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Studies on Purified Polyphenol Oxidase from Red Cocoyam (*Xanthosomamafafa*)

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ABSTRACT

Polyphenol oxidase (PPO) catalyzes oxidative conversion of phenolic substrates to their respective quinones, which further polymerize to form macromolecules resulting to browning reactions in various organisms. Here, the presence, purification and physicochemical properties of PPO from *Xanthosomamafafa* are described. Polyphenol oxidase from *X. mafafa* (*xm*PPO) is purified using a combination of ion-exchange (cation and anion exchanger) and gel filtration chromatography. The enzyme appeared to be homodimeric. The subunit molecular weight obtained on SDS-PAGE was 24.5 ± 0.3 kDa while that of native molecular weight on Sephadex G-100 was 44.3 ± 1.8 kDa. The K_m and V_{max} obtained for the purified *xm*PPO using L-DOPA as substrate were 6.5 ± 1.0 mM and 35.4 ± 2.0 units/mg protein respectively leading to first–order rate constant (k_{cat}/K_m) value of 3.9×10^3 s^{-1} M^{-1}. The optimum pH and temperature for the purified enzyme were 6.5 and 50 ^oC respectively. The enzyme was stable to heat at up to 60 ^oC retaining close to 70% residual activity. Substrates specificity revealed the enzyme had both monophenolase and diphenolase activities. In conclusion, the combination of properties of PPO from *X. mafafa* revealed that, it could also be enzyme of interest in several biotechnological applications.

Keywords: Polyphenol oxidase, L-DOPA, physicochemical properties, *Xanthosomamafafa*

1. Introduction

Polyphenol oxidases (PPOs) are copper-containing enzymes existing widely in microorganisms, fungi, plants, and mammals (Halaouli *et al*., 2006). They are ubiquitous copper-containing enzymes which use molecular oxygen to oxidize common *ortho*-diphenolic compounds such as caffeic acid and catechol to their respective quinones. PPO-generated quinones are highly reactive and may cross-link or alkylate proteins, leading to the commonly observed brown pigments in damaged plant tissues and plant extracts. Based on its association with browning reactions in crop plants, PPO has been characterized in a wide variety of food plants including banana, wheat, quince, and avocado, and a number of chemical inhibitors have been identified (Mayer, 2006). Although PPO is found at significant levels in a variety of fruits, vegetables and grains, its biological function in these tissues has rarely been studied. PPO is expressed in many different tissues and organs, including roots, leaves, flowers, and vascular tissue (Constabel *et al*., 1996). Polyphenol oxidase has been purified and characterized from a wide range of plant species and a variety of tissues (Mayer, 2006) and activity levels using common substrates vary widely. Key features of PPOs are two conserved copper-binding domains, and N-terminal chloroplast and thylakoid transit peptides.

Many PPOs are predicted to contain a proteolytic processing site near the C-terminus of the polypeptide (Marusek *et al*., 2006). There are mainly three types of polyphenol oxidases classified according to their substrate specificities and mechanism of actions.

However, polyphenol oxidase is essential for many living organisms to carry out various functions, including melanin biosynthesis as defense against the harmful effects of UV light (Claus and Decker, 2006; Halaouli *et al*., 2006). In plants, it is required for the biosynthesis of phenolic polymers such as lignin, flavonoids, and tannins (Marusek *et al*., 2006). PPO also play an important role in the regulation of the oxidation-reduction potential of cell respiration and in wound healing in plants (Mayer, 2006). Due to the ability of PPO to react with phenolic compounds, these enzymes have been proposed for use in a variety of biotechnological, biosensor and biocatalysis applications (Jus *et al*., 2009). It can be applied in detoxification of phenol-containing wastewater and contaminant soils (Martorell *et al*., 2012), synthesis of L-3, 4-dihydroxyphenylalanine (L-DOPA), one of the preferred drugs for the treatment of Parkinson's disease (Ates*et al*., 2007) or as additives in food processes due to their cross-linking abilities (Selinheimo*et al*., 2007). Tailoring polymers, e.g. grafting of silk proteins onto chitosan via tyrosinase reactions have also been reported (Anghileri *et al*., 2007). PPO has been exploited in plants, animals, fruits and vegetables (Zekiri*et al*., 2014; Ilesanmi and Adewale, 2020).

Red cocoyam (*Xanthosomamafafa*) is a genus of flowering plants in the arum family, Araceae. The genus is native and widely cultivated and neutralized in other tropical regions. Several are grown for their starchy corms, an important food staple of tropical regions. Many other species, including especially *Xanthosomaroseum* are used as ornamental plants. It is grown in West Africa, now a major producer, where it can be used as a replacement, for yams in a popular regional. Besides its losses during the cultivation and gathering periods, there are also losses of between 20 and 30% during post-harvest periods because the methods and infrastructure currently in use are rudimentary (Guzmán and Buitrago, 2000). During this period, it undergoes a rapid darkening when damages are inflicted and tissues are cut, peeled or crushed which could be associated with the presence of PPO (Kahn, 1985).PPO (tyrosinase) has been studied in different species of yam (*Dioscorea praehensilis, Dioscorea alata, Dioscorea rotundata and Colocasia esculenta*) (Ilesanmi *et al*., 2014). In that work, only the specific activity of enzyme from *X. mafafa* was reported. There was need to further investigate the characteristics and properties of the enzyme from *X. mafafa*. The present work was carried out to establish the presence of the enzyme in *X. mafafa* and document its properties for further applications.

2. Materials and Methods

2.1 Materials

Tubers from *X. mafafa* were obtained from farms around Owo environs, Southwestern Nigeria. The yam cultivars were authenticated at the FUTA Herbarium, Department of Botany, Federal University of Technology, Akure, Nigeria.

Chemicals

3,4-dihydroxyphenyl-L-alanine (L-DOPA), catechol, resorcinol, caffeic acid, DL-tyrosine, phenol, 1-naphthol, pyrogallol, blue dextran, trizma base, trizmaHCl, acetic acid, citric acid, sodium citrate, bovine serum albumin (BSA), sodium phosphate dibasic (Na_2HPO_4), anhydrous sodium phosphate monobasic ($NaHPO_4$), Coomassie brilliant blue R-250, glutathione were obtained from Sigma Chemical Company, St Louis, USA. Molecular weight standard for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was obtained from Carl Roth GmbH, Karlsruhe, Germany. CM-Sepharose CL-6B, QAE-Sephadex A-50, Sephadex G-100 were purchased from GE Healthcare Bio-sciences, Uppsala, Sweden. All other reagents were of analytical grade.

2.2 Methods

2.2.1 Preparation of red cocoyam homogenate

The red cocoyam tuber was peeled and homogenized in 50 mMphosphate buffer at 4ºC to obtain 30% homogenates. The homogenate was centrifuged at 10,000×g for 20 min at 4 ˚C in order to obtain clear crude supernatants. The supernatants were assayed for PPO activity using L-3, 4-

dihydroxyphenyalanine (L-DOPA) as substrate in a spectrophotometer. The supernatants were stored at -20 ˚C when not used immediately.

2.2.2 Polyphenol oxidase activity assay

Polyphenol oxidase activity with L-3, 4-dihydroxyphenylalanine (L-DOPA) was determined in crude soluble red cocoyam supernatants and routinely during purification according to the method of Altunkaya and Gökmen (2010) as modified by Ilesanmi *et al*. (2014). Appropriate volume of enzyme was added to 1 mM L-DOPA solution in 50 mM phosphate buffer, pH 6.5 to initiate the reaction. Initial rate of product formation was monitored as an increase in the absorbance at 475nm using UV-VIS spectrophotometer.

2.2.3Determination of protein concentration

The protein concentrations in the crude homogenates, partially purified tyrosinase and purified tyrosinase were determined as described by Bradford in (1976) using BSA as the standard protein.

2.2.4 Enzyme purification

2.2.4.1 Purification by ion-exchange chromatography on CM-sepharose and QAE-sephadex

Appropriate volume of supernatant obtained from *X. mafafa* was layered on CM-sepharose ion-exchange column (1.0 × 10 cm) previously equilibrated with 50 mM phosphate buffer, pH 6.5. Elution was done and 1 ml fractions were collected. Bound proteins were also eluted gradiently with 0-1M NaCl. The fractions were assayed for PPO activity. Active fractions were pooled for further analysis. CM-sepharose pools were layered on QAE-sephadex ion-exchange column (2.5 × 10 cm) previously equilibrated with 10 mMtris-buffer, pH 7.0. The eluted fractions were assayed for PPO activity. Active fractions were pooled together for further use.

2.2.4.2 Purification by gel filtration chromatography on Sephadex G-100

The lyophilized re-dissolved samples from QAE-Sephadexpool was layered on Sephadex G-100 gel filtration column (1.0 × 40 cm) equilibrated with 50 mM phosphate buffer pH 6.5. Elution was done with the equilibration buffer and fractions with PPO activity were pooled and freeze-dried. Again, the lyophilized samples were re-dissolved in minimal volume of 50 mM phosphate buffer pH 6.5.

2.2.5 Determination of native and subunit molecular weights

The native molecular weight of the *xm*PPO was determined on Sephadex G-100 column. The k_{av} of the standard proteins were plotted against their respective molecular weights to obtain a standard calibration curve. The native molecular weight of PPO from *X. mafafa* was obtained by interpolation of k_{av} values on the standard curve. The subunit molecular weight was determined according to the method of Laemmli (1970) as modified by Weber and Osborn, 1975 using tris-glycine buffer system at pH 8.3.The molecular weights of standard proteins were plotted against their respective relative mobility. The subunit molecular weights of the purified PPO from *X. mafafa* was obtained by interpolation of the R_m values on the standard curve.

2.2.6 Determination of Kinetic Parameters

The effect of various concentrations of L-3,4-dihydroxylphenylalanine (L-DOPA) on the purified PPO from *X. mafafa* was determined. The apparent kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) of the tyrosinase were determined in aqueous system by varying the concentrations of L-DOPA between 1-30 mM in 50 mM phosphate buffer, pH 6.5. The data obtained were analysed using non-linear regression software (Graph pad prism 5).

2.2.7 Effect of temperature on PPO activity

The effect of temperature on purified PPO activity from *X. mafafa* were studied by incubating the reaction mixtures containing 50 mM phosphate buffer, pH 6.5 and 1.5 mM L-DOPA at temperatures ranging from 10 to 80 ˚C for 5 min.

2.2.8 Heat Stability of polyphenol oxidase from X. mafafa

For the thermal stability tests, the enzyme was incubated at different temperatures (20 - 70 ˚C) and the residual activity was determined under the standard reaction conditions by taking aliquots at 10 min intervals for 1 h. The residual activities were expressed as a percentage of the activity at zero time which was taken to be 100%. The percentage residual activity was plotted against time of incubation.

2.2.9 Effect of pH on PPO activity from X. mafafa

The effect of pH on the PPO activity was determined in the pH range of 3.0 – 10.0 at room temperature. The following buffer systems at the indicated pH ranges were used: 50 mMcitrate buffer, pH 3.0 - 5.0; 50 mM MES buffer, pH 5.5 - 6.5; 50 mM HEPES buffer, pH 7.0 - 8.5 and 50 mM glycine-NaOH buffer, pH 9.0 – 11.0.

3. Results

3.1 Enzyme Purification

A single peak (Figure 1) of activity was recovered for *xmPPO* with a yield and purification fold of 66% and 1.4 respectively. On the QAE-Sephadex ion-exchange column, a single peak (Figure 2) of activity was recovered for the *xmPPO* with a yield and purification fold of 52% and 5.6 respectively. The concentrated, partially purified PPO from *X. mafafa* obtained from the previous step were further purified on Sephadex G-100 as represented in Figure 3. A single activity peak was obtained. The overall level of recovery of PPO activity was 41% and purification fold of 7.2 was achieved. The purification summary is shown in Table 1.

3.2 Purity test and molecular weight determination

Polyacrylamide gel electrophoresis of *xm*PPO in the presence of SDS gave a single band equivalent to a protein of 24.5 ± 0.3 kDa on 12% slab gel as shown in the electrophoretogram (Figure 4). The molecular weights of the native enzyme was estimated to be 44.3 ± 1.5kDaon Sephadex G-100 column.

3.3 Kinetic parameters of purified PPO from X. mafafa

The kinetic parameters were determined from non-linear regression plot of activity against substrate concentrations. The apparent K_m of PPO from *X. mafafa*for L-DOPAwas 6.5 ± 0.8 mM. Apparent V_{max} for the enzyme was 35 ± 0.7 units/mg protein. The specificity constant, k_{cat}/K_m estimated was $3.9 \times 10^3 M^{-1}s^{-1}$(Table 2).

3.4 Effect of temperature on the activity of purified PPO from X. mafafa

Figure 4 is a summary of the effect of temperature on purifiedPPO*X. Mafafa* at temperatures ranging from 10 to 80 °C. According to the results, the optimal temperature for this enzyme is approximately 50 °C. The enzyme was fully stable below 50 °C, but gradually lost its activity at temperature above 50 °C. The activation energy, E_a, for the oxidation of L-DOPA by the purified PPO from *X. mafafa*was2.3×10^{-2} kJ/mol respectively.

3.5 Thermal stability studies of purified PPO from X. mafafa

When the residual activity was plotted against the time of incubation (Figure 5), about 80% activity was retained at 60 °C for the PPO from *X. mafafa* after 1 h of incubation. About 35% activity was retained at 70 °C for the same period.

3.6 Effect of pH on the activity of purified PPO from X. mafafa

When the activities of PPO from *X. mafafa* were plotted against the pH, (Figure 6) highest activity was found to be between pH 5 and 8.The optimum activity was obtained at pH 6.5.

3.7Substrate specificity studies

Substrate utilization characteristics of the purified PPO from *X. mafafa*. All the enzyme had the highest activity with L-DOPA. This was followed closely by catechol, DL-tyrosine (a monophenol) and caffeic acid. None of the enzymes could oxidize resorcinol, phenol or 2-naphthol due to the position of their hydroxyl groups.

4.0 Discussion

Polyphenol oxidase is widely distributed in all organisms from lower to higher life forms (van Gelder, 1997). Based on its association with browning reactions in crop plants, PPO has been characterized in a wide variety of food plants including banana, wheat, quince, and avocado, and a number of chemical inhibitors have been identified (Yoruk and Marshall, 2003; Mayer, 2006). The presence of PPO in *X. mafafa* and the properties of the purified enzyme has been investigated in this study.

PPO from *X. mafafa* was purified by a combination of three steps using ion- exchange

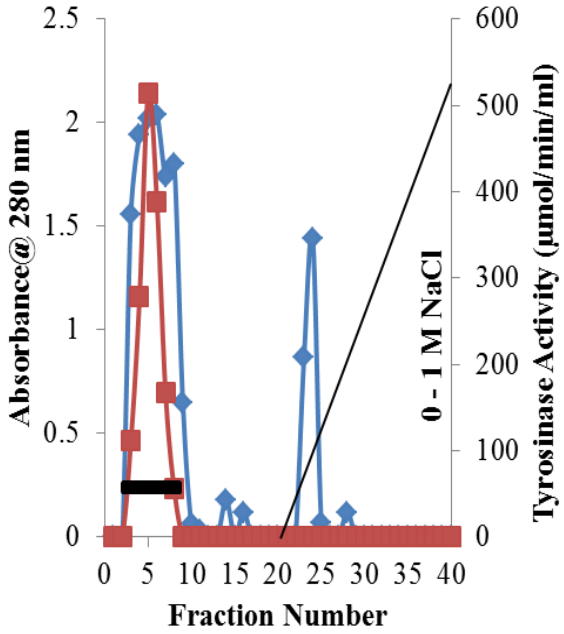

Figure 1: Elution profile of crude PPO obtained from *X. mafafa*on CM-sepharose column.Crude extract from *X. mafafa* were applied to a column (1.0 × 10 cm) of CM-Sepharose previously equilibrated with 50 mM phosphate buffer, pH 6.5. The column was washed until all the non-adhering proteins were removed.The flow rate was 12 mL/h and the bound proteins were eluted with a linear 0 – 1.0 M NaCl gradient. Fractions of 1ml each were collected and assayed for PPO activity. The protein profile was read at 280 nm using UV-Vis spectrophotometer. Active fractions ( ) were pooled. Absorbance @ 280nm( ) and PPO activity ( ).

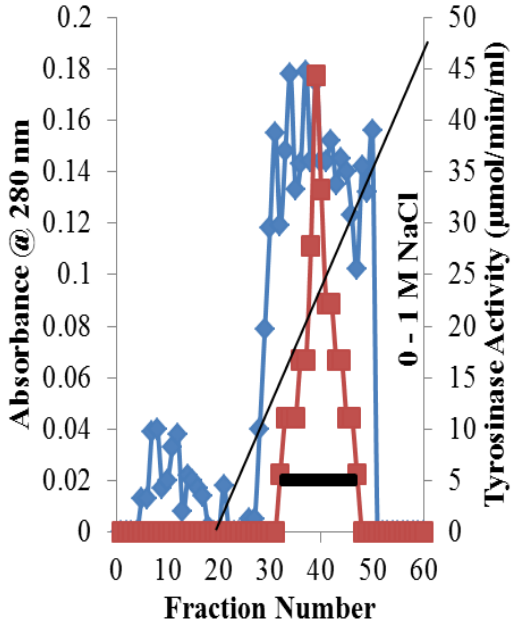

Figure 2: Elution profile of the partially purified PPO obtained from *X. mafafa* on QAE-Sephadex column. The CM-Sepharose pool was layered in batches on the QAE-Sephadex ion-exchange column (2.5 × 10 cm). Prior to layering, the column was thoroughly washed with distilled water, 1 M NaCl and equilibrated with 10 mMTris-HCl buffer, pH 7.0. Fractions of 2 mL each were collected at a flow rate of 18mL/h, and elution was done with the equilibration buffer. Linear gradient of 0 - 1 M NaCl gradient in the elution buffer was applied to the column to elute bound proteins. The protein profiles of the fractions were measured at 280 nm using UV-VIS spectrophotometer and each fraction was assayed for PPO activity. Fractions with PPO activity were pooled ( ). Absorbance @ 280nm( ) and PPO activity ( ).

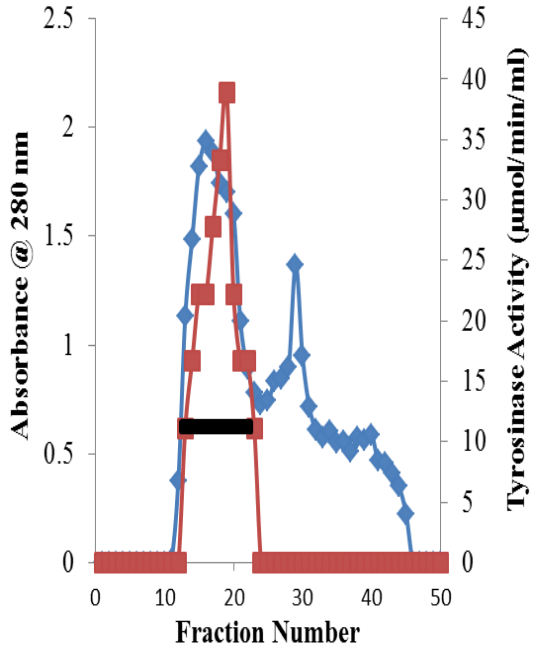

Figure 3: Elution profile of the partially purified PPO from *X. mafafa* on Sephadex G-100. The QAE-Sephadex pool obtained from *X. mafafa* was freeze-dried and re-dissolved in minimal volume of 50 mM phosphate buffer, pH 6.5 and then layered on Sephadex G-100 column (1.0 cm × 50.0 cm). The proteins were eluted with the equilibration buffer at a flow rate of 12 mL/h. Fractions of 1mL each were collected and active fractions were pooled and concentrated. Fractions with PPO activity were pooled (—). Absorbance @ 280nm(—◆—) and PPO activity (—■—).

chromatographyon CM-Sepharose column and QAE-Sephadex A-50 and then on size exclusion chromatography. The proteins gave an activity peak that came out with the flow-through fractions on CM-Sepharose ion-exchanger; this indicates that PPO from red cocoyam may not be a cationic protein. The active pool bound to the QAE-Sephadex, an anion- exchanger which suggested red cocoyam PPO to be anionic proteins. The addition of a gel filtration step increased the purity of the proteins and as well removed dark brown impurities. Polyphenol oxidase from plant origins are not usually expressed as isoforms unlike those obtained from microorganisms. Fewer reports had indicated the existence of the protein isoforms in plant origins.

The native and subunit molecular weights forPPO from *X. mafafa* showed that the native enzyme preparationishomodimeric proteins. This is consistent with the molecular weights reported for PPO from other plant sources. The molecular mass of the purified *B. megaterium* tyrosinase was approximately 35 kDa as determined by SDS-PAGE and 31 kDa as determined by size exclusion chromatography which suggested a monomeric protein (Shuster and Fishman, 2009).

Red cocoyam PPO displayed activity across a broader pH range of 4.5 – 8.5 with an optimum at 6.5. Activity was completely lost at pH 10.0 and pH below 4.0. Anosike and Ayaebene (1981) reported similar optimum pH of 7.0 for the polyphenol oxidase from *D. bulbifera*. The optimum pH also correspond to that obtained for polyphenol oxidase from *D. bulbifera*, bacterial tyrosinases such as *P. putida*F6 (pH 7.0), *Streptomycessp.* (pH 6.8) (McMahon *et al*., 2007), gill tissue of portabella mushrooms (pH 7.0) (Fan and Flurkey, 2004);*B. megaterium* (pH 7.0) (Shuster and Fishman, 2009) and fungal tyrosinase from *Pycnoporus*strains (Halaouli *et al*., 2005). However, alkaline pH were reported for some tyrosinases obtained from other sources such as *B. thuringiensis* and *T. roseum* which were 9.0 and 9.5respectively, reflecting the alkaline environments from which these bacteria were isolated (Liu *et al*., 2004).Little discrepancies in the reported values can be explained as being due to the nature of the source of enzyme, the substrate

used for the activity measurement and the purity of the enzyme. However, most results confirm that PPO is not significantly active under basic conditions.

Table 1: Purification Summary of PPO from *X. mafafa*

Sample	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	% Yield	Purification fold
Crude	18	14752	369	40	100	1.0
CM-Sepharose	30	9201	158	58	62	1.5
QAE-Sephadex	5.4	7198	32	224	52	5.6
Sephadex G-100	2.7	5700	19.7	289	41	7.2

Table 2: Kinetic parameters of PPO from *X. mafafa*.

Species	K_m (mM)	V_{max} (units/mg protein)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
*xm*PPO	6.5 ± 0.8	35 ± 0.7	0.26×10^2	3.9×10^3

The data are the mean ± standard deviation (SD) of three independent determinations.

PPO from *X. mafafa* was active at temperature range of 10 ˚C – 80 ˚C. The enzymes retained 65% of their maximum activity at temperatures between 10 ˚C – 40 ˚C and about 60% of their maximum activity at 80 ˚C. The optimal temperature for this enzyme is approximately 50 °C. The enzyme was fully stable below 60 ° C, but rapidly lost its activity above 75 °C. Thus, PPO from this specie is quite stable at high temperatures in comparison with PPO from other plant. This data could be helpful for industrial application of the enzyme at high temperature. The thermal stability could be a lead in its application to wastewater treatment. The value obtained for the activation energy in this study was much lower compared to the value for palmito (*Acanthophoenixrubra*) tyrosinase using 4-methylcatechol as a substrate (5.41 kJ mol^{-1}) (Robert *et al*., 1995), potato polyphenol oxidase using pyrogallol as a substrate (54.5 $kJmol^{-1}$) and for banana polyphenol oxidase using catechol as a substrate (18.6 kJ mol^{-1}), potato tyrosinase using L-DOPA (13.6 kJ mol^{-1}). The lower activation energy of the enzyme implies that a low minimum energy is required to initiate a reaction with the substrate and thus suggesting higher values of k_{cat}.

The kinetic parameters (K_m, V_{max}, k_{cat} and k_{cat}/K_m)were determined in aqueous system. The K_m values for L –DOPA obtained in this study is in good agreement with that of Dolashki *et al*. 2009, reporting a K_m of 7.8mM for L-DOPAfor tyrosinase from *Streptomyces albus*. Since the K_m reflects the affinity of enzyme for a substrate, the value of K_m, Michael is constant obtained for *xm*PPO revealed lower affinity for the substrate but improved product release due to decrease in tightness of the substrates thereby increasing the maximum catalytic efficiency, k_{cat}/K_m attained.

Polyphenol oxidase from *X. mafafa* had very low activity towards monohydroxyphenols but substantial activity towards dihydroxyphenols. No oxidation activity was observed for 2-naphthol, resorcinol and phenol in this work which is in accordance with the reports regarding ortho-phenolic compounds, which are poor substrates for PPO presumably because of steric hindrance (Selinheimo *et al*., 2007). The high oxidation activity towards L-DOPA, and catechol is remarkably consistent with most tyrosinases from other sources such as *S. glaucesens, N. crassa*and *T. roseum* (Kong *et al*., 2000). Plant PPO is typically found to have low or no monophenolase activity (Ilesanmi*et al*., 2014). The results may denote differences in the substrate binding pockets of these groups of tyrosinases (Selinheimo *et al*., 2007).It could be that the catecholase activity of PPO had less geometric and electronic requirements than the cresolase activity.

5. Conclusion

The presence of PPO in *X. mafafa* has been established in this report. The enzyme could thrive in high temperature. Stability against high temperature exhibited by this enzyme may be useful model for proper understanding of its stability and

could be an advantage in the use for several industrial processes. The kinetic properties of the enzyme will not only lead to a more efficient enzyme but also increase the economic potential in existing enzymatic processes. The combination of properties could be exploited for technical applications.

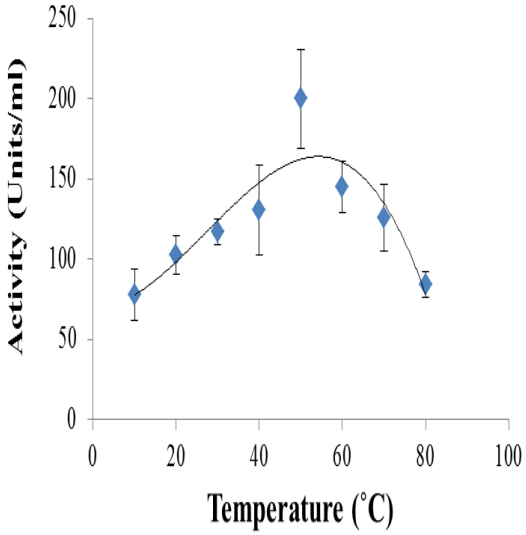

Figure 4: Effect of temperature on the activity of purified PPO from *X. mafafa*. Activity at each temperature was determined under the standard reaction conditions. The activities of the PPO from *X. mafafa* was plotted against temperature. From the plot, the optimum was estimated to be 50 ºC.

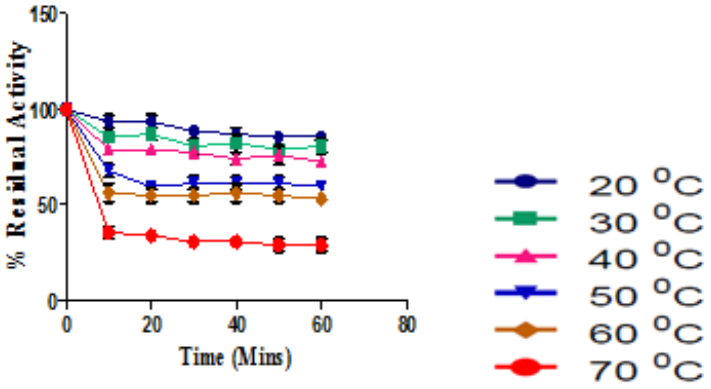

Figure 5: Thermal stability studies of purified PPO from *X. mafafa*. Aliquot of PPO from *X. mafafa* was incubated at different temperatures (20 ºC – 70 ºC) for 1 h. An aliquot of the enzyme solution (30 µl) was taken at 10 min interval and assayed for PPO activity and the residual activity was determined under the standard reaction conditions. The activity at zero time was taken as 100%. The residual activity was plotted against the time of incubation.

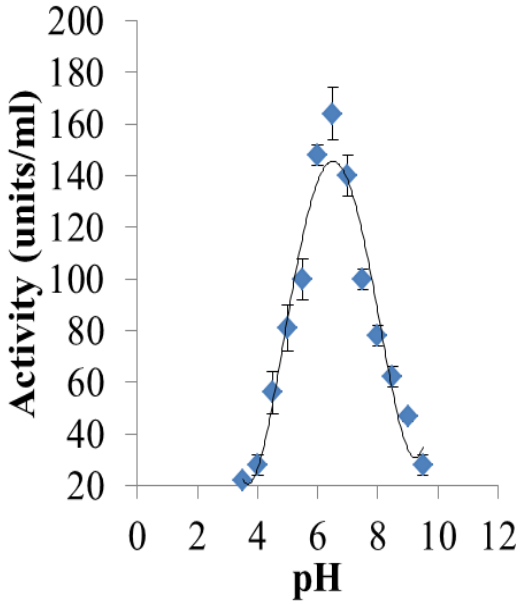

Figure 6: Effect of pH on the activity of purified PPO from *X. mafafa*. The enzyme was assayed for activity at the indicated pH values. The highest activity was obtained at pH of 6.5 for all the samples.

References

Altunkaya, A. and Gökmen, V. (2011). Purification and characterization of polyphenol oxidase,peroxidase and lipoxygenase from freshly cut lettuce(*L. sativa*). *Food Technol. Biotechnol. 49* (2) 249–256.

Anghileri, A., Lantto, R., Kruus, K., Arosio, C. and Freddi, G. (2007).Tyrosinase-catalyzed grafting of sericin peptides onto chitosan and production of proteinpolysaccharide bioconjugates.*Journal of Biotechnology* 127(3):508–519.

Anosike, E. O. and Ayaebene, A. O. (1981). Purification and some properties of polyphenol oxidase from yam tubers, *Dioscoreabulbifera*.*Phytochemistry*20: 2625 – 2628.

Ates, S., Cortenlioglu, E., Bayraktar, E. and Mehmetoglu, U. (2007). Production of L -DOPA using Cu-alginate gel immobilized tyrosinase in a batch and packed bed reactor. *Enzyme and Microbial Technology*.40: 683–687.

Bradford, M. M. (1976).A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein-dye binding.*Analytical Biochemistry*.72: 248–254.

Claus, H. and Decker, H. (2006). Bacterial tyrosinases.*Systematic and Applied Microbiology* 29(1): 3–14.

Constabel, C. P., Bergey, D. R. and Ryan, C. A. (1996). Polyphenol oxidase as a component of the inducible defense response in tomato against herbivores. In: Romeo, J. T., Saunders, J. A., and Barbosa, P. (eds) Phytochemical diversity and redundancy in ecological interactions. Plenum Press, New York. 231–252.

Dolashki, A., Gushterova, A., Voelter, W. and Tchorbanov, B. (2009). Purification and characterization of tyrosinases from *Streptomyces albus*.64:724-732.

Fan, Y. and Flurkey, W. H. (2004). Purification and characterisation of tyrosinase from gill tissue of Portabella mushrooms.*Phytochemistry*65: 671–678.

Guzman, M. and Buitrago, G. (2000).Yam: Production of Seed for Biotechnology.*Bogotá Unibiblos*.

Halaouli, S., Asther, M., Kruus, K., Guo, L., Hamdi, M., Sigoillot, J. C. and Lomascolo, A. (2005).Characterization of a new tyrosinase from *Pycnoporus* species with high potential for food technological applications. *Journal of Applied Microbiology* 98:332-343.

Halaouli, S., Asther, M., Sigoillot, J. C., Hamdi, M., and Lomascolo, A. (2006).Fungal tyrosinases: new

prospects in molecular characteristics, bioengineering and biotechnological applications.*Journal of Applied Microbiology* 100(2):219–232.

Huber, M. and Lerch, K. (1985). Primary structure of tyrosinase from *Streptomyces glaucescens*.*Biochemistry*24: 6038–6044.

Ilesanmi, O. S. and Adewale, I. O. (2020). Physicochemical properties of free and immobilized tyrosinase from different species of yam (*Dioscorea*spp). *Biotechnology Reports*, e00499.

Ilesanmi O. S., Ojopagogo Y. A. and Adewale, I. O. (2014). Kinetic characteristics of purified tyrosinasefrom different species of *Dioscorea* (Yam) in aqueous and non-aqueous systems. *Journal of Molecular Catalysis B: Enzymatic*108:111-117.

Jus, S., Kokol, V. and Guebitz, G. M. (2009). Tyrosinase-catalysed coating of wool fibres with different protein-based biomaterials.*Journal of Biomaterials, Science Polymers E*. 20(2): 253–269.

Kahn, V. (1985). Effect of proteins, protein hydrolyzates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana.*Journal of Food Science* 50: 111- 115.

Kong, K. H., Park, S. Y., Hong, M. P. and Cho, S. H. (2000). Expression and characterization of human tyrosinase from a bacterial expression system. *Comparative Biochemistry and Physiology Biochemical. Molecular Biology* 125(4): 563–569.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4.*Nature*227: 680 – 685.

Lerch, K. (1983). *Neurospora*tyrosinase: structural, spectroscopic and catalytic properties. *Molecular and Cellular Biochemistry*52: 125–138.

Liu, N., Zhang, T., Wang, Y. J., Huang, Y. P., Ou, J. H.andShen, P. (2004). A heat inducible tyrosinase with distinct properties from Bacillus thuringiensis. *Letters Applied Microbiology* 39(5): 407–412.

Martorell, M. M., Pajot, H. F., Rovati, J. I. and Figueroa, L. I. C. (2012).Optimization of culture medium composition for manganese peroxidase and tyrosinase production during reactive black 5 decolourization by the yeast *Trichosporonakiyoshidainum*.*Yeast.*29: 137–144.

Marusek, C. M., Trobaugh, N. M., Flurkey, W. H. and Inlow, J. K. (2006). Comparative analysis of polyphenol oxidase from plant and fungal species.*Journal of Inorganic Biochemistry* 100: 108–123.

Mayer, A. M. (2006). Polyphenol oxidases in plants and fungi: going places? A review.*Phytochemistry*67: 2318–2331.

McMahon, A. M., Doyle, E. M., Brooks, S. J. and O'Connor, K. E. (2007). Biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida*F6.*Enzyme and Microbial Technology* 40: 1435–1441.

Roberts, S. M., Turner, N. J., Willetts, A. J. and Turner, M. K. (1995).Introduction to biocatalysis using enzymes and microorganisms.*Cambridge University Press, New York.*

Selinheimo, E., Autio, K., Krijus, K. and Buchert, J. (2007). Elucidating the mechanism of laccase and tyrosinase in wheat bread making. *Journal of Agriculture and Food Chemistry* 55(15)**:** 6357–6365.

Shuster, V. and Fishman, A. (2009).Isolation, cloning and characterization of a tyrosinase with improved activity in organic solvents from *Bacillus megaterium.Journal of Molecular Microbiology and Biotechnology* 17:188–200.

Strothkemp, K. G., Jolley, R. L. and Mason, H. S. (1976). Quaternary structure of mushroom tyrosinase.*Biochemical and Biophysical Research Communication* 70: 519-524.

Van Gelder, C. W. G., Flurkey, W. H. and Wichers, H. J. (1997). Sequence and structural features of plant and fungal tyrosinases.*Phytochemistry*45: 1309-1323.

Weber, K. and Osborn M. (1975).Proteins and sodium dodecylsulfate, molecular weight determination on polyacrylamide gels and other procedures. In the proteins (Neurath H, Hill R. eds.), 3rd ed,1, 179-223. Academic press.

Westerholm-Parvinen, A., Selinheimo, E., Boer, H., Kalkkinen, N., Mattinen, M. and Saloheimo, M. (2007). Expression of the *Trichodermareesei*tyrosinase 2 in *Pichiapastoris*: isotopic labeling and physicochemical

characterization. *Protein Expression and Purification*55: 147–158.

Zekiri, F., Molitor, C. Mauracher, S. G., Michael, C. Mayer, R. L. Gerner, C. Rompel. A. (2104). Purification and characterization of tyrosinase from walnut leaves (*Juglansregia*). *Phytochemistry*101:5–15.

Zhang, J. P., Chen, Q. X., Song, K. K. and Xie, J. J. (2006). Inhibitory effects of salicylic acid family compounds on the diphenolase activity of mushroom tyrosinase.*Food Chemistry*95:579-584.