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RESEARCH ARTICLE

Extraction and characterization of peroxidase from *Camellia sinensis*

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Abstract The state Assam located in the eastern part of India is well known in the world as a major tea grower. Peroxidase is an oxidative enzyme known to be involved in defence reactions in plants. Peroxidase enzyme extracted from *Camellia sinensis* (tea) leaves collected from 5 leading tea estates located in district Dibrugarh of Assam, India was characterized and optimization study was carried out. Its effect on varying substrate concentration, temperature, pH and salt was studied. Its activity was found to be inhibited by ethylene-diamine-tetra-acetic acid. The optimum temperature and pH was 30 °C and 6.5 respectively. The enzyme was thermostable showing activity up to 60 °C. Its specific activity was 6.19 U/mg protein and k_m and V_{max} values were 0.01 mg/mL (0.04 mM) and 0.03 mg/mL (0.12 mM) respectively. With all the above features, the enzyme peroxidase is proved to be useful for industrial purposes.

Keywords Peroxidase characterization · Thermostable · *Camellia sinensis*

Introduction

Peroxidase is an oxidative enzyme which is involved in defence reactions in plants [1]. Peroxidases reduce hydrogen peroxide and oxidize a wide number of compounds including phenols, aromatic amines, thioanisoles, halide ions, thiocyanate ions, fatty acids and also degrade hydrogen peroxide [2]. Plant peroxidases are induced by stress for example, pathogen attack, wounding, heat, cold or draught and UV light [3]. Products from these enzymes are also commonly used in pharmaceutical industry, waste water treatment, bio-sensor construction and food industry [4–7]. They also serve many purposes, including lignifications, suberization, wound healing, protection against pathogen attack and the scavenging of damaging hydrogen peroxide from the cell [8]. The characterisation of these enzymes may be of interest, not only for their negative effects on colour, flavour and nutritional values, but also for their positive effects on food [9, 10]. Peroxidase is widely used in health sciences, food industry and for diagnostic purposes [11]. Peroxidase isolated from soyabean was involved in the defence reaction of plant against pathogen attack [12]. Being the most heat resistant enzyme, it is used in food industry as an index in blanching procedures [13].

Peroxidases have received extensive attention in the recent years as biocatalysts for synthetic applications in biotransformation [14–17]. In plant kingdom, they are omnipresent, catalyzing a reaction in which the acceptor is hydrogen peroxide and the hydrogen donor is another compound [18]. Their industrial application has been demonstrated by several authors. Some of them perform peroxide removal from industrial wastes [19]. They are used in ELISA system [20, 21] and waste decolourization [22] and waste water treatment containing phenolic compounds [23, 24]. India is currently the foremost producer, consumer and exporter of commercial tea. The state of Assam is the world's largest tea growing region.

According to the pre-existing literature, the characterisation of peroxidase enzyme of tea was very limited.

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Fig. 1 Leaves of *Camellia sinensis* for experimental analysis

Considering this fact in mind, the present research is carried out to characterize the use of the enzyme for industrial purposes. This study optimizes peroxidase enzyme from tea leaves as this will explore a new idea about its use for industrial and agricultural purposes.

Material and Methods

Sample Collection

Tea leaves (Fig. 1) were collected from 5 leading tea gardens, Ethelwood, Moran, Lepetkotta, Borbarooah and Anandabag tea estates located in Dibrugarh district, Assam, India (Fig. 2).

Extraction of Peroxidase

1 g of fresh green tea leaves were washed thoroughly with deionised water at room temperature and were homogenised in 4.5 mL of 0.1 M phosphate buffer of pH 6.5 for 15 min [25]. The homogenate was filtered using cheese cloth to remove suspended particles. The clear filtrate was centrifuged at 10,000 rpm for 20 min at 4 °C (Sigma 3–30 K, Germany). The collected supernatant was stored at 4 °C.

Enzyme Assay

The enzyme assay was carried out by taking 0.1 mL of above extract in 0.1 mL o-dianisidine (Conc. 1 mg/mL)

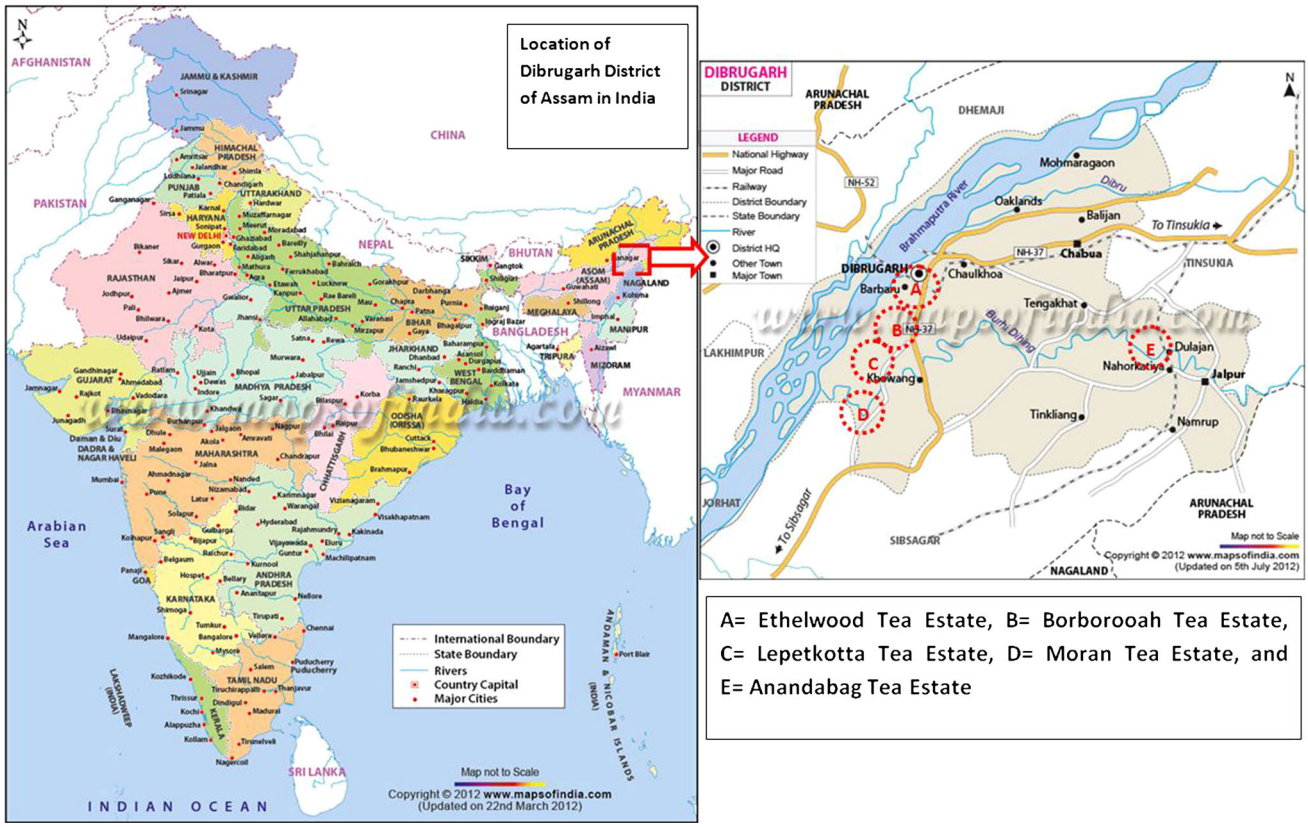

Fig. 2 Location of Dibrugarh district in India as well as the location of the five tea estates from where the samples were collected

maintained at 28 °C for 2 min (Sartorius Stedim biotech, Certomat BS-1, Germany) to carry out the reaction [25]. The reaction was stopped by adding 0.2 mL of H_2O_2 (30 %). The oxygen liberated reacts with *o*-dianisidine to give a red chromophore, which can be measured at 430 nm. The reaction is shown as follows:

$$H_2O_2 \rightarrow H_2O + [O]$$

$$o\text{-dianisidine} + [O] \rightarrow \text{oxidised } o\text{-dianisidine (coloured)}$$

The change in absorbance at 430 nm was recorded for every 30 s for 5 min during the reaction by UV–Vis Spectrophotometer (TCC-240A, Shimadzu corporation, Kyoto, Japan) (Fig. 3).

The specific activity of the enzyme was expressed in terms of units/mg of protein/mL of reaction mixture according to the following equation:

$$\text{Specific activity} = \frac{\text{enzyme activity}}{\text{protein content}\left(\frac{\text{mg}}{\text{mL}}\right)}$$

Characterisation of Peroxidase Enzyme

The thermal stability of the enzyme was assayed spectrophotometrically (at 430 nm) by incubating the reaction mixture for 25–30 min at the temperature ranging from 10, 20,…,80 °C against suitable blank. The effect of pH was monitored spectrophotometrically by carrying out the reaction at pH 5, 5.5, 6,…,9. The effect of substrate concentration was determined by carrying out the reaction at a substrate (*o*-dianisidine) concentration ranging from 0.002, 0.006, 0.010,…,0.050 mg/mL at an interval of 30 s for 5 min.

The effect of inhibitor was studied by assaying the reaction by adding 100 µL of 0.1, 0.4, 0.8,…,13 mM EDTA (ethylenediaminetetraacetic acid) solution for the above mentioned duration.

Effect of salt concentration on the isolated enzyme was determined by carrying out the reaction by adding 100 µL of 0.5, 1.0,…,8.0 % (w/v) of NaCl.

Kinetic Constants

Apparent k_m and V_{max} was determined by Lineweaver–Burk equation taking *o*-dianisidine as substrate. The lower or higher k_m value indicates higher or lower affinity of tea (*Camellia sinensis*) leaf peroxidase for substrate *o*-dianisidine.

All the chemicals used in the study were procured from Merck India Pvt. Ltd. All the tests were performed in triplicate.

Statistical Analysis

The values reported are the mean of at least three independent determinants. The significance of the study was checked by performing *t* test and *f* test.

Results and Discussion

Peroxidase was extracted and characterized from tea leaves collected from 5 leading tea estates located in Dibrugarh district of Assam, India. The optimum temperature for the enzyme activity was 30 °C, but the activity decreased significantly after 60 °C (Fig. 4). The optimum pH was 6.5 for the above reaction but the reaction rate significantly decreased after pH 7.5 (Fig. 5). Optimum substrate concentration for the reaction mixture was 0.044 mg/mL. The enzyme activity decreased after adding 4 % NaCl (Fig. 6). The reaction rate decreased gradually after adding 10–13 mM EDTA as inhibitor (Fig. 7).

The enzyme kinetics was determined with the help of Lineweaver–Burk equation (Fig. 8). K_m value was 0.01 mg/mL (0.04 mM) and V_{max} value was 0.03. The enzyme specific activity was 6.197 U/mg of protein.

The temperature effect on enzyme activity was studied at 10–90 °C. The optimum activity in terms of optical density was at 30 °C but its decreasing trend was not so

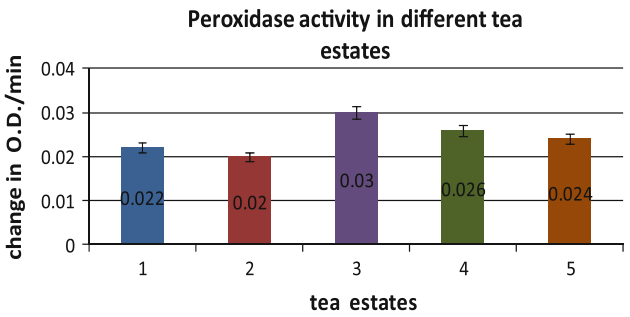

Fig. 3 Peroxidase activity of leaves of *Camellia sinensis* collected from different tea estates (*1* Ethelwood, *2* Borborooah, *3* Lepetkotta, *4* Moran, *5* Anandabag). The data is presented as mean ± SD

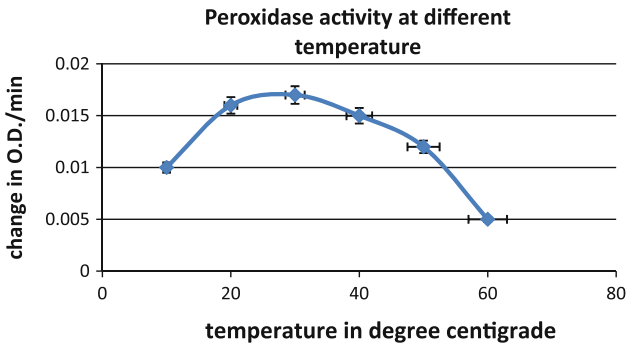

Fig. 4 Change in the activity of enzyme peroxidase in the leaves of *Camellia sinensis* pooled from above mentioned five different tea estates of Dibrugarh district at different temperature. The data is presented as mean ± SD

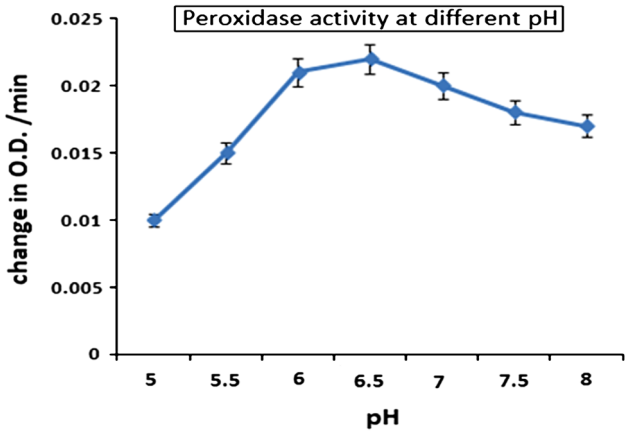

Fig. 5 Change in the activity of enzyme peroxidase in the leaves of *Camellia sinensis* at different pH. The data is presented as mean ± SD

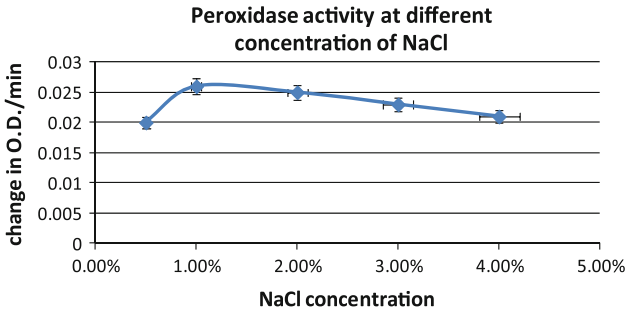

Fig. 6 Change in the activity of enzyme peroxidase in the leaves of *Camellia sinensis* at different NaCl concentration. The data is presented as mean ± SD

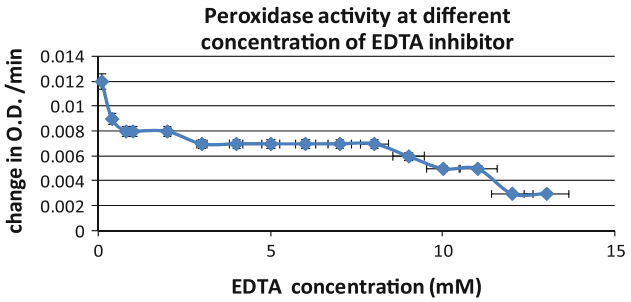

Fig. 7 Change in the activity of enzyme peroxidase in the leaves of *Camellia sinensis* at different EDTA concentration. The data is presented as mean ± SD

pronounced up to 50 °C. The fall was much sharp from 60 °C onwards. The result was similar to that of Burnette [26] who reported peroxidases to be heat stable.

pH is the key factor for enzyme activity as it changes ionisation state of protein and substrate [27–29]. In the present study, the optimum pH was 6.5. The result was in accordance with the findings of Halpin et al. [30], Civello et al. [31] and Rehman et al. [32] who found optimum pH for different vegetables to be between 6 and 6.5.

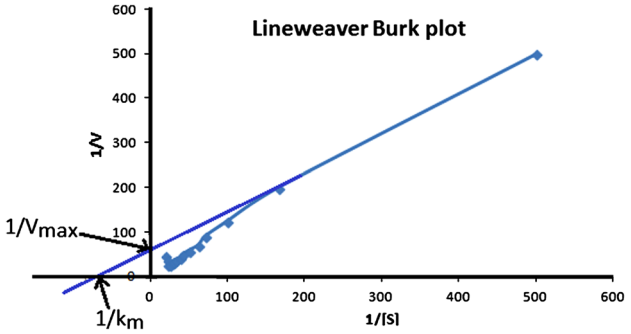

Fig. 8 Lineweaver–Burk plot for the determination of k_m and V_{max} (where, $1/V_{max} = 28.5$ or $V_{max} = 0.03$, $1/k_m = 55$ or $k_m = 0.01$, $V_{max}/k_m = 0.03/0.01 = 3$)

The optimum activity was found at 0.6 M salt concentration. This activity was almost at par (80 % activity) with the activity in the absence of salt indicating that this amount does not hamper the enzyme activity. This finding was in accordance with the peroxidase activity of radish [33].

The K_m value of H_2O_2 using guaiacol as reducing substrate was 0.9 mM for peroxidase II [34]. The significance of low K_m for H_2O_2 reflects higher hydrophobic interactions between substrate and the heme group at the enzyme active site [35]. In the current study, the K_m of *o*-dianisidine was 0.04 mM for peroxidase enzyme, which also indicated higher interaction between substrate and enzyme active site as reported with turnip peroxidase isoenzyme Cl [36].

EDTA has moderate inhibitory effect on peroxidase [37]. EDTA inhibits the enzyme activity by reacting with the peroxidase active center [38]. In the present study, the peroxidase activity was inhibited by EDTA from 10 mM and was quite prominently decreased at 13 mM.

Conclusion

Plant enzymes such as peroxidases are widely used in medicine as diagnostic tools and in the bioremediation and biobleaching industries among others [39]. Now a days, hazardous chemicals viz; formaldehyde sulphoxylate has been replaced with eco-friendly horseradish peroxidase enzyme in textile discharge printing. Advantages such as elimination of formaldehyde, energy saving, environmental friendly and reduction of strength loss have been observed by using the peroxidase enzyme discharge printing method [40]. Horseradish peroxidase immobilised on colloidal gold modified screen-printed carbon electrode displayed fast amperometric response and an electrocatalytic activity to the reduction of hydrogen peroxide (H_2O_2) without the

help of an electron mediator. The biosensor exhibited high sensitivity, good reproducibility and long term stability for the determination of H_2O_2 [41]. Recently, peroxidases from bitter gourd (*M. charantia*) immobilised on some cheaper supports have been found highly effective in decolourising reactive textile dyes as compared to its soluble counterpart as the immobilised enzyme looses only 50 % activity even after 10 cycles of usage [42].

Peroxidase enzyme has attained a crucial position in biotechnology as well as in industrial applications. Knowledge on the characterisation of enzyme is therefore very essential to know its stability in temperature, pH, substrate concentration, salt concentration, inhibitors, enzyme kinetics and enzyme specific activity so that such enzyme can be used for the industrial purposes from readily available sources present in particular area such as the case of tea plants in Assam. This will help reduce people dependency on a particular plant for industrial purposes and help in future to explore more from it.

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Conflict of Interest The authors declare that they have no conflict of interest and do not have any financial relationship with the organization that sponsored the research in the manuscript.

References

1. Chen C, Be langer RR, Behamou N, Pautitz T (2000) Defence enzyme induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol Mol Plant Pathol 56:13–23
2. Vojinovic V, Carvalho RH, Lemos F, Cabral JMS, Fonseca LP, Ferreir BS (2007) Kinetics of soluble and immobilized horseradish peroxidase-mediated oxidation of phenolic compounds. Biochem Eng J 35(2):126–135
3. O'Brien PJ (2000) Peroxidases. Chem Biol Interact 129:113–139
4. Jia J, Wang B, Wu A, Cheng G, Li Z, Dong S (2002) A method to construct a third-generation horseradish peroxidase biosensor self-assembling gold nanoparticles to three-dimensional sol–gel network. Anal Chem 74:2217–2223
5. Köksal E, Gülçin I (2008) Purification and characterization of peroxidase from cauliflower (*Brassica oleracea* L. var. botrytis) buds. Protein Pept Lett 15:320–326
6. Lavery CB, Macinnis MC, Macdonald MJ, Williams JB, Spencer CA, Burke AA, Irwin DJ, D'Cunha GB (2010) Purification of peroxidase from horseradish (*Armoracia rusticana*) roots. J Agric Food Chem 58:8471–8476
7. Prakash D, Nawani N, Kapadnis B (2013) Cloning, expression and characterisation of thermophilic alkalophilic N-acetylglucosaminidase from *Streptomyces* sp. NK 52 for the targeted production of N-acetylglucosamine. Proc Natl Acad Sci India Sect B 83(3):431–437
8. Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. Plant Cell Physiol 42:462–468
9. Matheis G, Whitaker JR (1984) Modification of proteins by polyphenol oxidase and peroxidase and their products. J Food Biochem 8:137–162
10. Matheis G, Whitaker JR (1987) A review enzymatic cross-linking of proteins applicable to foods. J Food Biochem 11:309–327
11. Kawak SS, Kim SK, Lee MS, Jung KH, Park IH, Liu JR (1995) Acidic peroxidases from suspension culture of sweet potato. Phytochemistry 39(5):981–984
12. Staehelin CM, Mellor RB, Weiemken A, Boller T (1992) Chitinase and peroxidase in the effective (fix+) and ineffective (fix−) soybean nodules. Planta 187(3):295–300
13. Reed G (1975) Oxidoreductase. In: Enzymes in food processing. Academic Press, New York, USA, p 216
14. Adam W, Lazarus M, Saha-Mollenr CR, Weichold O, Hoch U, Haring D, Schreirer CR (1999) Biotransformations with peroxidases. Adv Biochemical Eng Biotechnol 63:73–108
15. Van Deurzen MPJ, Van Rantwijk F, Sheldon RA (1997) Purification of a peroxidase from *Solanum melongena* fruit juice. Tetrahedron 53:13183–13220
16. Colonna S, Gaggaro N, Richelmi C, Pasta P (1999) Recent biotechnological developments in the use of peroxidases. Trends Biotechnol 17:163–168
17. Van de Velde F, Van Rantwijk F, Sheldon RA (2001) Improving the catalytic performance of peroxidises in organic synthesis. Trends Biotechnol 19:73–80
18. Rodrigo C, Rodrigo M, Alvarruiz A, Frigola A (1996) Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. J Food Prot 59:1065–1071
19. Saitou T, Kamada H, Harad AH (1991) Isoperoxidase in hairy roots and regenerated plants of horseradish (*Armoracia lapathifolia*). Plant Sci 75:195–201
20. Castillo L, Alpeeva I, Chubar T, Galaev I, Csoregi E, Sakharov I (2002) Purification and substrate of peroxidase from sweet potato tubers. Plant Sci 4(2):122–124
21. Deepa S, Arumughan C (2002) Purification and characterisation of soluble peroxidase from oil palm (*Elaeis guinensis* Jacq.) leaf. Phytochemistry 61:503–511
22. Jadhav UU, Dawkar VV, Telke AA, Govindwar SP (2009) Decolorization of direct blue GLL with enhanced lignin peroxidase enzyme production in *Comanonas* sp. UVS. Chem Technol Biotechnol 84:126–132
23. Lai YC, Lin SC (2005) Application of immobilised horseradish peroxidase for the removal of *p*-chlorophenol from aqueous solution. Process Biochem 40:1167–1174
24. Dalal S, Gupta MN (2007) Treatment of phenolic wastewater by horseradish peroxidase immobilised by bioaffinity layering. Chemosphere 67:741–747
25. Malik CP, Singh MB (1980) Plant enzymology and histoenzymology. Kalyani Publishers, New Delhi, p 431
26. Burnette FS (1977) Peroxidase and its relationship to food flavour and quality: a review. J Food Sci 42:1–6
27. Gawlik-Dziki U, Zlotek U, Swieca M (2008) Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var capitata L.). Food Chem 107:129–135
28. Makhija P, Nigam VK, Mohan MK, Ghosh P, Sasamal D (2006) Characterisation of extracellular alkaline proteases produced by halophilic bacteria. Proc Natl Acad Sci India 76((B) IV):362–367
29. Azmi MA, Akhtar K, Naqvi SNH, Rizvi S, Khan MFU, Shahid M (2006) Toxicity of carbofuran and determination of alkaline phosphatise in red flour beetle. Proc Natl Acad Sci India 76((B) I):85–88

30. Halpin BR, Pressey J, Sen, Mondy N (1989) Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). J Food Sci 54:644–649
31. Civello PM, Martinez GA, Chaves AR, Anon MC (1995) Peroxidase from strawberry fruit partial purification and determination of some properties. J Agric Food Chem 43:2596–2601
32. Rehman KU, Yaqub M and Arshad M (1997) Studies on comparative evaluation of peroxidase extracted from tomatoes and horseradish legume. Proceedings of the 1st National Symposim on Biotechnology for sustainable development, University of Agriculture, Faisalabad, Pakistan, p 87
33. Rehman K, Yaqub M, Sheikh MA, Arshad M (1999) Extraction and evaluation of peroxidase from vegetable sources. Int J Agric Biol 3:170–173
34. Saleh MA, Malki AR, Khan JA, Sulaiman IM, Kumosani AT (2012) Properties of peroxidase from chewing stick miswak. Afr J Pharma Pharmacol 6(9):660–670
35. Richard-Forget FC, Gauillard FA (1997) Oxidation of chlorogenic acid, catechins and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* cv. Williams) polyphenol oxidase and peroxidase, a possible involvement of peroxidase in enzymatic browning. J Agric Food Chem 45:2472–2476
36. Duarte-Vazquez MA, Garcia-Almendarez BE, Regalado C, Whitaker JR (2000) Purification and partial characterization of three turnip (*Brassica napus* L. var. *esculenta* D C) peroxidases. J Agric Food Chem 48:1574–1579
37. Marquez O, Waliszewski KN, Oliarta RM, Pardio VT (2008) Purification and characterization of cell wall-bound peroxidase from vanilla bean. LWT Food Sci Technol 41:1372–1379
38. Debowska R, Podstolski A (2001) Properties of diphenolase from *Vanilla planifolia* (Andr.) shoot primordial cultured in vitro. J Agric Food chem 49:3432–3437
39. Nuria GR, Maria OS, Edgar SM, Jesus BC (2012) Invitro production of plant peroxidases. Appl Biochem Biotechnol 66:1644–1660
40. Karthikeyan K, Durai B (2011) New method of discharge printing on cotton fabrics using horseradish peroxidase. AUTEX Res J 11(2):61–65
41. Xu X, Liu S, Ju H (2003) A novel hydrogen peroxide sensor via the direct electrochemistry of horseradish peroxidase immobilised on colloidal gold modified screen-printed electrode. Sensors 2003:350–360
42. Akhtar S, Khan A, Hussain Q (2005) Potential of immobilised bitter gourd (*Momordica charantia*) peroxidises in the decolorization and removal of textile dyes from polluted waste water and dyeing effluent. Chemosphere 60(3):291–301