stable and suggests that it could be exploited for industrial applications that occur at high temperature without easily being denatured. The pH studies reported that this enzyme perform maximally at pH close to neutrality which is good for biotechnological applications. The wide application of peroxidase due to its ever growing need in analytical and biotechnological applications has necessitated its exploration and isolation beyond horseradish. Since the chemical and kinetic parameters (K_m and V_{max}) of peroxidase from maturing *Solanum lycopersicum* fruits as seen from this study are close to that of horseraddish (HRP), then it becomes imperative to source peroxidase from maturing *Solanum lycopersicum* fruits to boost its commercial availability, which is currently in short supply. Also, the procedure enumerated in this study to obtain this enzyme and to study its kinetic activities is effective and suitable for analytical applications.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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