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Purification and Characterization of Sweet Potato (*Ipomoea Batatas*) Peroxidase

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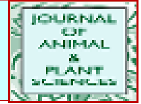
1 ABSTRACT

Peroxidase (POX), a biotechnologically important enzyme was purified from bulb of *Ipomoea batatas* and characterized for use in the bioconversion of phenolic compounds from industrial wastewater. Purified peroxidase (POXp) was obtained using the gel-filtration chromatography and anion exchange chromatography. A final yield of 18 % was obtained with 20.5 as purification fold. Electrophoresis on SDS-PAGE and native-PAGE showed that POXp is monomeric with a molecular weight of 44 kDa. The optimum pH and optimum temperature were respectively 6.0 and 45 ° C. All enzyme activity was retained for 2 hours at pH values between 4.0 to 9.0. While the enzyme was partially inhibited by SDS, PCMB and sodium disulfide, it was completely inhibited by ascorbic acid, citric acid, sodium azide and sodium thiosulfate. The presence of Calcium (Ca^{2+}), Copper (Cu^{2+}), Barium (Ba^{2+}), Manganese (Mn^{2+}) and polyethylene glycol increased POXp activity but it was reduced by Magnesium (Mg^{2+}), Sodium (Na^{+}), Potassium (K^{+}) and EDTA. However, Zinc (Zn^{2+}) had no effect on POXp. The enzyme oxidized a wide range of phenolic substrates, the greatest rate of oxidation being obtained with guaiacol. These properties may allow the use of these enzymes for bioremediation of industrial wastewater containing phenolic compounds.

2 INTRODUCTION

Plant peroxidases are heme-containing enzymes that catalyze oxidation of various organic and inorganic substrates in the presence of hydrogen peroxide (Bozzo *et al.*, 2004; Köksal, 2011). They are ubiquitous and can be obtained from plant, animal and fungus (Boeuf *et al.*, 2000; Veitch, 2004; Sunde and Thompson, 2009). In plant kingdom they are involved in many processes of plant development such as seed germination (Lewak, 1986), fruit ripening (Rothan and Nicola, 1989), abscission (Gaspar *et al.*, 1978), senescence (Abeles *et al.*, 1988), sexual differentiation (Ghosh and Basu, 1984), floral induction (Kay and Basile, 1987), lignin and suberin biosynthesis (Wakamatsu and Takahama, 1993; Bernards *et al.*, 1999; Boerjan *et al.*, 2003), auxin oxidation (Veitch, 2004), disease resistance (McLusky *et al.*, 1999) and regulation of hormone (Gaspar, 1986). POXs have enormous applications. They have been used as biocatalysts in the bioremediation of wastewater containing phenolic compounds and aromatic amines (Wagner and Nicell 2001; Alemzadeh and Nejati, 2009; Ashraf and Husain, 2010). Peroxidases have also been used as



catalysts in organic and polymer synthesis (Hutterman *et al*., 2001), decolorization of synthetic dyes (Bhunia *et al*., 2002; Akhtar *et al*., 2005) and food technology (Lavery *et al*., 2010). Moreover they are used in bio-sensor construction (Ruzgas *et al*., 1996; Jia *et al*., 2002) and application in analysis and diagnostic kits (Agostini *et al*., 2002).
Several peroxidases isoenzymes from plant sources, including sweet potato (Leon *et al*., 2002) have been isolated; however these investigations have been unsuccessful. Horseradish peroxidase (HRP) is the most extensively studied peroxidase. The availability and cost of commercially available HRP restricts its applications (Fatima *et al*., 2007). For example, HRP Type I, essentially salt-free, lyophilized powder, 50-150 units/mg solid (using pyrogallol), costs more than 45 USD/5000U (*SIGMA-ALDRICH* product). Obtaining the cheapest new source of peroxidase, to replace the HRP would be an alternative. The aim of the present study was to extract, purify and characterize a novel source of peroxidase from an economical affordable source such as *Ipomoea batatas*, which can be utilized as biocatalyst.

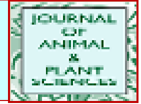
3 METHODOLOGY

3.1 Chemicals and reagents: Phenyl-Sepharose CL-4B, Sephacryl S-200 HR, DEAE-Sepharose CL-6B and CM-Sepharose CL-6B gels, BSA and H_2O_2 were obtained from Sigma Chemical Co. Protein standards and reagents used for electrophoresis were purchased from Bio-Rad. All other chemicals and reagents were of analytical grade.

3.2 Plant Material: *Ipomoea batatas* bulbs were grown in Ouagadougou (Burkina Faso) during 2008. They were stored at -20 ° C (Diao *et al*., 2011).

3.3 Peroxidase activity and total protein determination: Crude enzyme extracts were prepared by grinding the bulbs of sweet potato **(**300 g) in 450 ml of distilled water containing 0.9 % NaCl (w/v). The crushed extract was centrifuged at 6000 rpm for 30 min. The supernatant was filtered through Whatman paper N°1, stored at -20 ° C and used as crude enzyme extract. POX activity was measured with a spectrophotometric according to Dicko *et al.* (2002). The reaction mixture consisted of 10 µL of enzyme extract, 20 µL of 50 mM guaiacol, 10 µL of 100 mM H_20_2 and 160 µL of 100 mM citrate-phosphate buffer (CPB) pH 6.0. Control assays in which the enzyme extracts or substrates were replaced by buffer were performed. The reaction was monitored at 470 nm. One unit of POX activity (U) is defined as the amount of enzyme releasing 1 µmol of guaiacol radical/min under the assay conditions (Dicko *et al*., 2002). Protein concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

3.4 Purification procedure: Prior to purification, pH's effects on enzyme activities were determined in order to identify the best buffer to use**.** Results showed that 100 mM CPB, pH 6.0 is the best buffer for enzyme stability. All purification steps were made at 4 °C, pH 6. The crude extract was concentrated with 80 % of ammonium sulphate. After centrifugation (6,000 rpm, 30 min, 4 °C), the precipitate was dispersed in 100 mM CPB and again centrifuged. Supernatant was dialyzed with the same buffer and applied to sephacryl S-200 HR column (1.6X65 cm) previously equilibrated with 100 mM CPB. The column was eluted with the CPB at flow rate of 0.17 mL/min, 1 mL/fractions. The eluted fractions were collected and peroxidase activity was measured. All fractions containing strong peroxidase activity were pooled and subjected to DEAE-Sepharose column (2.6 x 6.0). Unbound proteins were removed by washing the gel with two volumes of equilibration buffer. Bound proteins were then eluted in a stepwise NaCl gradient (flow rate of 1 ml / min, 2.0 ml fractions). The pooled active fractions were dialyzed with 100 mM CPB pH 6.0, constituted the purified sweet potato peroxidase (POXp), and stored at 4 °C, prior to biochemical characterization.
Protein concentration and enzyme activities were determined at each purification step**.**



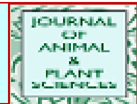
3.5 Protein electrophoresis: SDS-PAGE was performed by first denaturing protein samples by treatment in 125 mM Tris-HCl buffer, pH 6.8 containing 4 % (w/v) SDS, 1 % (v/v) β-mercaptoethanol, 20 % (v/v) glycerol and 0.025 % (w/v) bromophenol blue, at 100 °C, for 5 min. Electrophoresis was performed according to Laemmli (1970) on 1.5 mm thick slab gels (7 X 8 cm) containing 12 % of acrylamide, Tris-HCl buffer pH 8.8; 375 mM and 0.1 % (w/v) SDS. Electrophoresis was carried out in 25 mM Tris, 192 mM glycine buffer containing 0.1 % (w/v) SDS as electrode buffer, applying an intensity of 10 mA. Proteins were stained with silver nitrate according to Blum *et al* (1987). Molecular-mass standard markers (Bio-Rad) were phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soya bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). For non-denaturing PAGE, samples were mixed just before electrophoresis with sample buffer without β-mercaptoethanol and SDS. Proteins were revealed as mentioned above.

3.6 Effects of pH and temperature on enzyme activities: The effect of pH on enzyme activity was determined by measuring the oxidation of guaiacol in a set of buffers at various pH values ranging from pH 2.6 to 10.0. The used buffers were 100 mM citrate-phosphate from pH 2.6-7.0, 100 mM sodium acetate from pH 4.0 to 5.6, 100 mM sodium phosphate from pH 5.6-8.0, 100 mM tris-HCl from pH 7.0-9.0 and 100 mM glycine from pH 8.0-10.0. The pH values of each buffer were determined at 25 °C. The pH stability of the purified enzyme was studied in pH range 2.6 to 10.0 using the buffers described above. After 2 h pre-incubation at 25 °C, aliquots were taken and immediately assayed for residual POX activity. The effect of temperature on POX activity was performed in the best buffer at optimum pH over a temperature range of 10 to 80 °C using the routine POX assay. For thermal denaturation tests, the aliquots of enzyme were preincubated at different temperatures ranging from 10 to 80 °C for 30 min. Residual activities, determined at 37 °C under the standard assay conditions, were expressed as activity percentage compared to the untreated control enzyme activity.

3.7 Determination of substrate specificity: The substrate specificity of POX was determined by incubating the enzyme with various phenolic substrates (50 mM) at 37 °C in 100 mM citrate-phosphate buffer pH 5.6 for 5 min. The oxidation of these substrates was determined by monitoring the change in absorbance using a spectrophotometer (DU 7500, Beckmann, Munich). The oxidation rates of substrates were measured as a decrease in absorption (substrate disappearance) or an increase of absorbance (product appearance). The same reaction mixture and routine assay conditions were used by replacing guaiacol with ABTS (A_{405}; ε= 36.8 mm^{-1} cm^{-1}), pyrogallol (A_{420}; ε=2640 M^{-1} cm^{-1}), catechol (A_{295} ε=1700 M−1 cm−1), ferulic acid (A_{318}; ε=6000 M^{-1} cm^{-1}), or other phenolic compounds (A_{420}).

3.8 Effect of some chemicals on POX activities: To determine the effect of various compounds such as metal ions, detergents and dithiol-reducing agents as possible activators or inhibitors of the purified POX, the enzymatic solutions were preincubated at 37 °C for 2 hours with the compounds and then the remaining activity was assayed. The final concentration of ions or chemicals agents in the reaction mixture was 5 mM. The substrate guaiacol was added to the medium and incubated at 37 °C for 5 min. The residual activity was assayed as in the standard conditions.

3.9 Statistical analysis: All spectrophotometric assays were performed using a MRX 96-well microplate reader coupled to a computer (Hewlett Packard). Absorbances were automatically recorded. All assays were performed in triplicate. Data were expressed as means ± standard errors using SPSS software package.



4 RESULTS AND DISCUSSION

4.1 Purification of peroxidase: The procedure for enzyme purification is summarized in Table 1. For the partial purification of peroxidase, crude extract was precipitated by using solid ammonium sulphate. An 83 % recovery of peroxidase activity was obtained on this step. Further separation was made by gel filtration chromatography resulting in one peak (Fig. 1A). From this step, a purification factor of 3.9 was obtained, with a yield of 38 % (Table 1). The last step was obtained by ion-exchange chromatography, resulting in one peak eluted with a 0-1 M NaCl gradient (Fig. 1B). From this end-step, a final purification factor of 20.5 was obtained, with a yield of 18 % (Table1). This yield is significantly better than those (2.5 %) obtained by Bari *et al.* (2013) with the purification of a peroxidase from papaya. Nevertheless it was lower than those obtained by Goyal and Chugh (2013) with the POX of pearl millet grains (41 %), those obtained by Mall *et al.* (2013) with the POX from *Citrus medica* leaf (28.6 %) and those of apple seed POXs (39.99 %) obtained by Zia *et al.* (2011). His specific activity was 828, 77 U/mg (Table 1). Which was higher than 136.10 U/mg reported by Hu *et al.* for peroxidase from lettuce stems (2012).

Table 1. Purification of POX from Burkina potato

Purification step	Total Protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification factor	Yield (%)
Crude extract	1019±1.3	41100±1.8	40	1.0	100
Ammonium sulphate	443±1.2	34330±1.1	77	1.9	83
Sephacryl-S100 H	99±0.4	2225±0.9	157	3.9	38
DEAE-Sepharose CL-6B	9±0.2	7500±0.3	828	20.5	18

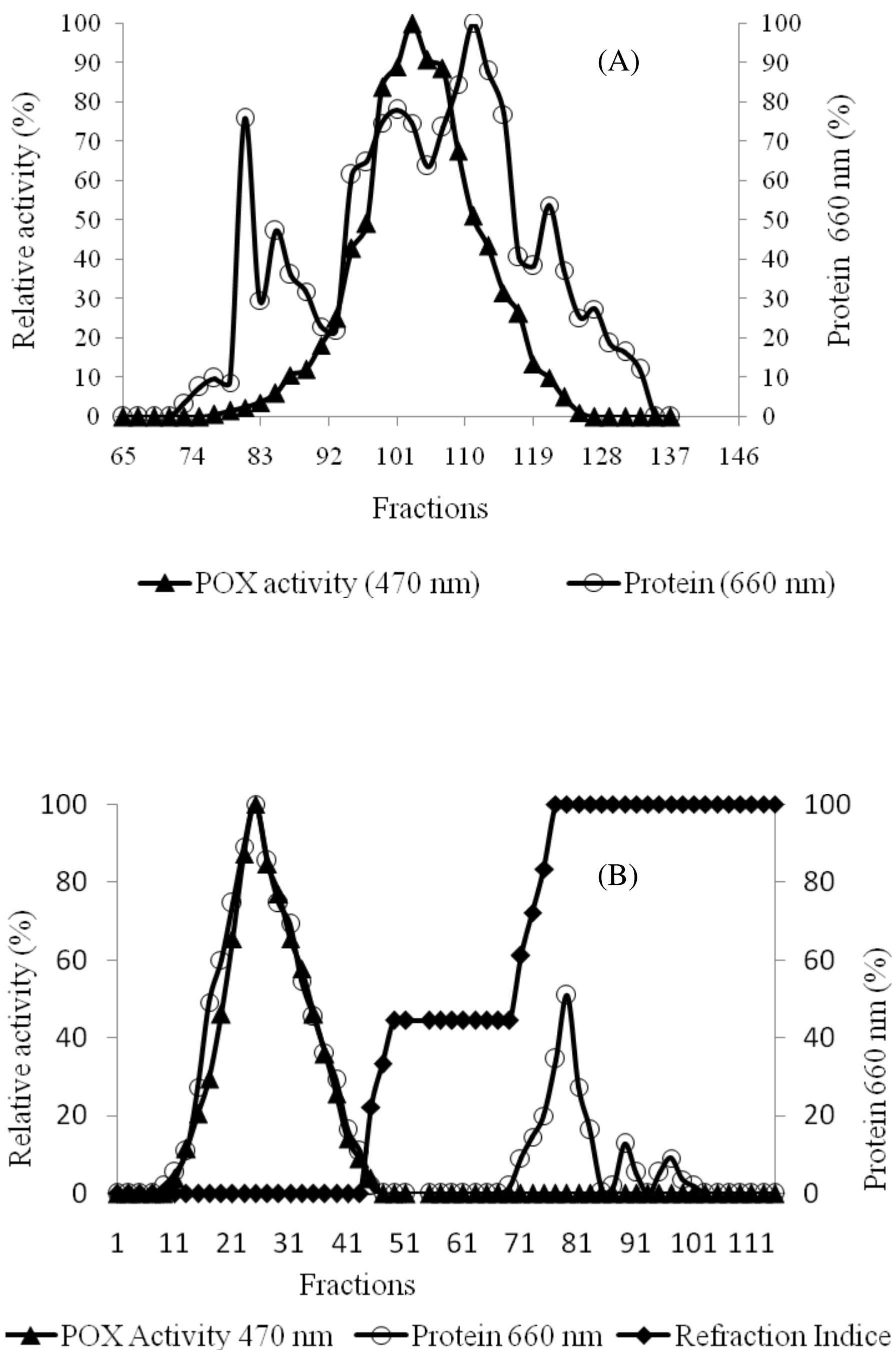


Figure 1. Purification of peroxidase from sweet potato. (A) Gel filtration on Sephacryl S-200 HR column. (B) Anion exchange chromatography on DEAE-Sepharose CL-6B column

4.2 Molecular properties: POXp appears as a single protein band of molecular weight about 44 kDa by SDS-PAGE analysis (Fig.2, lane C). The native-PAGE of the same enzyme gave also a single band, confirming the homogeneity of the enzyme and that a single isoenzyme was present at the last purification step (Fig.2, lane A). This enzyme had a monomeric structure as most of

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plant secretory peroxidases (Vitali *et al.*, 1998; Johri *et al.*, 2005; Kim and Lee, 2005; Hermelinda *et al.*, 2007). In comparison to other molecular weights of purified plant peroxidase, POXp from Burkina' sweet potato was similar to those of cationic peroxidase from *Raphanus sativus* (Kim and Lee, 2005) and those of *Moringa oleifera* leaves (Khatun *et al.*, 2012). These data are lower than peroxidase from cabbage leaves (67 kDa) (Kharatmol and Pandit, 2012) and those (70 kDa) of *Hevea brasiliensis* cell (Chanwun *et al.*, 2013). Nevertheless this POX has a molecular weight higher than peroxidase from Sweden sweet potato (*I. batatas*) tubers (37 kDa) (Leon *et al.*, 2002); *Sorghum bicolor* grain (38 kDa) (Dicko *et al.*, 2006); *Zizania latifolia* (31 kDa) (Luo *et al.*, 2012) and avocado (40KDa) (Rojas-Reyes *et al.*, 2014).

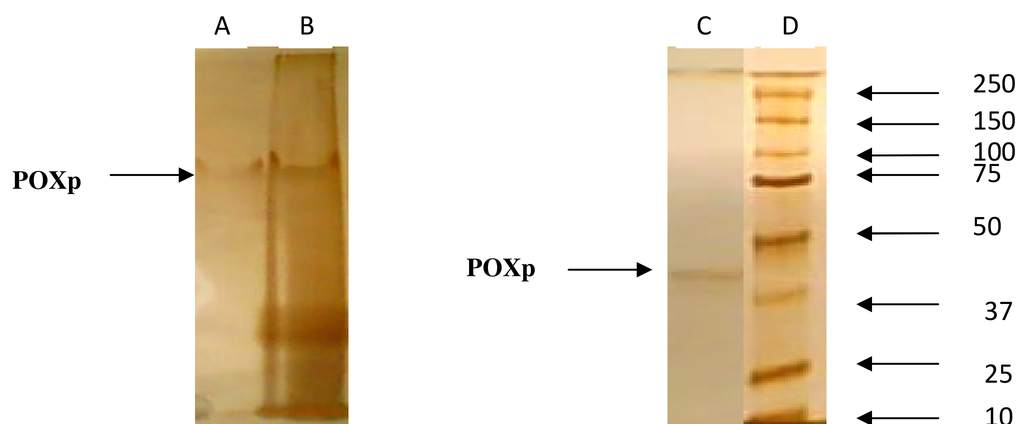

Figure 2. Electrophoresis of purified peroxidase from sweet potato. Lane A, Native-PAGE of purified POXp. Lane B, Native-PAGE of total protein. Lane C, SDS-PAGE of purified POXp. Lane D, molecular weight markers (values in kDa). Proteins were stained by silver nitrate.

4.3 pH and temperature effects: The pH effect on guaiacol oxidation is shown in Figure 3. It was found that purified POXp from sweet potato had optimum pH at 6.0. This is similar to those obtained on POX from Turkish black radish (Sisecioglu *et al.*, 2010). However, these results are lower than those of *Solanum melongena* fruit (Vernwal *et al.*, 2006) and leaves of spinach (*Spinacia oleracea*) (Köksal, 2011). After 2 hours incubation, the enzyme retained more than 80 % of its activity at pH ranging from 4.6-8.6 (Fig.4). It is important to notice that the enzyme activity is not only dependent on the pH but also on the chemical nature of the buffering substances. At extreme pHs, below 4.0 and above 9.0, POXp activity drastically decreased. At low pH, the decrease of activity might be attributed to the release of heme prosthetic group from the polypeptide chain, which resulted in the loss of enzyme activity (Lopez and Burgos, 1995, Deepa and Arumughan, 2002). The decrease of activity at high pH could be attributed to the formation of phenol-conjugated base. Therefore, the basic form did not allow the phenolic compounds to act as hydrogen donors. It is known that the active site of POX is mainly composed of ionic groups (prosthetic group) that must be in the proper ionic form in order to maintain the conformation of the active site of enzyme for substrate binding and reaction catalysis (Whitaker, 1995). Hence, the best conditions for POXp activities were obtained at pH 4.6-8.0 (Fig.4). This is quite interesting because the use of pH close to neutrality may be an advantage for biological and industrial applications. The peroxidase from *Ipomoea batatas* was optimally active at 45 °C (Fig. 5). This optimum temperature was lower than those reported for hyperthermostable peroxidase from the *Solanum melongena* (84 °C) (Vernwal *et al.*, 2006) and POX of Turnip root (50 °C) (*Brassica napus var. okapi*) reported by Saboora *et al.* (2012). However, the

optimum temperature for the enzyme activity was higher than POXs from fruits of *Mallus pumilus* (Singh *et al*., 2010) and Turkish black radish (*Raphanus sativus* L.) (Sisecioglu *et al*., 2010).

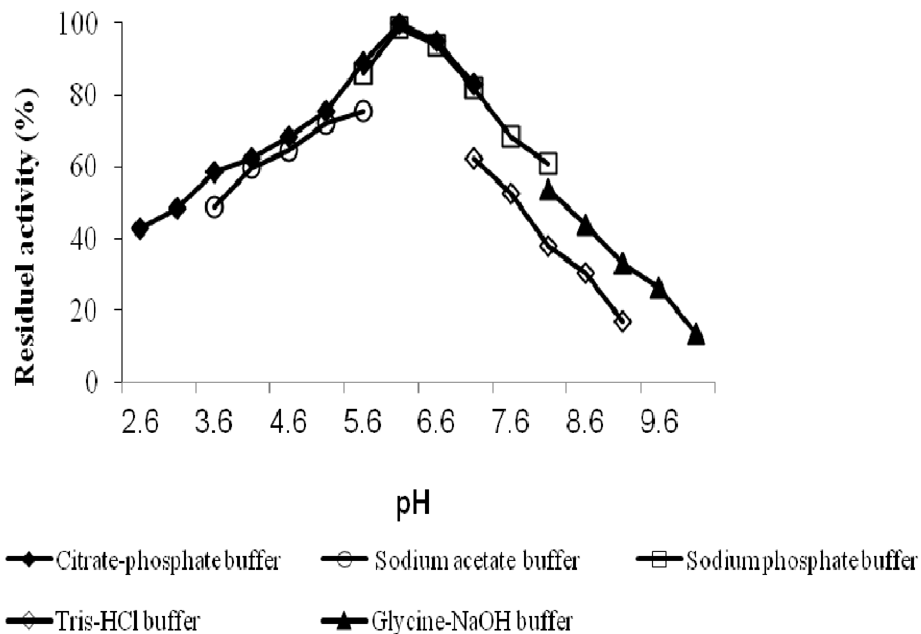

Figure 3. The effect of pH on the enzyme activity. Activities were determined by measuring the oxidation of the guaiacol in a series of buffers at various pH values ranging from pH 2.6 to 10.0.

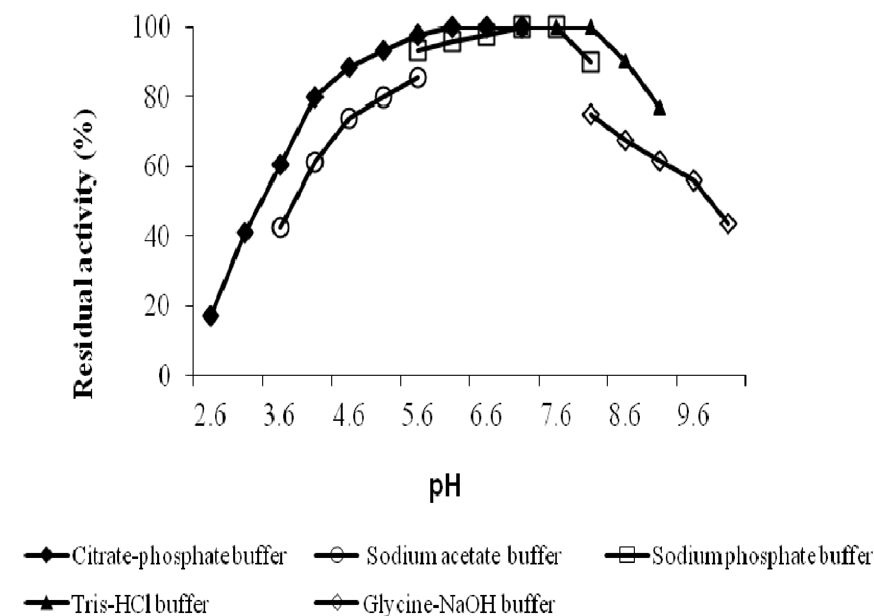

Figure 4. The pH stability of POXp. Enzyme activity was carried with guaiacol as substrate, after 2h incubation at 25 °C in series of buffers at various pH values ranging from pH 2.6 to 10.0.

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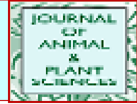


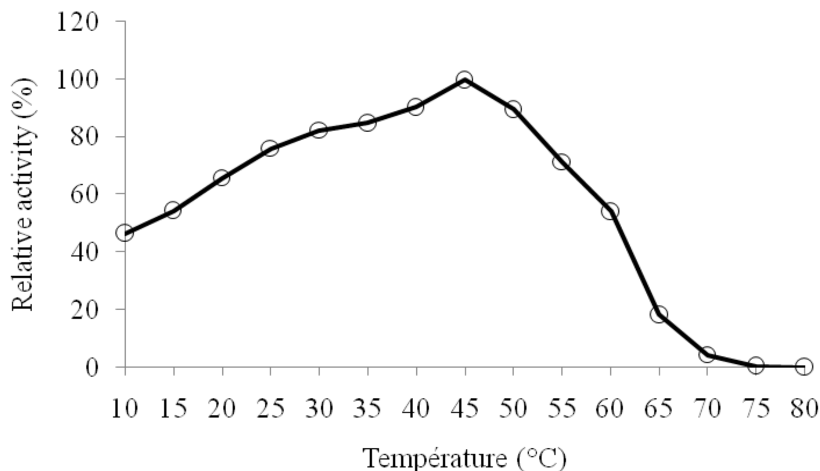
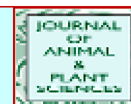


Figure 5. Effect of temperature on the POXp activities. Enzyme activities were determinate at the indicated temperature in 100 mM citrate-phosphate buffer pH 6.0 using guaiacol as substrate under the standard test conditions.

4.4 Effect of metal ions, reducing and detergent agents: The influence of various metal ions and chelating agents on the purified enzyme is reported in Table 2. POXp was inhibited by EDTA, K^+, Mg^{2+} and Na^+. Cu^{2+} ions increased the activity of POXp, thereby, indicating their potential role as cofactor. For DNA polymerase, it was found that Cu^{2+} at low concentrations increased the affinity of the enzyme to its substrate, whereas at high Cu^{2+} concentrations, it can result on the enzyme inhibition (Zaalishvili *et al.*, 1990). While Zn^{2+} inhibited POX isolated from *Mallus pumilus* (Singh *et al.*, 2010) or from *H. tuberosus* (Wang *et al.*, 2008), it has no effect on the POXp from this study. In the other hand, it was reported that Zn^{2+} activated POXs from bean seeds (Wang *et al.*, 2008). The purified enzyme was activated by Mn^{2+}, which is similar to data obtained on peroxidases from *Cocos nucifera* (Murugesan and Rathnam, 2013) and *Citrus reticulata var. Kinnow* (Nouren *et al.*, 2013). However, Dubey *et al.* (2007) observed an inhibition with Mn^{2+} for apple peroxidases. POXp was also activated by Ba^{2+} and Ca^{2+}. POX activation with divalent cations such as Ca^{2+} is quite usual because the latter is present in the structure of POXs, and it plays a role in the stability and activity of POX during the catalysis. POXp was inhibited by K^+. Goyal and Chugh (2013) observed also an inhibition with K^+, on peroxidase from pearl millet (*Pennisetum glaucum)* grains. The influence of other chemicals such as detergent and other organic compounds was also studied (Table 3). All the tested detergents inhibited POXp, but to different extends. Results show that POXp activities were totally inhibited by sodium azide. Sodium azide binds the heme iron atom to the distal site thus giving six-coordinate peroxidase complexes that inactivate the enzyme (Veitch, 2004). POXp was also totally inhibited by reducing agent such as sodium disulfide, sodium thiosulfate and ascorbic acid. Sodium dodecyl sulfate (SDS) and *p*-chloro-mercuro-benzene (*p*CMB) showed 33 % and 59 % inhibition, respectively. The inhibition by *p*CMB indicated that thiol groups are important for POX catalysis. Ethylene diamine tetra acetic acid (EDTA), a chelating agent also exerted an inhibitory effect. The same effect of EDTA was found for POXs from *Jatropha curcas* and *Viscum angulatum* (Cai *et al.*, 2012; Das *et al.*, 2011). Sodium dodecyl sulphate (SDS) which is a strong anionic detergent inhibited POXp activity probably due to a conformational change of the enzyme. However, polyethylene glycol (PEG) enhanced the enzyme activities. This non ionic polymer has been shown to be efficient in improving POX



catalysis notably during the degradation of phenolic compounds (Diao *et al*., 2011). PEG might exert positive effect on POX with the protection of the enzyme by interacting with the reaction products (Kinsley and Nicell, 2000).

Table 2: Effect of metals ions on POX activities. Enzymatic activity was assayed with guaiacol as substrate.

Metal ion	Ba^{2+}	Zn^{2+}	Ca^{2+}	Mn^{2+}	Cu^{2+}	K^{+}	Na+	Mg^{2+}	EDTA
Relative activity (%)	146	102	140	105	147	25	64	27	16

Table 3: Effect of detergents reducing agents and other compounds on POX activities. Enzymatic activity* was assayed with guaiacol as substrate

Chemical compound	ascorbic acid	sodium thiosulfate	sodium azide	citric Acid	sodium disulfite	PCMB	SDS	PEG
Relative activity (%)	0.00	0.00	0.00	0.00	7	59	33	556

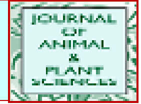
*Enzymatic activity was assayed with guaiacol as substrate

4.5 Substrate Specificity: Data show that POX oxidized a wide range of phenolic substrates (Table 4). This is of great importance since many industrial effluents contain a variety of phenolic contaminants; of which some are more amenable to enzymatic treatment than others. However, the efficiency of POX catalysis depends on the chemical nature of the reducing substrates. Guaiacol was the best substrate for POXp following by ABTS. The oxidation's degree of 4-methoxyphenol acid and pyrogallol were respectively 82.56 % and 82.34 %.

Table 4: Substrate specificity of the purified sweet potato peroxidase (POXp)

Phenolic compound	Specific activity (U/mg protein)	Relative activity (%)
Guaiacol	828.00±9.08	100.00
ABTS	772.36±8.22	93.28
4-Methoxyphenol Acid	683.60±9.6	82.56
Pyrogallol	681.77±11.24	82.34
Gallic Acid	652.38±9.16	78.79
Catechol	643.27±6.07	77.69
Dopamine	625.06±7.22	75.49
4-hydroxybenzoic Acid	597.73±4.16	72 .19
1-Naphtol	555.59±9.21	67.10
Ferulic Acid	504.10±1.9	60.99
Vanillin	456.47±7.98	55.13
1,4-Tyrosol	444.30±7.65	53.66
Phloroglucinol	441.24±12.42	53.29
Para-coumaric Acid	438.26±3.46	52.93
Caffeine	432.13±7.26	52.19
4-Hydroxyphenylacetic Acid	432.13±4.08	52.19
Syringic Acid	415.90±3.89	50.23
Tannic acid	00	0.00

Gallic Acid, catechol and dopamine were over 75 % oxidized by POXp. Except tannic acid, all other hydroxylated-aromatic compounds were over 50 % oxidized by POXp. The outcome of



such studies might help to use POX from sweet potato grown in Burkina Faso to detoxify phenolic compounds from wastewaters (Diao *et al*., 2011) and might therefore have important application with respect to phytoremediation.

CONCLUSION

In conclusion, peroxidase from sweet potato grown in Burkina Faso can be extracted and purified by means of few classical purification steps with relative high yields. Biochemical parameters with respect to enzyme activity and stability revealed its wide possible utilization in biotechnological process involving peroxidases. Moreover, the study on substrate specificity showed that POX is very important in the oxidation of phenolic compounds including those found in polluted environment. This property is of interest because it could be useful in finding solution to the thorny problem of degradation of recalcitrant phenolic compounds that resist on conventional methods for removal. The study opened a new perspective for the use of potato peroxidase in environmental biotechnology.

5 ACKNOWLEDGEMENTS

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6 LIST OF ABBREVIATION:

ABTS: 2, 2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid,
CPB: Citrate Phosphate Buffer,
EDTA: ethylene diamine tetra acetic acid.
HRP: Horseradish peroxidase
PEG: Polyethylene glycol,
*P*CMB: para-chloromercuro-benzoic acid**,**
POX: Peroxidase,
POXp: purified peroxidase of sweet potato,
SDS: Sodium Dodecyl Sulfate.

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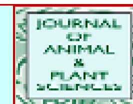

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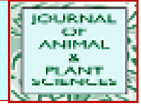

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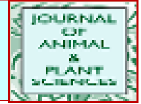

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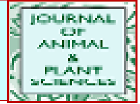

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