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ABSTRACT

Objectives: Peroxidases (POX), isoenzymes were purified to homogeneity from bulbs of *Raphanus sativus* (Radish) grown in Burkina Faso and characterized for use as an alternative source of POX for biotechnological applications.

Methodology and Results: The enzymes were purified using cation exchange, anion-exchange and hydrophobic interaction chromatography. Two isoperoxidases, cationic POX1 and anionic POX2, were isolated and purified 18.5 and 27.2 fold, respectively. Purified enzymes were found to be monomeric proteins with molecular masses of 70 kDa and 47 kDa for POX1 and POX2, respectively, as determined by SDS-PAGE. The effect of pH and temperature was done using guaicol as a substrate. The optimal pH of the both purified POX was 5.6 and 80 % of its activity was retained at pH values between 4.0–8.0 after incubation for 2 h, but POX2 appeared to be highly stable than POX1. POX1 and POX2 had optimum temperatures of activity at 35° and 40 °C, respectively. Activities of these isoenzymes were determined using different phenolic substrates in the presence and absence of ionic effectors. The results show that both POX activities were activated by bivalent cations such as Mg^{2+} and Ca^{2+} but were inhibited by K^{+}, Na^{+}, Zn^{2+}, EDTA and reducing compounds. While Ba^{2+} is an activator for POX2, it had no effect on POX1. They oxidize all the phenolic compounds used. The greatest rate was obtained with ABTS and guaiacol, for POX1 and POX2, respectively.

Conclusions and application of findings: POXs oxidize a wide range of phenolic substrates and have high stability at a wide range of pH. These properties make these enzymes potential biotechnological tools. They could be use in industrial applications to produce food dyes, phenolic resins from natural phenolic compounds or in the construction of biosensors and kits for analyses and diagnostics. They could play also an important role in the oxidation of phenolic compounds in polluted water. This is interesting because it could be useful in finding solutions to the thorny problem of recalcitrant phenolic compounds that resist to conventional methods for bioremediation.

Keywords: peroxidase, purification, characterization, *Raphanus sativus,* phenolic compound.

INTRODUCTION

Plant secretory peroxidases (POXs) are heme-containing proteins, which are member of oxidoreductases [donor: hydrogene peroxide oxidoreductase, EC 1.11.1.7.] and catalyze the oxidation of a wide variety of organic and inorganic substrates in the presence of hydrogen peroxide (H_2O_2). POX activities have been characterized in plants, microorganisms and animals, where they play several roles. Plant peroxidases are found in tonoplast and plasmalemma, inner and outer side of the cellular wall both in soluble as well as, ionically in bound forms (Chen *et al.*, 2002). It is one of the key enzymes controlling plant growth and development. POXs are involved in diverse physiological functions such as lignin biosynthesis (Gross, 2008), suberization (Bernards *et al.*, 1999), wound healing (Kumar *et al.*, 2007), fruit ripening (Huang *et al.*, 2007), auxin metabolism and disease resistance (Veitch, 2004). They can also catalyze the oxidative polymerization of phenols, anilines and other aromatic substrates to insoluble oligomers in the presence of H_2O_2 (Dunford and Stillman, 1976). Due to the high-redox potential required for phenolic compound oxidation, POXs are of very interest as biocatalysts. POXs from different sources are used for extensive potential applications in the clinical, biochemical, biotechnological and industrial fields, and in the synthesis of specific compounds (e.g. various aromatic chemicals) (Srinivas *et al.*, 2002). POXs coupled with other enzymes in polyenzymatic systems producing H_2O_2 is used in the quantitation of many compounds, such as glucose in blood. Because of the oxidative nature of POXs, there are several areas where they could replace current chemical oxidant techniques. It is probably the most well-suited enzyme for the preparation of enzyme-conjugated antibodies which are used in enzyme linked immunosorbent assay (ELISA) tests, due to its ability to yield chromogenic products at low concentrations, and low temperature (Krell, 1991). POXs are used for developing reliable methods of the determination of H_2O_2, which are of great importance both for analytical and industrial fields (Sergeyeva *et al.*, 1999). This enzyme is still used in the determination of lipid peroxidation in cell membranes (Kokçam and Naziroglu, 1999) and in meat food products (Halliwell and Chirico, 1993; Medeiros *et al.*, 1996). POXs play important roles in food quality, because they are involved in colour and flavour deterioration (Ashie *et al.*, 1996). Some modern applications of POXs include treatment of wastewater containing phenolic compounds, the synthesis of several different aromatic chemicals and polymeric materials (Jadhav *et al.*, 2009; Diao *et al.*, 2011). Horseradish peroxidase (HRP) is the most extensively studied peroxidase. It has proven to be efficient in diagnostic, biosensing and other biotechnological applications (Regalado *et al.*, 2004). The availability and cost of commercially available HRP restrict its applications. Peroxidases from other plant sources have also been explored. However, these investigations have been unsuccessful in terms of identifying peroxidases able to knock out HRP as the preferred plant peroxidase in biotechnology. The availability of highly stable and active peroxidases from sources other than horseradish would go a long way towards the development of a catalytic enzyme with broad commercial and environmental applications. Radish (*Raphanus sativus*) is known to contain POX activities. However, no investigation has been carried out on the radish cultivated in the environment of Burkina Faso. The present work was undertaken to isolate and characterize POX isoenzymes from radish grown in Burkina Faso as alternative source of POX for technological applications.

MATERIALS AND METHODS

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

Plant Material: *Raphanus sativus* bulbs (Figure 1) were collected from Ouagadougou (Burkina Faso) and then stored at -30°C prior to POX extraction.

Figure 1: Radish bulbs

Peroxidase activity and total protein determination: **C**rude enzyme extracts were prepared by grinding the bulbs of radish (20 g) in 10 ml of distilled water containing 0.9 % NaCl (w/v). The homogenate was centrifuged at 6000 rpm for 30 min. The supernatant filtered through Whatman paper N°1 was used as the crude extract and conserved at 4°C. POX activity was determined in triplicate by monitoring hydrogen peroxide dependent oxidation of guaiacol. Appearance of oxidation products was followed at 470 nm. POX activity was measured with a spectrophotometric assay by monitoring the H_2O_2-dependent oxidation of guaiacol, at 25 °C. 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 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. Total protein concentration was determined in triplicate by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Purification procedures: Prior to purification, the effects of pH on enzyme activities and stabilities were determined in order to identify the best buffer to use**.** Results showed that 20 mM citrate-phosphate buffer (CPB) pH 6 is the best buffer for enzyme stability. All purification steps were performed at 18°C. The crude enzyme extract from radish was loaded onto a CM-Sepharose CL-6B column (2.6 x 4.0 cm) equilibrated with 20 mM CPB pH 6. The column was washed with the same buffer at flow rate of 1 ml/min. Bound proteins were then eluted over stepwise gradient of NaCl (0.3, 0.5 and 0.7 M), in 20 mM CPB, pH 6 (flow rate 1 ml/min, 3.0 ml/fraction). The first fraction of pooled unbound POX activity (Peak 1) was loaded onto a DEAE-Sepharose CL-6B column (2.6 x 6.0 cm) equilibrated with 20 mM CPB pH 6. Unbound proteins were removed by washing the gel with two bed volumes of equilibration buffer. Bound proteins were then eluted over stepwise gradient NaCl (0.3, 0.5 and 1 M), in 20 mM CPB, pH 6.0 (flow rate of 1 ml/min, 2.0 ml per fractions). The active fractions were pooled and dialyzed overnight against 20 mM CPB, pH 6.0. No further purification steps were necessary. On the other hand, the second fraction of the bound POX activity (Peak 2) eluted from CM-Sepharose CL-6B column at the first step was saturated with ammonium sulphate to a final concentration of 1.7 M and loaded onto a phenyl-sepharose HP column for hydrophobic interaction chromatography. The active fractions were pooled and dialyzed overnight against 20 mM sodium acetate buffer pH 6. No further purification steps were necessary.

Gel electrophoresis: Zymography: For in-gel enzyme staining after incubation with substrate, referred as zymography the crude enzyme extract was subjected to native-PAGE to identify POX isoenzymes. After electrophoresis, POX isoenzymes were revealed by monitoring the H_2O_2-dependent oxidation of guaiacol.

SDS-PAGE: Prior to electrophoresis, the samples were denatured by treatment in 125 mM Tris-HCl buffer, pH 6.8 containing 4 % (w/v) SDS, 1 % (v/v) 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). The 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). Proteins were stained with silver nitrate according to Blum *et al.* (1987).

Native-PAGE: Prior to non-denaturing PAGE, extract samples were mixed with electrophoresis buffer without 2-mercaptoethanol and SDS. Afterwards, gel electrophoresis was performed as mentioned above. Proteins were stained with silver nitrate according to Blum and collaborators (1987).

Effects of pH and temperature on enzyme activities and stabilities: Effect of pH: 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 buffer from pH 2.6-7, 100 mM sodium acetate buffer from pH 4 to 5.6, 100 mM sodium phosphate from pH 5.6-8, 100 mM tris-HCl from pH 7-9 and 100 mM glycine from pH 8-10. The pH values of each buffer were determined at 25°C. The pH stability of each purified enzyme was studied in pH range 2.6 to 10.0 in

the same above buffers. After 2 h pre-incubation at 25°C, aliquots were taken and immediately assayed for residual POX activity.

Effect of temperature: The effect of temperature on POX activity was performed over temperatures ranging from 10 to 80 °C using guaiacol as hydrogen donor under the routine POX assay. The enzyme was always conserved in the buffer displaying the best conditions of stability with respect to the chemical nature of the buffer and its pH. For thermal denaturation tests, the aliquots of enzyme were preincubated at different temperatures ranging from 10 to 80 °C for 10 min. Residual activities, determined at 37 °C under the standard assay conditions, were expressed proportionally to the control untreated enzyme.

Determination of substrate specificity: The substrate specificity of POX isoenzymes was determined by incubating each enzyme with various phenolic substrates (10 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 of the substrate or an increase of absorbance of the products. 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}).

Effect of some chemicals on POX activities: To determine the effect of 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 residual activity was assayed. The final concentration of each chemical compound in the reaction mixture was 5 mM. Afterwards, the substrate guaiacol was added to the medium, and the reaction was started by addition of H_2O_2. The residual activity and control assays were carried out as the standard conditions.

Statistical Analysis: All spectrophotometric assays were performed using a MRX 96-well microplate reader coupled to a computer (Hewlett Packard). Absorbance was automatically recorded. All assays were performed in triplicate. Data were expressed as means ± standard errors using SPSS software package. Significant differences between POX1 and POX2 were tested by student's t-test, with 95 % confidence interval ($p < 0.001$ and 0.05).

RESULTS AND DISCUSSION

Purification: Zymography of POX activity after native polyacrylamide gel electrophoresis (PAGE) from a radish (*Raphanus sativus*) extract showed two major activities referred as POX1 and POX2 (Figure 2). These two POXs were purified using three column chromatographic steps (Figures 3A, 3B and 3C). The first step was cation-exchange chromatography (Figure 3A) over a column of CM-Sepharose. It allowed separating two main isoenzymes POX1 and POX2. POX1 was bound to the column at pH 6, suggesting that its p*I* is lower than pH 6, thus it is an acidic POX. However, POX2 was bound to the column at pH 6, and was further eluted with 0.3 M NaCL in citrate-phosphate buffer. This isoenzyme is considered as cationic POX. The most active fractions of POX1 peak were subjected to anion-exchange chromatography over a DEAE-Sepharose CL-6B column (Figure 3B). A single peak of POX1 activity was obtained. This isoenzyme was purified at 18.5 fold with a yield of 22.1 % (Table 1).

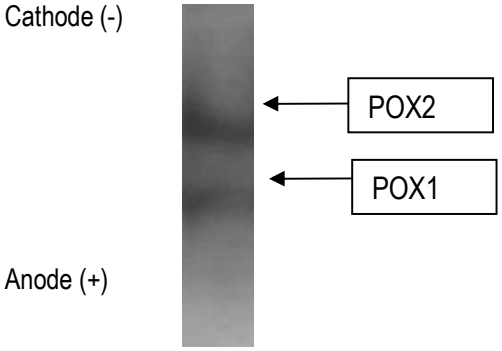

Figure 2: Zymography of POX activities from Burkina radish using gaïacol as hydrogen donor.

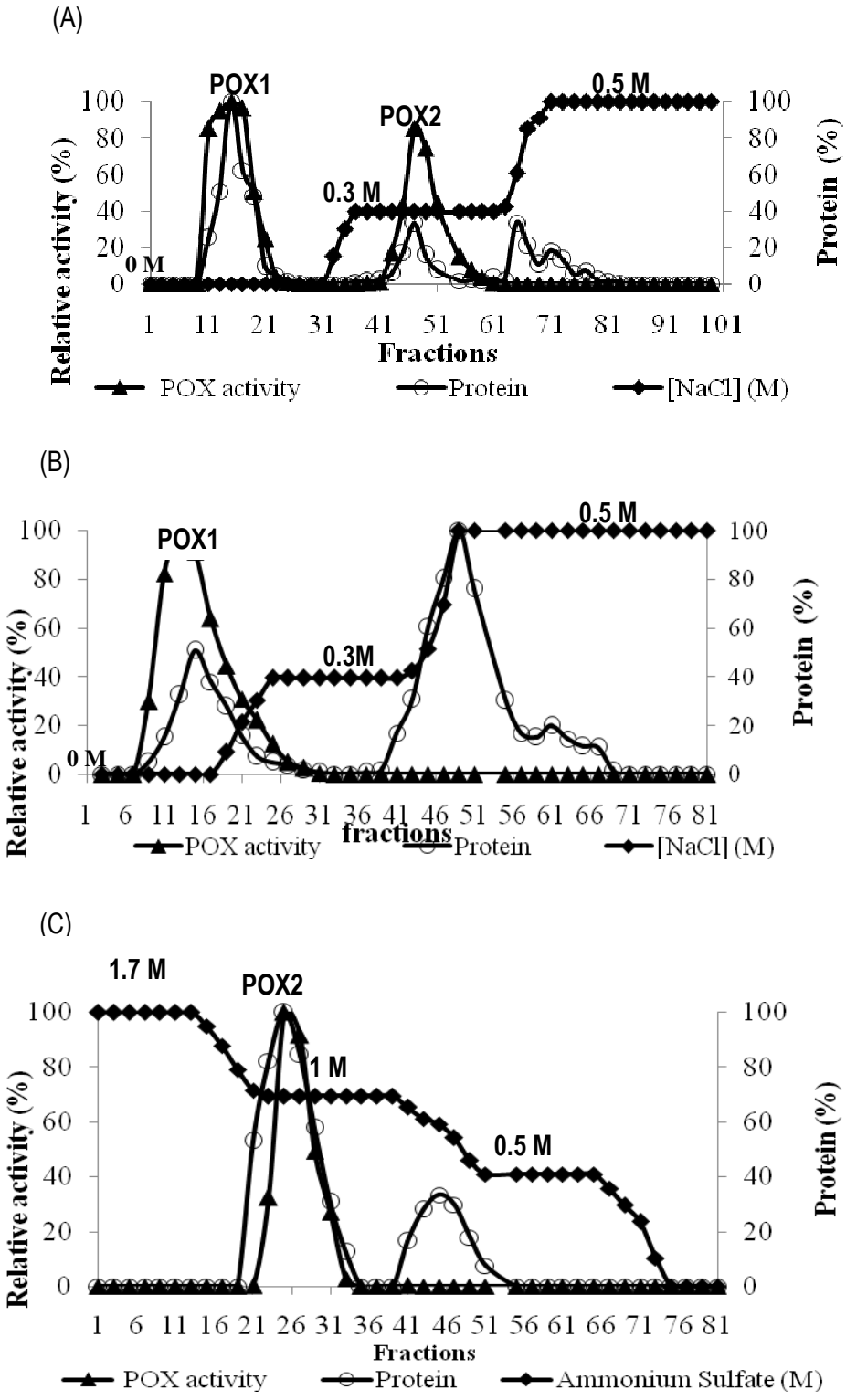

Figure 3: Purification of POX from Burkina radish. (A) Cation-exchange chromatography. (B) Anion-exchange chromatography, (C) Hydrophobic interaction chromatography

Table 1: Purification summary of POX1 and POX2 from Burkina radish.

Purification step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification factor	Yield (%)
Crude extract	176.06±2.9	38530±1.7	226	1	100
CM- Sepharose CL-6B	15.36±0.5	29074±1.3	1892	8.4	75.5
DEAE-Sepharose CL-6B (POX1)	2.03±0.6	8519±0.9	4187	18.5	22.1
Phenyl sepharose CL-4B (POX2)	0.97±0.1	5969±1.1	6151	27.2	15.5

This purification of anionic POX1 by DEAE cellulose column was a determinant step for removing several contaminant proteins. Indeed, anionic POXs purification by DEAE cellulose column is very common (Mohamed *et al.*, 2008; Zia *et al.*, 2011)**.** The peak of POX2 resolved on the CM-Sepharose CL-6B column step was applied to phenyl-Sepharose HP column for hydrophobic interaction chromatography (Figure 3c). A single peak showing POX2 activity was eluted at 1.5 M $(NH_4)_2SO_4$. From this end-step in the purification, a final purification factor of 27.2 was obtained, with a yield of 15.5 % (Table 1). Both isoperoxidases gave final yields significantly lower than those previously obtained (28 %) (Dicko *et al.*, 2006) for the purification of cationic POX from sorghum. However, the yields are 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).

Molecular properties: The purity of POX isoenzymes was supported by SDS-PAGE, in which each isoenzyme appeared as single polypeptide band (Fig. 4). SDS-PAGE allowed estimating molecular weights of POX1 and POX2 to be 70 kDa and 47 kDa, respectively. Thus, the molecular weight of POX1 is largely superior to that of POX2. These weights are different to those (45 kDa) obtained by Wang *et al.* (2004) on cationic peroxidase from Chinese radish. Furthermore, both POXs had a molecular mass higher than other plant POXs such as, horseradish root (40 kDa) (Lavery *et al.*, 2010), *Lactuca sativa* (35 kDa) (Hu *et al.*, 2012), avocado (40 kDa) (Rojas-Reyes *et al.*, 2014), fresh-cut *Zizania latifolia* (31 kDa) (Luo *et al.*, 2012), five isoperoxidases of Korean radish A1, A2, C1, C3 (44 kDa) and A3n (31 kDa) (Lee and Kim, 1994), and *Sorghum bicolor* grain (38 kDa) (Dicko *et al.*, 2006). Molecular mass of POX1 was higher than that of POX from *Cytisus Multiflorus* (49 KDa) (Galende *et al.*, 2016), POXs from cabbage leaves (67 kDa) (Kharatmol and Pandit, 2012) and POXs from leaves of royal palm tree *Roystonea regia* (51 kDa) (Sakharov *et al.*, 2001). Molecular masses of POXs from various sources have been reported to range from 30 to 60 kDa (Thongsook and Barrett, 2005). Although relatively high, this molecular is identical to that of POX from *Hevea brasiliensis* cells (Chanwun *et al.*, 2013). The observed high molecular mass of POX1 may be attributed to post-translational modifications of the polypeptide chain including the number and compositions of glycan chains commonly present in plant POXs (Sakharov *et al.*, 2000; Dicko *et al.*, 2006). Nevertheless, these POXs from Burkina radish are monomeric proteins as found for most plant secretory POXs (Johri *et al.*, 2005; Kim and Lee, 2005; Hermelinda *et al.*, 2007; Hu *et al.*, 2012; Vishwasrao *et al.*, 2017).

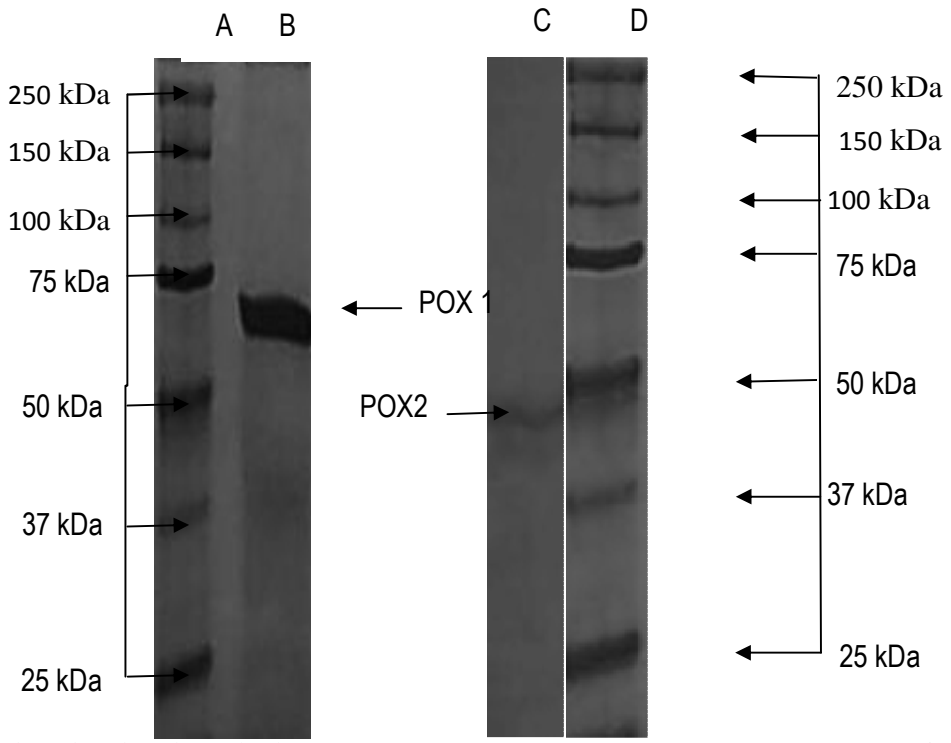

Figure 4: SDS-PAGE of purified POX from Burkina radish. Lane A and D, molecular weight markers (values in kDa). Lane B purified POX1. Lane C purified POX2. Proteins were stained by silver nitrate.

Effects of pH and temperature: The effect of different pH on the oxidation of guaiacol by POX1 and POX2 is illustrated in Figures 5 and 6. For both POXs, maximum activity was obtained at pH 5.6. This optimum pH was similar to those of POX from *Solanum melongena* (Vernwal *et al*., 2006) and POX from *Vigna radiata* (Basha and Prasada, 2017) but it was lower than the one reported (pH 7) for POX from *L. sativa* (Altunkaya and Gökmen, 2011) and that (6.0) of *Rosmarinus officinalis L*. leaves (Aghelan and Shariat, 2015). The pH dependence of the stability of POX2 (fig.6B) showed a maximal stability at pH range of 4.6 to 7.6 in citrate-phosphate buffer (4.6-7), sodium acetate buffer (5.0-6) and sodium phosphate buffer (6-7.6) buffer when it was preincubated for 2 h at ambient temperature. However, in these cases, the activity of POX1 was reduced by about 10 to 20 % (fig.6A), when compared to that obtained with POX2 in sodium acetate buffer and sodium phosphate buffer. Both POXs conserved their full activities in citrate-phosphate buffer at pH 6-6.6. At pH below 4 and above 8.0, enzymes activities of both POX1 and POX2 drastically decreased (Figures 5 and 6). 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 (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 permit the phenolic compounds to act as hydrogen donors. Hence, the best conditions for the POX activities were obtained at pH 4.6-8.0. This is quite interesting because the use of pH close to neutrality may be interesting for biological and industrial applications. POX1 and POX2 were optimally active at 35 °C and 40 °C, respectively. Those optimum temperatures are lower than those reported for hyperthermostable POX from *Solanum melongena* (84°C) (Vernwal *et al*., 2003) and those (50 °C) from POX of Turnip (*Brassica napus var. okapi*) Root, reported by Saboora *et al.* (2012) and those (55) from *Citrus reticulata (*Nouren *et al*., 2013). However, optimum temperatures of activity of both POXs were higher than those of *L. sativa* (30°C) (Altunkaya and Gökmen, 2011). Nevertheless, POX2 has similar optimum thermal activity to a copper-induced anionic POX from sunflower roots (Hager *et al.*, 2008), those from fruits of *Mallus pumilus* (Singh *et al.*, 2010), from Horseradish Peroxidase (Sarika *et al.*, 2015) and from rosemary (*Rosmarinus officinalis* L.) leaves (Aghelan and Shariat, 2015). The thermal denaturation investigated as described in Materials and methods revealed that both POX1 and POX2 were stable at

temperature up to 30 and 40 °C, respectively. Above these temperatures, their activities declined rapidly as temperature increased and were completely inactivated at 75-80 °C.

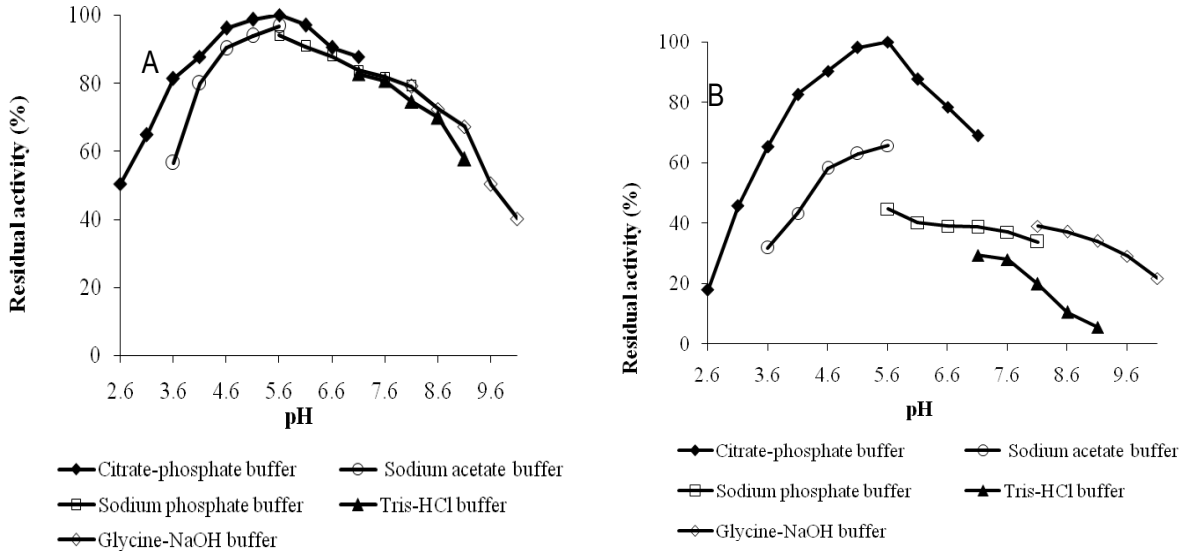

Figure 5: Optimum pH for guaiacol oxidation by Burkina radish POXs. (A) Optimum pH of POX1; (B) optimum pH of POX2.

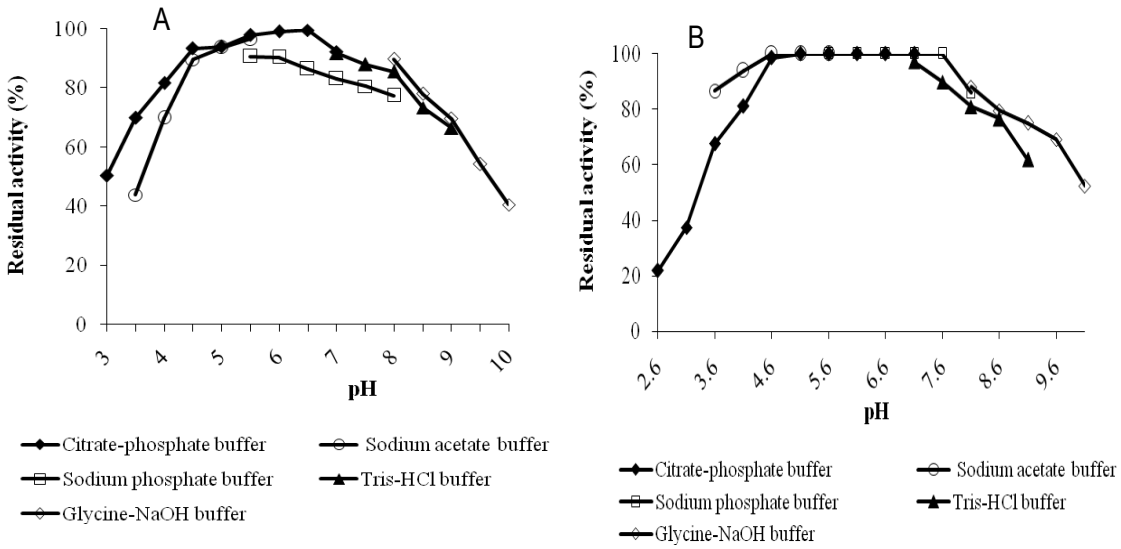

Figure 6: Influence of pH and the buffer on Burkina radish POXs. (A) Effect on POX1; (B) effect on POX2.

Substrate Specificity: Varieties of phenolic compounds were tested for their prominence to be oxidized by POX1 and POX2 in the presence of hydrogen peroxide (Table 2). POX1 and POX2 both oxidized a wide range of phenolic substrates such as monophenol, di and polyphenol. These results are similar to those obtained by Basha and Prasada (2017) and Yadav *et al.* (2017). 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. The results in Table 2 show that ABTS and guaiacol were the best substrates for POX1 and POX2, respectively. Dopamine

degrees of oxidation by POX1 and POX2 were 86.9 and 91.3 %, respectively. Gallic acid, 4-methoxyphenol, pyrogallol, and vanillin were over 50 % oxidized by POX1 compared to ABTS. The level of oxidation of tested phenolic compounds by POX1 is higher than POX2 except for ferulic, caffeic and 4-hydroxybenzoic acids. The outcome of such data may help to use Burkina radish POX to detoxify phenolic compounds from wastewater (Diao *et al.*, 2011) and therefore might have important implications with respect to phytoremediation.

Table 2: Substrate specificity of the purified POXs from Burkina radish

Phenolic compound	Specific activity (Units/mg)		Relative activity (%)	
	POX1	POX2	POX1	POX2
ABTS	4187±0.56	5611±1.06	100	91.23
Guaiacol	3606±0.89	6151±1.03	86.19	100
4-hydroxybenzoic Acid	1195±0.77	1885±0.85	28.56	30.65
Ferulic Acid	732±0.79	2473±0.67	17.5	40.22
Gallic Acid	2233±1.02	2123±1.11	53.34	34.52
Para-coumaric Acid	104±0.82	960±0.83	2.5	15.61
4-methoxyphenol Acid	2581±0.48	3409±1.26	61.66	55.43
Syringic Acid	1328±0.54	1834±0.89	31.74	29.82
1-Naphtol	1133±1.15	567±0.32	27.08	9.22
Phloroglucinol	901±0.86	1914±1.47	21.52	31.13
1,4-tyrosol	385 ±0.41	75±0.76	9.21	1.23
Caffeine	27±0.57	601±0.82	0.65	9.78
Vanillin	2132±1.53	906±1.08	50.94	14.74
4-hydroxyphenylacetic acid	38±0.25	42±0.09	0.91	0.69
Pyrogallol	3467±0.92	4064±1.20	82.82	66.08
Catechol	1738±0.84	2032±0.86	41.52	33.04
Dopamine	3638±1.33	5615±2.01	86.91	91.30
Tannic acid	154±0.29	205±0.51	3.69	3.34

Effects of chemical compounds: Since, industrial effluents contain several chemicals, which can impair enzyme activities, it was important to assess them *in vitro* effect for better prediction of *in situ* efficiency. The impact of various metal ions and chelating agents on the purified POX1 and POX2 activities is shown in Table 3. The two enzymes showed different behaviour in the presence of chemical compounds. POX2 was activated by Ba^{2+} and inhibited by Mn^{2+}. However, Ba^{2+} had no effect on POX1. The common activator ions of both POXs were Mg^{2+}, Ca^{2+}, and Cu^{2+} and the common inhibitor ions were K^{+}, Na^{+}, and Zn^{2+}. Cu^{2+} ions increased the activity, thereby, indicating their potential role as cofactor. Zaalishvili *et al.* (1990) observed that at low Cu^{2+} concentrations, the binding of these ions to the high-affinity sites of the enzyme increases the polymerase activity, whereas at high Cu^{2+} concentrations, the binding may also occur to sites with a lower affinity for the metal, which results in the inhibition of the enzyme activity. Both POXs were inhibited by Zn^{2+}. It has been reported that heavy metals sach as Zn^{2+}, were potent inhibitors of POX from Jerusalem artichoke (*H. tuberosus*) (Şat, 2008). Previous analysis has shown that Zn^{2+} inhibited peroxidases from *Mallus pumilus* varieties (Singh *et al.*, 2010). Other investigations revealed that Zn^{2+} has an inhibitor effect on POX from Jerusalem artichoke, e.g. *H. tuberosus*) (Şat, 2008). Contrary to our findings, it was reported that Zn^{2+} stimulated lima bean seed peroxidases (Wang *et al.*, 2008). POX2 was inhibited by Mn^{2+}, which is similar to those observed by Dubey *et al.* (2007) on apple peroxidases. However, activation effects of Mn^{2+} were observed for peroxidases from *Cocos nucifera (*Murugesan and Rathnam, 2013) and *Citrus reticulatavar.* Kinnow (Nouren *et al.*, 2013). As found in this study, an inhibition effect of K^{+} was found for peroxidase from pearl millet (*Pennisetum glaucum*) (Goyal and Chugh, 2013). Student's t test shows that the difference between POX1 and POX2 is not significant ($P > 0.05$) although both POX have a different behaviour with respect to ions.

Table 3: Effect of metal ions on POX activities. Enzymatic activity was assayed with guaiacol as substrate.

Metal ion 5 mM	**Relative activity (%),**	
	POX1	POX2
Ba^{2+}	100	156 .21
Zn^{2+}	30.7	30.56
Ca^{2+}	115.2	108.1
Mg^{2+}	124.2	111.36
Cu^{2+}	161.4	165.45
K^{+}	59.3	53.46
Na+	73.4	64.02
Mn^{2+}	91.2	88.18

The influence of other chemicals rather than metal ions was also studied (Table 4). All the detergents tested inhibited POX1 and POX2 but at different degrees. Results show that POX activities were inhibited by reducing agent such as, sodium azid, sodium thiosulfate and ascorbic acid, as well as with sodium disulfide and parachloro-mercuro benzoate (*P*CMB). However, POX activities were not very sensitive to SDS. Both POX1 and POX2 were highly inhibited by *P*CMB showing the importance of thiol groups in the POX catalysis. EDTA, an ion-chelating compound also exerted an inhibitory effect (table 4). This shows that calcium is necessary for the activity of POXs. EDTA also exerted an inhibitory effect of POXs from *Viscuman gulatum* (Das *et al.*, 2011) and *Jatropha curcas* (Cai *et al.*, 2012). Polyethylene glycol (PEG) enhanced the activities of both POXs. 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 4: Effect of detergents reducing agents and other compounds on POX activities.

Chemical compound (5 mM)	**Relative activity (%)**	
	POX1	**POX2**
Sodium disulfide	1.97	2.55
Ascorbic acid	0.67	0.53
Sodium thiosulfate	1.88	0.22
Parachloro_mercuro benzoate	36.01	30.38
Sodium azide	1.34	00
EDTA	52.45	60.31
Citric Acid	26.04	32.12
SDS	93.22	90.89
Polyéthylène glycol	256.23	261.16

Although the molecular weight of the two peroxidases are different, statistical analysis of all character shows that there is no significant differences (P >0.05) between both, on catalytically terms.

CONCLUSION

This study showed that Burkina radish contains two major peroxidase isoenzymes. Both peroxidases have some identical physico-chemical and catalytical properties. However, their molecular properties are different. They have a high stability over a wide range of pH. Moreover, the study on substrate specificity showed that both POXs oxidize a wide range of phenolic substrates. That could play an important role in the oxidation of phenolic compounds including those found in polluted water effluents containing phenolic compounds. 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 bioremediation. It can also be exploited in other industrial applications to produce food dyes, phenolic resins and polymers with natural phenolic compounds or in the construction of biosensors and kits for analyses and diagnosis. Our study introduces a new source of POXs

that can be used as an industrial enzyme for biotechnological applications. Nevertheless, later studies will be carried out to compare the efficiency of purified POX1, POX2 and the crude extract in the elimination of phenolic compounds from industrial wastewater. Technological approaches using activators such as PEG, Mg2+, Ca2+ and Cu2+ will be employed to optimize yields.

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List of abbreviation

ABTS: 2, 2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid,
EDTA: ethylene diamine tetra acetic acid.
PCMB: para-chloromercuro-benzoic acid,
PEG: Polyethylene glycol,
POX: Peroxidase
SDS: Sodium Dodecyl Sulfate

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