American-Eurasian Journal of Scientific Research 10 (4): 221-227, 2015
ISSN 1818-6785

DOI: 10.5829/idosi.aejsr.2015.10.4.1154

Partial Purification and Characterizaton of Peroxidase Extracted from *Gongronema latifolium*

Ogana Joy and S.O.O. Eze

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

Abstract: Peroxidases (EC. 1.11.1.7) are ubiquitous enzymes inthe living world, where they catalyse the oxidation of agreat diversity of substrates while reducing hydrogen peroxide (H_2O_2). We investigated here, the enzyme assay, the best concentration of hydrogen peroxide and *o*-dianisidine for the assay, the optimum pH and temperature and the determination of Vmax and km using hydrogen peroxide and *o*-dianisidine, of peroxidase from *Gongronema latifolium* from our result the best concentration of hydrogen peroxide and *o*-dianisidine for the assay is 5mM and 0.4mM respectively, optimum pH and temperature 7 and and 30°C. The apparent Vmax and km is 20u/ml and 1.8mM for hydrogen peroxide and 3.3u/ml and 0.12mM for *o*- dianisidine. From this result the purification of peroxidase from *Gongronema latifolium* using Guiacol as substrate yielded a higher purification fold when compared with *o*-dianisidine.

Key words: Hydrogen peroxide · peroxidases · *o*-dianisidine and Guaiacol

INTRODUCTION

Peroxidases are known to occur in different tissues and the pattern of expression and properties of these peroxidases vary between them. Peroxidases are haem-containing oxidoreductases (EC 1.11.1.7) that reduce peroxides, mainly hydrogen peroxide, to water and subsequently oxidize small molecules, often aromatic oxygen donor [1, 2 and 3].They are ubiquitous in nature and are involved in various physiological processes in plants. Studies have suggested that peroxidases play a role in lignification, suberization, cross-linking of cell wall structural proteins, auxin catabolism, self-defense against pathogens and senescence [4, 5 and 6].Plant peroxidases contain two-calcium ions (Ca^{2+}), which are essential for the structural stability and thermal stability of the enzyme as well as its *in vitro* activation during analysis [7, 8, 9]. Peroxidases are widely used in clinical laboratories, industries and in environmental conservation [10-13]. Several peroxidases have been isolatedsequenced and characterized. They has essentially been classified in three classes,supported in the first instance by comparison ofamino acid sequence data and confirmed bymore recent crystal structure data (class I, intracellular prokaryotic peroxidases; class II,extracellular fungal peroxidases and class III,secretory plant peroxidases) [14]. *Gongronema latifolium* is known as '*utazi*' in the southeastern and '*arokeke*' in the south-western part of Nigeria. Also *Gongronema latifolium* is called "madumaro" by Yoruba ethnic group in Nigeria It is a perennial edible plant with soft and pliable stem. It is a tropical rainforest plant which belongs to the family of *Aslepiadaceae* [15, 16, 17]. It is a climber with tuberous base found in deciduous forest from Guinea Bissau and western Cameroons. Various parts of these plants, particularly the stems and leaves are used as chewing sticks or liquor and in places such as Sierra Leone they are also used as a decoction or cold infusion of pounded stem is used for colic and intestinal symptoms usually associated with worm [18, 19, 20, 21]. The liquor, usually obtained after the plant is sliced and boiled with lime juice or infused in water over three days is usually taken as a purge for colic and stomach pains as well as to treat symptoms connected with worm infections [7, 8]. In Ghana the boiled fruit are used as laxative. In Eastern State of Nigeria, the leave are used to prepare food for mother that have recently put to bed, where it is believed to stimulate appetite, reduce post-partum contraction and enhance the return of the menstrual cycle [12, 13]. Since previous studies highlighted the environmental importance of plant peroxidase, there is no information published about *Gongronema latifolium* leaf peroxidase. We describe here the optimum pH, temperature, the best concentrations of hydrogen peroxide and *o*-dianisidine to be use for the assay and determination of Vmax and Km of peroxidase from *Gongronema latifolium* leaf.

Corresponding Author: Ogana Joy, Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.

MATERIALS AND METHODS

Sample Collection and Location: Matured fresh leaves of *Gongronema latifolium* were purchased from Ogige Market in Nsukka Senatorial Zone of Enugu State Bovine serum albumin (BSA) (Merck, England.) Folin-Ciocalteau Phenol Reagent (Sigma-Aldrich, Germany.) Guaiacol Hydrogen peroxide (BDH pool, England.) *O*-Dianisidine.

Preparation of Enzyme Extract: The leaves of *G. latifolium* were left under room temperature to dry for 24 hours. A known weight, 35g was weighed out and finely ground with pestle and mortar. The powdered sample was put into a beaker, after which 200ml of 0.01M ice cold phosphate buffer (pH 6.5) was added. The mixture was stirred and the solution was filtered using cheese cloth. The filtrate was collected and centrifuged at 4000 rpm for 30minutes in order to remove chlorophyll. The supernatant was measured and found to be 160ml. The enzyme extract was stored in the refrigerator.

Peroxidase Assay Using *O*-Dianisidine as Substrate: Peroxidase activity was assayed using the modified method of Eze *et al.* [5]. The change in absorbance at 460nm due to the oxidation of *o*-dianisidine in the presence of hydrogen peroxide and ezyme extract at 30°C was monitored using Jenway 6405 UV/VIS Spectophotometer. The standard assay solution contained 0.3ml of 0.1% *o*-dianisidine, 0.2ml of hydrogen peroxidase, 2.4ml of sodium phosphate buffer pH 6.5 and 0.1ml of enzyme extract in total of 3.0 ml.

One unit of enzyme activity was defined as the amount of enzyme that gave an absorbance change. = 0.1/min at 30°C.

Peroxidase Assay Using Guaiacol as Substrate: Peroxidase activity was measured using the method reported by Melda *et al*. [15]. Peroxidase substrate solution was prepared daily by mixing 0.1 ml guaiacol, 0.1 ml hydrogen peroxide (30%) and 99.8 ml 0.1M sodium phosphate buffer (pH 6.5). Peroxidase assays were conducted by pipetting 0.12 ml of enzyme extract and 3.48 ml of substrate solution in the cuvette. The peroxidase activities were measured from the increase in absorbance at 470 nm using an UV/VIS spectrophotometer (Jenway 6406). The reaction was monitored for 5 min at 30sec intervals at 25°C. All experiments were run in triplicates.

Determination of Optimum pH: The activity of peroxidase was examined within the pH range of 4.0 - 9.0 using the following buffer systems: Sodium-acetate buffer (0.1M, pH 4 - 4.5); sodium phosphate buffer (0.1M, PH 5.0 – 7.5); Tris – HCl buffer (0.1M, PH 8 – 9.0) The residual activity was then determined using o-dianisidine as the reducing substrate for the assay method.

Optimum Temperature: The optimum temperature was determined at the optimum pH by measuring the activity of the enzyme in temperatures ranging from 10to 90°C .

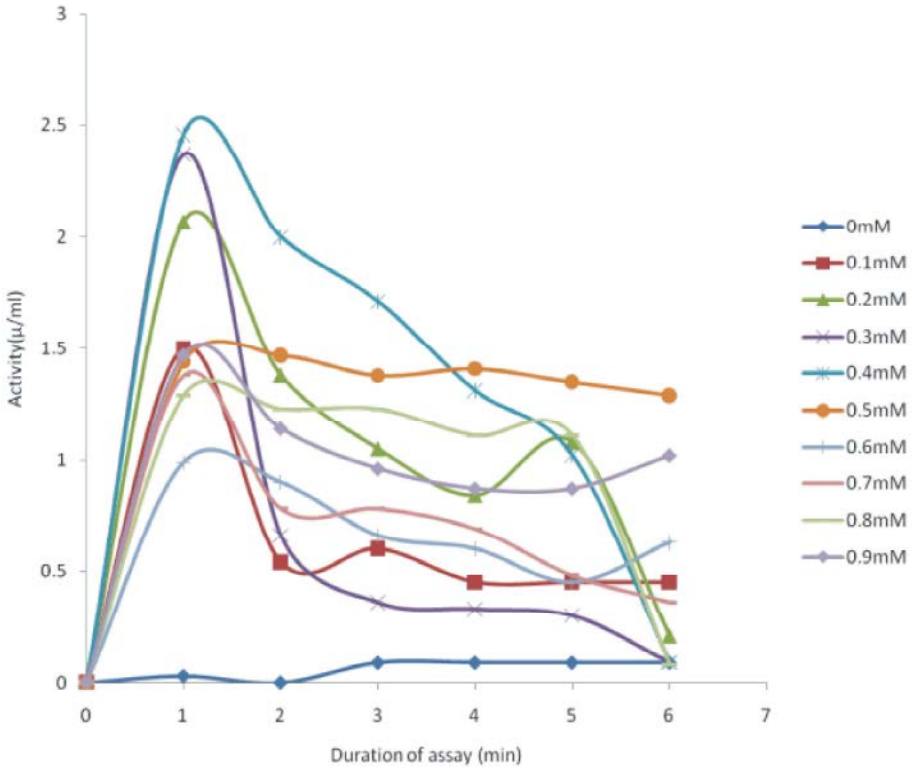
RESULTS

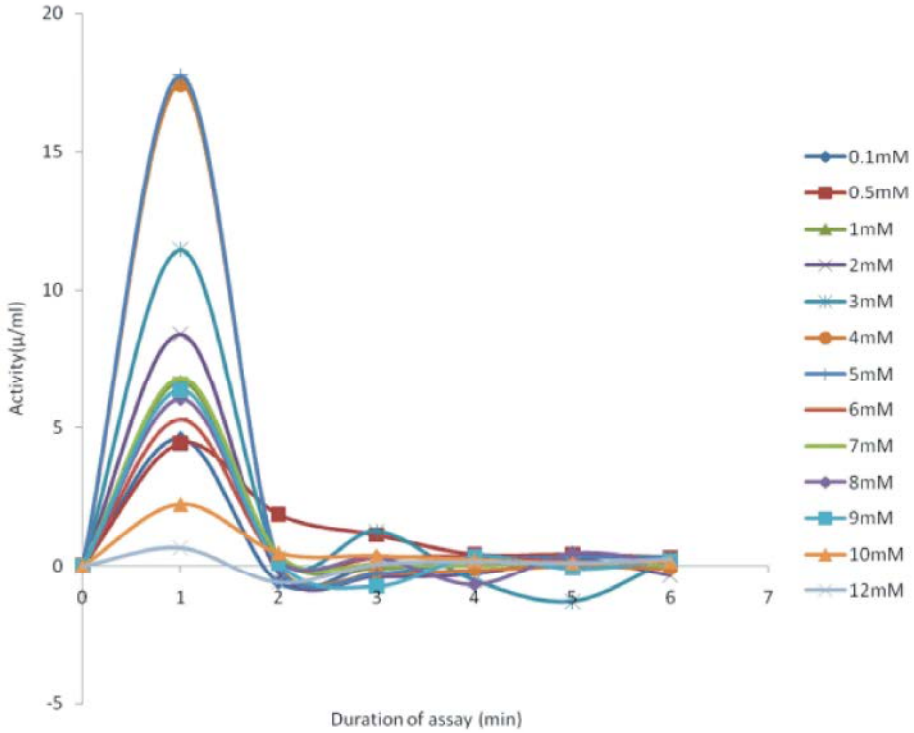
Purification of Peroxidase: Table 1 shows the purification profile of peroxidase from *Gongronema latifolium* on a two-step purification process of ammonium sulphate precipitation followed by dialysis. The enzyme was purified 2.49 fold with a specific activity of 3.71 when guaiacol was used as substrate and a purification fold of 6.8 and specific activity of 2.04 when *o*-dianisidine was used as the substrate.

The Effect of Different Concentration of *O*-Dianisidine on Peroxidase: Figure 1 shows the effect of *o*-dianisidine on peroxidase activity. Peroxidase activity varies with time (of assay) and with concentration of the substrate used for the assay. At concentrations of 0.5mM to 0.9mM, the activity plot tends to stabilize after 1minute while following the same pattern at concentration of 0.2, 0.3 and 0.4mM, the decrease in activity was sharper after 1minute of assay. From the graph the peak activity was observed at 0.4mM at 1minute of assay.

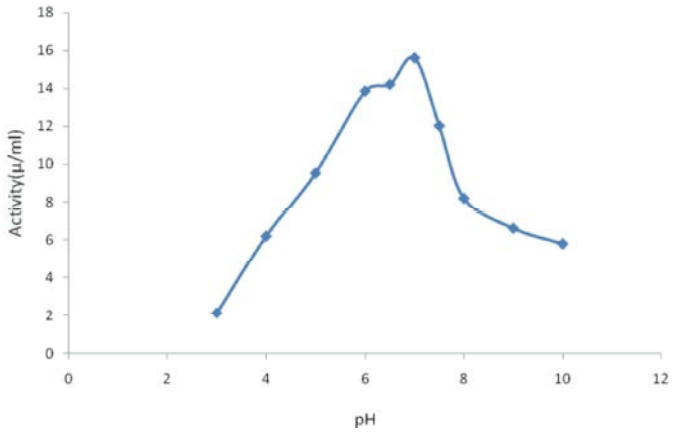
Table 1: Purification Table

Procedure	Total Protein		Specific Activity		Total Activity		Activity yield		Purification factor	
S	A	B	α	B	A	B	α	B	α	B
Crude Enzyme	6.0	7.53	0.30	1.30	1.36	5.0	100	100	1.0	1.0
70% $NH_4(SO_4)_2$ ppt	6.0	31.09	1.02	1.23	196	230	34	9.45	3.4	0.95
Dialysed Enzyme	8.0	8.02	2.04	3.71	131	238	90	37.98	6.8	2.49

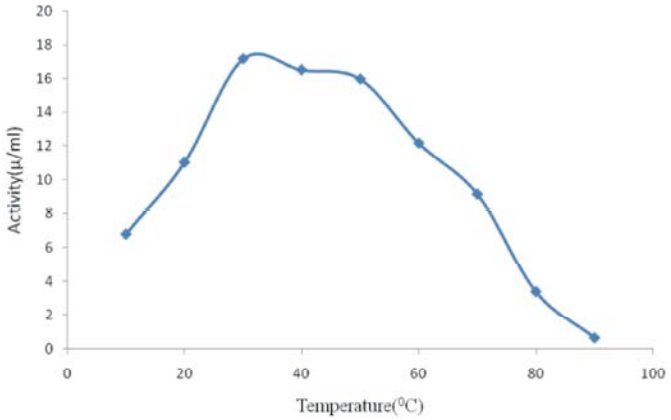




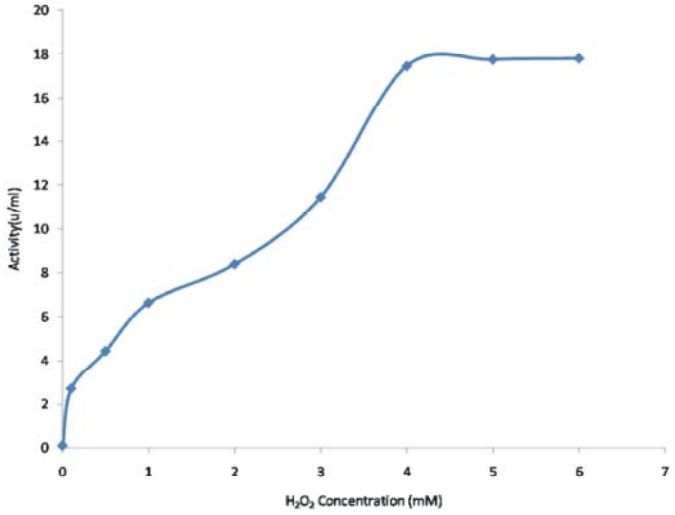
The Effect of Different Concentration of Hydrogen Peroxide on Peroxidase: Figure 2 shows the effect of hydrogen peroxidase on peroxidase activity. Peroxidase activity varies with time of assay and concentration of hydrogen peroxide. All the concentrations tends to stabilize after 1minute of assay. From the graph the peak activity was observed at 5mM at 1minute of assay.



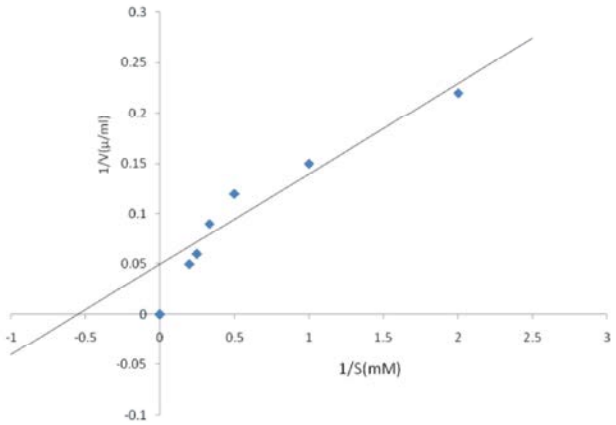
Effect of pH on Peroxidase Activity: Figure 3 shows that peroxidase activity was pH-dependent, a maximum enzyme activity of 17.15 µ/ml was observed at pH 7.0, there were decreases in activity after pH 7.



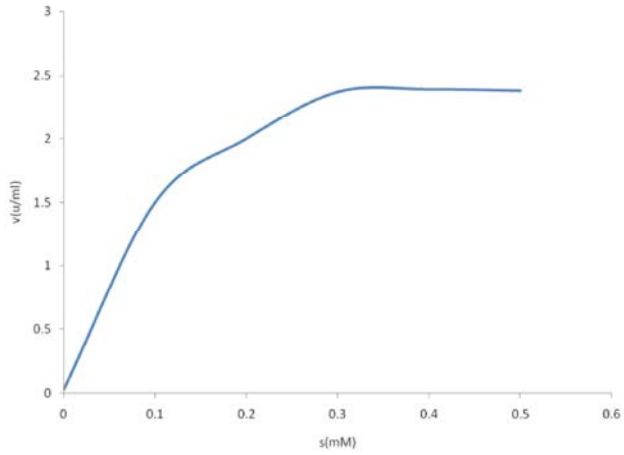
Study on Temperature Optimum: From Figure 4, the optimum temperature was observed at 30°C with the highest activity of 17.15 µ/ml, after which the activity of the enzyme began to decrease with almost complete inactivation at 90°C



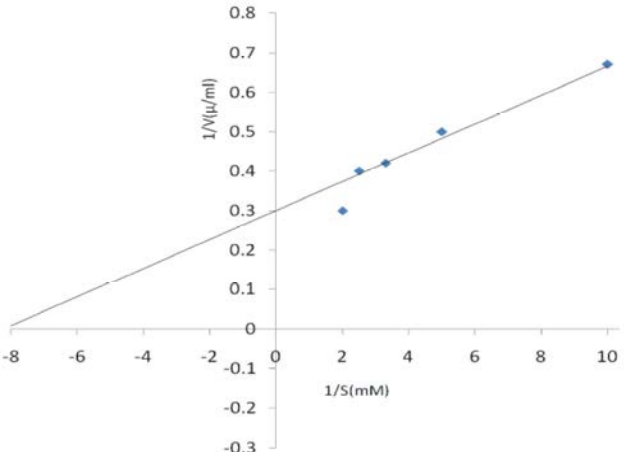
Variation of Peroxidase Activity with Different Concentrations Hydrogen Peroxide: Figure 5 shows the effect of different concentration of hydrogen peroxide (0.1,0.5, 1, 2, 3, 4, 5 and 6 mM) on peroxidase activity. As the concentration of hydrogen peroxide increases, the activity of the enzyme also increases, until it reached 4mM. At that concentration, the activity became constant indicating hyperbolic curve of Michaelis-Menten plot.



Determination of Km and Vmax: Figure 6 is the Lineweaver-Burk plot from variation of peroxidase activity with hydrogen peroxide. From this plot the Vmax is 20 u/ml and the Km is 1.8mM



Variation of Peroxidase Activity with Varying Concentration of *O*-Dainisidine: Figure 7: shows the Michealis–Menten plot of *o*-dianisidine on peroxidase. As the concentration of o-dianisidine increases the activity of peroxidase also increases until the concentration reached 0.3mM. At this point increase in the concentration of *o*-dianisidine does not lead to corresponding increase in the velocity of the reaction



The Lineweaver-burk Plot of Effect of Different Concentrations of *O*-dianisidine on Peroxidase Activity: Figure 8 shows the Lineweaver-Burk plot, from Michealis-Menten plot of *o*-dianisidine on peroxidase. The Km is 0.12mM and Vmax is 3.3 u/ml.

DISCUSSION

The purification profile of peroxidase from *Gongronema latifolium* on a two-step purification process of ammonium sulphate precipitation and dialysis yielded enzyme with 2.49 fold of purification and specific activity of 3.71 when guaiacol was used as a substrate, while a purification fold of 6.8 and specific activity of 2.04 was obtained when *o*-dianisidine was used as substrate. Yihong *et al*. [20] reported a purification fold of 17.92 on peroxidase from lettuce stems on a three-step purification of ammonium sulphate precipitation, G-100 filtration and concanavalin A affinity chromatography. Also Melda *et al*. [15] reported a purification fold of 9.7 with a three-step purification process of ammonium sulphate precipitation, dialysis and a CM Sephadex ion exchange chromatography on peroxidase from Turkish black radish. On a four-step purification process of Sephadex G-25, ammonium sulphate precipitation, DEAE Sepharose and cancanavalin A Sepharose, peroxidase from horseradish was purified 2692 fold. The low purification fold from this result was as a result of only two-step purification process used, when compared to the three or four-step purification reported on other sources of peroxidases.

It was known that pH is a key factor for enzyme activity, as it changes ionization states of the protein and substrate (Gawlik-Dziki *et al*., 2008) [6]. Peroxidase from *Gongronema latifolium* has an optimum pH of 7.0. Mamounata *et al*. [12] reported optimum pH of four different sources of peroxidase, pH 5.5 to 6.5 for *Allium sativum,* pH 6 for *Ipomoea batatas,* pH 5 for *Raphanus sativus* and pH 3.5 to 4 for *Sorghum bicolor*. Also the optimum pH range 6.5-7.5 has been reported previously by Khalil-Ur-Rehaman *et al*.(1999) [8] on peroxidases from different kinds of vegetable. In the cases of horseradish peroxidase and beans cell peroxidase the pH optimum were 8.5 and 7.2 respectively (Bowell *et al.*, 2002) [1]. The result of this study is consistent with the findings of Majed and Mohammad (2005) [11] who reported that haem-peroxidase from palm tree leaves is stable over a broad pH range with optimum pH at pH 7.0. Also, Kim and Lee (2005) [9] reported optimum pH of 5 from cauliflower bud peroxidase when guaiacol was used as a substrate, pH 4 when ABTS and catechol were used and pH of 7.5 when pyrogallol and 4-methyl catechol were used as substrates. An optimum pH of 7.0 from this study will enable peroxidase from *Gongronema latifolium* to be applied widely in industrial processes.

Peroxidase is thought to be the most heat stable enzyme in plant, because plant peroxidases are glycosylated proteins. It was observed in this investigation that peroxidase from *Gongronema latifolium* has an optimum temperature of 30°C and the activity was at minimum at 90°C, showing inactivation of the enzyme. Optimum temperature 30°C correlated those of Civello *et al*. (1995) [2] who reported maximum enzyme activity at 30°C. It is also interesting to note that Mamounata *et al.* (2011) [12] also reported optimum temperature on four different sources of peroxidase as follows, 40°C for *Allium sativum* and *Sorghum bicolor,* 30°C for *Ipomoea batatas* and *Raphanus sativu* (which are consistent with these result). Also Yihong *et al*. (2002) [20] reported 45°C for peroxidase purified from lettuce stems. Optimum temperature varies among species of plant, also differences in optimum temperature may be as a result of different reducing substrates used for the assay. Most industrial applications of peroxidase use temperature ranges of 25 to 55°C; as such peroxidase from *Gongronema latifolium* with optimum temperature of 30°C can be applied in some of these processes.

The effect of the substrates (hydrogen peroxide and *o*-dianisidine) concentration on peroxidase activity showed that the activity of peroxidase increased with corresponding increase in substrates concentration until a saturation point of about 5mM for hydrogen peroxide and 0.3mM for *o*-dianisidine, indicating that the active sites are saturated with the substrates. The Km from these results was 1.12mM for *o*-dianisidine and 1.8mM for hydrogen peroxide and Vmax value of 3.3u/ml for *o*-dianisidine and 20µ/ml for hydrogen peroxide. Similar to these results is the observation of Kim and Lee (2005) [9] that reported Km value of 1.18mM for *o*-dianisidine and 1.27mM for hydrogen peroxide, with Vmax 0.032 u/ml/min for *o*-dianisidine and 0.138 u/ml/min for hydrogen peroxide, for peroxidase from *Raphanus sativus*. Also, Melda *et al.* [15] reported that peroxidase from *Raphanus sativus* had Km values of 0.036mM for guaiacol and 0.0084mM for hydrogen peroxide, with Vmax values of 3512.23u/ ml/min and 38728.17u/ml/min respectively. From these reports it is evident that the nature of different reducing substrates affect the Km and Vmax of peroxidase.

REFERENCES

1. Bolwell, G.P., V.L. Bindschedler, K.A. Blee, V. Butt, D.R. Davies, S.L. Gardner, C. Gerrish and F. Minibayeva, 2002. The apoplastic oxidative burst in response to biotic stress in plant: A three component system. Journal Experimental Botany, 53: 1367-1376.

2. Civello, P.M., A.G. Martinez, A.R. Chaves and M.C. Anon, 1995. Peroxidase from strawberry fruit: Partially purification and determination of some properties. Journal of Agriculture and Food Chemistry, 43: 2596-2601.
3. Deighton, F.C., 1957. Vernacular Botanical Vocabulary for Sierra Leone. Crown Agents for Overseas Government and Administration, London, pp: 681.
4. Delannoy, E., P. Marmey, A. Jalloul, H. Etienne and M. Nicole, 2006. Molecular analysis of class III peroxidases from cotton. The Journal of Cotton Science, 10: 53-60.
5. Eze, S.O.O., F.C. Chilaka and B.C. Nwanguma, 2010. Studies on thermodynamics and kinetics of thermo-inactivation of some quality-related enzyme in white yam (*Dioscorea rotundat*). Journal of Thermodynamic Catalysis, 1: 104.
6. Gawlik-Dziki, U., U. Zlotek and M. Swieca, 2008. Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa*). Journal of Food Chemistry, 107: 129-135.
7. Hiraga, S., K. Sasaki, H. Ito, Y. Ohashi and H. Matsui, 2001. A large family of class III plant peroxidases. Cell Physiology, 42: 462-468.
8. Khali-Ur-Rehaman, M., M.A. Yaqub Sheikh and S. Arshad, 1999. Extraction and evalution of peroxidase from various vegetable sources. International Journal of Agriculture and Biology, 3: 170-173.
9. Kim, S.S. and D.J. Lee, 2005. Purification and characterization of a cationic peroxidase Cc in *Raphanus sativns*. Journal of Plant Physiology, 162: 609-617.
10. Lopez-Molina, D., A.N.P. Hiner, J. Tudela, F. Garci-Cànovas and J.N. Rodriguez-Lopez, 2003. Enzymic removal of phenols from aqueous solution by artichoke (*Cynana scolymus* L.) extracts. Enzyme and Microbial Technology, 33: 738-742.
11. Majed, S.A. and A.I. Mohammand, 2005. Thermostable characteristics of peroxidase from Arabian palm date (*Phoenix dactylifera L*). Saudi Journal of Biological Science, 12: 1.
12. Mamounata, D., H.K. Oumou, O. Nafissetou, G.B. Romaric, H.N.B. Imael and H.D. Mamoudou, 2011. Comparison of peroxidase activity from *Allium sativum, Ipomoea batatas,Raphanus sativus* and *Sorghum bicolor* grown in Burkina Faso. African Journal of Biochemistry Research, 5 124-128.
13. Manu, B.T. and U.J.S. Prasada Rao, 2009. Calcium modulated activity enhancement and thermal stability study of a cationic peroxidase purified from wheat bran. Journal of Food Chemistry, 114: 66-71.
14. McLellan, K.M. and D.S. Robinson, 1987. Purification and heat stability of brussels prout peroxidase isoenzymes. Food Chemistry, 23: 305-319.
15. Melda, S., G. Lhami, C. Murat, M. Ali, B.K. Hilal Habibe and O. Hasan, 2010. Purification and characterization of peroxidase from Turkish black radish (*Raphanus sativus* L.). Journal of Medicinal Plants Research, 4: 1187-1196.
16. Morebise, O., M.A. Fafunso, J.M. Makinde, O.A. Olajide and E.O. Awe, 2002. Anti-inflammatory property of the leaves of *Gongronema latifolium*. Phytotherapy. Reseach, 1: 75-77.
17. Ugochukwu, N.H. and N.E. Babady, 2002. Antioxidant effects of *Gongronema latifolium* in hepatocytes Insulin dependent diabetes mellitus. Filoterapia, 73: 612 -618.
18. Ugochukwu, N.H., N.E. Babady, M.K. Cobourne and S.R. Gasset, 2003. The Effect of *Gongronema latifolium* extract on serum lipid profile and oxidative stress in hepatocytes of diabetic rats. Journal of Biological Sciecnce, 28: 1-5.
19. Welinder, K.G. and M. Gajhede, 1993. Structure and evolution of peroxidases. Journal of Biochemistry and Physiology, 52: 35-42.
20. Yihong, H., W. Juan, L. Ping and M. Yiwei, 2012. Purification and partial characterization of peroxidase from lettuce stems. African Journal of Biotechnology, 11: 2752-2756.
21. Ozioko, Paul C., Ugwu, P.C. Okechukwu F.C. Chilaka and M.C. Ude Sylvester, 2013. Purification and Characterization of β-d-glucosidase from the Digestive Tracts of an African Giant Snail (*Achatina achatina*). World Journal of Pharmacy and Pharmaceutical Sciences, 2(3): 814-824.