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WIDE OCCURRENCE OF MANGANESE PEROXIDASE IN PLANTS

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ABSTRACT
The possibilities of the presence of MnP gene in 9 plants whose whole genome sequences are available have been predicted using bioinformatic techniques. The presence of MnP in the leave extracts of seven plants has been demonstrated. The communication reports wide occurrence of MnP in plants. It has paved the way for the search of suitable MnP containing plants which could be used for bioremediation for larger polluted areas with recalcitrant organic pollutants.

KEY WORDS: Key words: Plant peroxidase, Heme enzyme, Bioremediation, MnP genes.

INTRODUCTION

Manganese peroxidase, MnP [E.C.1.11.1.13], a heme containing enzyme, was first reported[1] in the lignolytic culture of *Phanerochaete chrysosporium,* (Kuwahara et al., 1984). Since then it has been reported in a number of fungi (Hatakka, 1994, Pelaez, 1995]. The structural and functional studies of this enzyme have been summarized in two reviews (Mertinez, 2012, Yadav and Yadav 2007). It is a biotechnologically important enzyme having potential applications in delignification of lignocellulosic materials, in biopulping and bio bleaching in paper industries and in degradation of recalcitrant organic pollutants (Kuhad and Singh, 2007). MnP genes from *Coriolus versicolor* has been expressed in transgenic tobacco plant and roots of the plants have been shown to remove recalcitrant organic pollutant pentachlorophenol from water (Iimura 2002) suggesting that such transgenic plants have great potential for the bioremediation of large areas of soils contaminated with recalcitrant organic pollutants. The reported studies opened the question whether the natural MnP containing plants are available on the earth which could be used for the above purpose. When the studies reported in this manuscript were initiated MnPs in organisms other than fungi except one plant (Yadav et al., 2012) were not known. The authors initiated search for the possibilities of the presence of MnP genes in the genomes of plants whose sequence were available in gene data bank (ncbi. nlm. nih. gov.) using bioinformatic techniques. The studies showed the probability of the presence of MnP genes in 9 plants out 29 whose genome sequences were known. The leave extracts of these plants were tested for the presence of MnP activity. The plant extracts showed the wide occurrence of MnP in plants. The results are reported in this short communication.

MATERIALS & METHODS

Chemicals

Lactic acid, sodium lactates, H_2O_2 were from s. d. fine chem. Ltd. Mumbai and 2, 6-dimethoxy phenol (DMP) and $MnSO_4$ were from Merck Ltd. Mumbai (India). All chemicals were of A.R. grade and used without further purifications.

Search for Mn genes in plant genomes

The available fungal gene were retrieved from Gene Bank, MnP genes with accession numbers CAA83148.1 and AAC8522.1 were selected and subjected to BLAST search with the available whole genome sequences of plants in the data base https://www.ncbi.nlm.nih.gov. The plant sequences to which MnP gene showed more than 50% identities were selected. The leave extract of seven such plants were tested for the presence of MnP enzyme.

Preparation of leaf extracts

The leaves of seven plants namely *Brassica juncea* (Brassicaceae), *Brassica oleracea* (Brassicaceae), *Citrus maxma* (Rutaceae), *Eucalyptus grandis* (Myrtaceae), *Ficus elastica* (Euphorbiaceae), *Hordeum vulgare* (Poaceae) and *Phaseolus vulgaris* (Fabaceae) were washed with milli Q water, dried, crushed in mortar with 4.0 ml of 100 mM sodium lactate-lactic acid buffer pH 4.5 at 25ºC using pestle and filtered through whatsmann filter paper. The filtered extract was assayed for the activity of MnP using the following method.

Assay of the MnP activity

The activity of MnP in leave extracts were assayed using the method reported by Kuan et al. (Kuan et al., 1993). The reaction solution 1 ml contained 200µM $MnSO_4$, 20 µM of H_2O_2, 10 µL of the leaf juice in 50 mM sodium lactate-lactic acid buffer pH 4.5 at 25ºC. The reference solution has the same composition as the sample solution except H_2O_2 which was added in the sample cuvette to initiate the enzymatic reaction. The reaction was

monitored spectrophotometrically by measuring the increase in absorbance at 270 nm due to the formation of Mn (III)–lactate. The molar extinction coefficient used for the calculation of the enzyme activity was 3500 $M^{-1}cm^{-1}$. UV/Vis spectrophotometer Hitachi (Japan) model U-2900 available in the Department of Chemistry, DDU Gorakhpur University, Gorakhpur, UP, India was used for spectrophotometric studies. The least count the spectrophotometer was 0.001 absorbance unit. The unit of the enzyme was the amount which transformed 1µM of Mn (II) to Mn (III) per minute under assay conditions reported above.

Assay of laccase activity

The extracts of leaves were tested for laccase activity by the method reported by the method reported by Coll et al. (Coll et al., 1993). The assay solution 1 mL contained 1.0 mM 2, 6-dimethoxy phenol [DMP] in 50 mM sodium malonate buffer pH4.5 at 25°C and 50 µL of leaf extract. The reaction was monitored by measuring the increase in absorbance at 468 nm spectrophotometrically and enzyme activity was calculated using the molar extinction coefficient value 49.6 mM^{-1} cm^{-1}.

Confirmation of presence of MnP activity in the leave extracts:

Confirmation of the presence of MnP activity in the leave extracts of plant leaves were done by oxidation of DMP by Mn (III) formed enzymatically. MnP in presence of H_2O_2 and Mn (II) generates quinone dimer from DMP which has absorption maximum at 469 nm with molar extinction coefficient value 4.96 x $10^4M^{-1}cm^{-1}$.This can be used for confirming the presence of MnP in leaves extracts. The spectra of reaction solutions 1.0 ml containing 1000 µM DMP, 200 µM MnSO4, 20 µL of leaf juice in 50 mM sodium lactate-lactic acid buffer pH 4.5 at 25ºC were recorded after 5 minute of the addition of 20 µM H_2O_2. The reference cuvette contained all the components of the sample solution except H_2O_2.

Determination of Km for Mn (II) and H_2O_2 for the plant leaves MnPs.

The apparent Km values of MnP present in plant leave extracts of *Thevetia peruviana, Azadirachta indica*, *Psidium guajava, Saraca asoka, Calotropis gigantean* for Mn(II) and H_2O_2 were determined by measuring steady state velocity at different concentrations of one substrate at a fixed enzyme saturating concentration the other. Triplicate measurements of the steady state velocity of the enzyme catalyzed reaction at a particular concentration of the substrate were made and data points are the average of the three measurements. The standard deviation was less than 5%. Michaelis –Menten plots for each substrate were drawn and Km were determined from the plots by calculating the substrates concentrations at which steady state velocities were half of their V_{max} values.

RESULTS & DISCUSSION

The results of bioinformatic studies for the search of the possibilities of the presence of MnP gene in plant genomes whole sequences for which were available in the data bank (ncbi. nlm. nih. gov.) are given in table 1.The table contains the plants names along with their plant families and the percent identity of MnP gene present in plant genome. The whole genome sequence for 29 plants were available, MnP gene showed more than 50% identities with 9 plant genomes indicating that these plants may contain MnP gene.

TABLE 1. Plants and percent identity of the MnP gene present in the genome of the plant

S.N.	Plants Name	% Identity
1.	*Boechera stricta*, (Brassicaceae)	56%
2.	*Brassica oleracea,* (Brassicaceae),	50%
3.	*Brassica oleracea,* (Brassicaceae)	55%
4.	*Brachypodium distachyan* (Poaceae)	56%
5.	*Eucalyptus grandis* (Myrtaceae),	50%
6.	*Eucalyptus nitens* (Myrtaceae),	50%
7.	*Havea brasillensis* (Euphorbiaceae),	69%
8.	*Hordeum vulgare* (Poaceae)	69%
9.	*Lablab purpureus* (Fabaceae)	50%

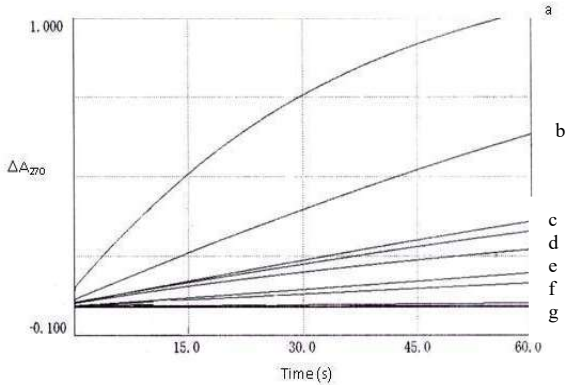

FIGURE1. Absorbance at 270 nm Vs time in the MnP oxidation of Mn (II).
The sample compsition is given in the text. The small letters refers to the following plants (a) *Citrus maxima* (b) *Brassica oleracea* (c) *Phaseolus vulgaris* (d) *Hordeum vulgare* (e) *Eucalyptus grandis* (f) *Brassica juncea* (g) *Ficus elastica*

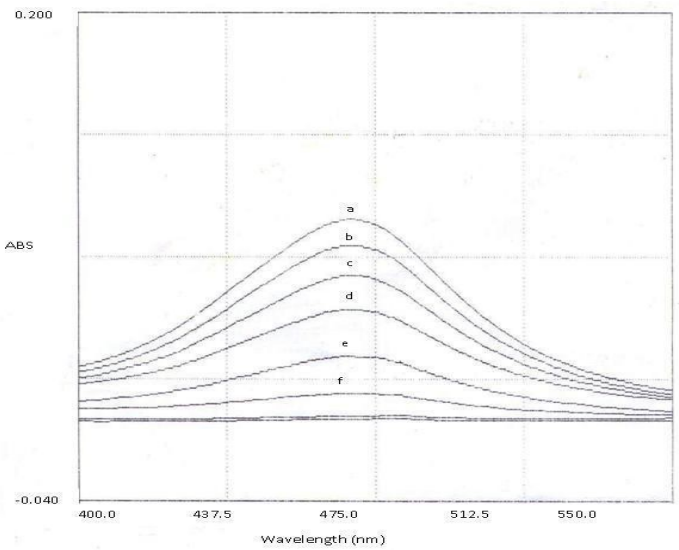

FIGURE 2. The formation of quinine dimer of oxidation product of 2, 6- di methoxy phenol as a result of oxidation by Mn (III). Solution composition given in the text. The small letters refer to (a) *Citrus maxima* (b) *Brassica oleracea* (c) *Phaseolus vulgaris* (d) *Hordeum vulgare* (e) *Eucalyptus grandis* (f) *Brassica juncea.*

The results of the assays of the leaves extracts of seven plants selected from table1 namely *Brassica juncea* (Brassicaceae), *Brassica oleracea* (Brassicaceae), *Citrus maxma* (Rutaceae), *Eucalyptus grandis* (Myrtaceae), *Ficus elastica* (Euphorbiaceae), *Hordeum vulgare* (Poaceae) and *Phaseolus vulgaris* (Fabaceae) for MnP activity are shown in fig.1 in which the increase in absorbance at 270 nm versus time are plotted. The increase in absorbance at 270 nm is due to the formation of Mn (III)-lactate by the enzymatic oxidation of Mn (II) to Mn(III) by MnP (Kuan et al.,1993). The enzymatic oxidation of Mn (II) to Mn (III) is evident from the figure showing the presence of MnP in the extracts of the leaves of the selected plants. The maximum MnP activity [14.10U/mL] was observed in the leaf of *Citrus maxima* belonging to the plant family Rutaceae followed by the leaf extract of cabbage [9.7U/ml] belonging to the family Brassicaceae. The possibility of the laccase catalyzed oxidation of Mn (II) to Mn (III) (Schlosser et al.,2002) was ruled out by testing the laccase activity of the leave extracts using 2, 6-dimethoxyphenol as the substrate((Coll et al., 1993) on the time scale of Mn (II) to Mn(III) oxidation by MnP. Further confirmation of the presence of MnP activity in the above plants were done using the oxidation of 2, 6-dimethoxyphenol. The MnP system in the presence of lactate generates a quinone dimer which has maximum absorption at 469 nm with molar extinction coefficient, $\varepsilon_{469,}$ value of 4.96 x 10^4 $M^{-1}cm^{-1}$ which can be used for the quantitative assay of MnP (Kamitsuji et al., 2004). The results of these studies are shown in figure 2 which shows the spectra of MnP system in presence of DMP in the range 400-550 nm after 5 minutes of addition of 20 µM of H_2O_2 in the reaction cuvette which contained 1000 µM DMP, 200 µM MnSO4, 20 µL of leaf juice in 1.0 mL of 50 mM sodium lactate-lactic acid buffer pH 4.5 at 25ºC. The reference solution contained every component except H_2O_2 which was added to the reaction cuvette only to initiate the reaction. The appearance of absorption maxima at 469 nm due to the formation of quinone dimer as a result of oxidation of DMP by Mn (III) is evident. In the absence of H_2O_2, these were flat lines. Different peak heights of absorption maximum in cases of juices of different plant leaves indicated the different levels of MnP present in them. In this case also maximum level of MnP was observed in case of leave extract of *Citrus maxima* followed by the leave extract of cabbage. It is worth mentioning that the above plants were selected on the basis of in silico analysis of plant genome sequences available in the databases whose numbers were only 29. There are a large number of plants whose genome sequences are not known. In order to take them into account, the authors also tested some plant leaves selecting randomly namely *Azadirachta indica* (Meliaceae), *Calotropis gigantean* (Apocynaceae) and *Thevetia peruviana* (Apocynaceae); *Psidium guajawa* (Myrtaceae) and *Saraca asoca* (Fabaceae). The leaves extract of these plants also showed the presence of MnP enzyme.

Michaelis –Menten curves of MnP present in the leaves of *P. guajawa* using MnSO4 and H_2O_2 as variable substrates are shown in fig3 (a) and 3(b), respectively. The Km values for MnSO4 and H_2O_2 of the MnPs of the above 5 plant leave extract were determined and are given in table 2. These Km values have been determined using crude enzyme extract and may differ from the Km values of the pure enzymes. Therefore these Km values have been termed apparent Michaelis constant denoted by K'm. Only one MnP from the plant source, *Musa paradiciaca* leaf has been purified (Yadav et al., 2012) and Km values of this MnP for MnSO4 and H_2O_2 have been found to be 21µM and 9.5µM respectively. The Km values of fungal MnPs for MnSO4 and H_2O_2 have been reported to be in the range of 14-200 µM and 6-39 µM, respectively [(Kuan et al.,1993, ((Coll et al., 1993, Schlosser et al.,2002, Warishi et al.,1992,Kamitsuji et al., 2004). Thus the value of apparent Michaelis constants of the MnPs of plant leaves extracts for Mn (II) and H_2O_2 are not far off from those reported for fungal MnPs.

TABLE 2. Apparent Michaelis constant Km for $MnSO_4$ and H_2O_2 of the MnPs present in plant leaves

S.N.	Name of the Plant	Km for $MnSO_4$ (mM)	Km for H_2O_2 (mM)
1.	*Azadirachta indica*	0.40	0.04
2.	*Calotropis gigantean*	0.32	0.02
3.	*Thevetia peruviana*	0.32	0.03
4.	*Psidium guajawa*	0.11	0.01
5.	*Saraca asoca*	0.23	0.02

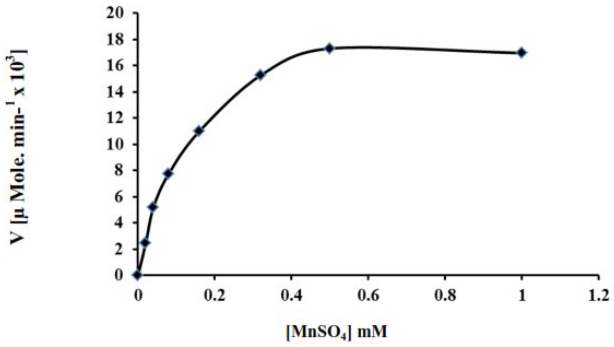

FIGURE 3a. Michaelis –Menten curves of MnP present in the leaves of *Psidium guajawa* (a) $MnSO_4$ as the variable substrate

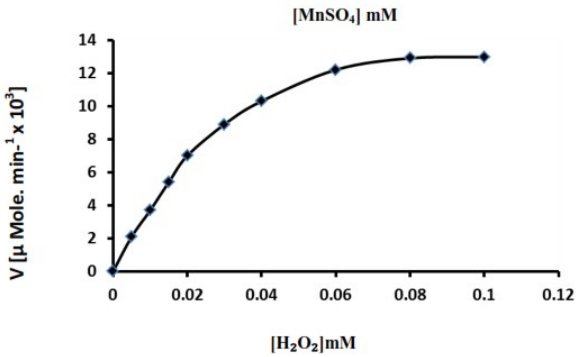

FIGURE 3b. Michaelis –Menten curves of MnP present in the leaves of *Psidium guajawa* (b) H_2O_2 as the variable substrate

CONCLUSION

The studies showed the probability of the presence of MnP genes in 9 plants out 29 whose genome sequences were known. So far MnP has been reported in fungi (Hatakka, A., 1994 and Pelaez, et al., 1995) and except for one report in a plant (Yadav et al., 2012) , the other sources of this enzyme are not known. This communication reports wide occurrence of MnP in plants. MnP is known to degrade a large number of recalcitrant organic pollutants and plants are better agents for bioremediation of larger polluted areas in comparison to fungi and bacteria, these studies pave the way for the search of suitable plants for phytoremediation of larger polluted areas with recalcitrant organic pollutants.

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