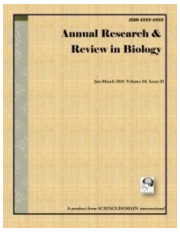

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Biochemical Characterization of Two Polyphenol Oxidase Purified from Edible Yam (*Dioscorea cayenensis-rotundata* cv. Kponan) Cultivated in Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration between all authors. Author LPK designed the study. Author APA wrote the protocol. Authors JCY and SNG made physicochemical analyzes and performed the statistical analysis. Author SD wrote the manuscript and managed the analyses of the data. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Yam (*Dioscorea cayenensis-rotundata* cv. Kponan) is a staple food in many tropical regions. A biochemical characterization study of crude polyphenol oxidase from yam (PPO) was carried out to provide useful information for food processing operations. Two polyphenol oxidases of edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire were purified to homogeneity. The purification procedure consisted of ammonium sulphate fractionation, ion-exchange, size exclusion and hydrophobic interaction chromatography. The enzymes designated PPO1 and PPO2 had native molecular weights of approximately 113.7±0.34 and 115.65 ±1.78 kDa, respectively, and functioned as dimeric (PPO2) and monomeric (PPO1) structures. The two isoforms isolated had different optimum pHs and temperatures. The maximal activity of PPO1 occurred at 35ºC and pH 6.0. On the other hand, PPO2 had a maximum activity at 25ºC and pH 6.6. The enzymes were stable at their optimal temperatures (25ºC and 35ºC) and their pH stability was in the range of 5.6–7.0. Polyphenol oxidases (PPO1 and PPO2) remained their full activity in the presence of ion Mn^{2+}, Fe^{2+}, Na^{+} and Cu^{2+} but were

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inhibited strongly by the reducing agents as beta-mercaptoethanol, L-ascorbic acid, sodium bisulphite and L-cysteine. The values Vmax/Km showed that the enzymes have the greatest reactivity towards dopamine among the substrates used. The present work is therefore the first report on the purification to homogeneity of a yam tuber PPOs

Keywords: Polyphenol oxidase; Dioscorea cayenensis-rotundata; dopamine; yam; browning.

1. INTRODUCTION

Yam is a staple food in many tropical and subtropical areas particularly in West Africa, south Asia and the Caribbean, where it also has a social and cultural importance [1]. It is an important food for about 300 million people throughout the world [2]. The most important species in Côte d'Ivoire are *Dioscorea cayenensis-rotundata* complex and *Dioscorea alata* [3]. Yam is an excellent source of starch, which provides calorific energy. It also provides protein contribution three times higher than cassava and sweet potato [4]. Yam is unfortunately hampered by a phenomenon of enzymatic browning when the tissues are cut, peeled or crushed during processing for food or for storage [5]. Browning reactions which occur after infliction of a mechanical injury to some plant tissues, live fruits, tubers and vegetables are initiated by the enzymes, polyphenol oxidase, peroxidase and to the production of polyphenols and derived products [6]. Polyphenol oxidase (PPO; EC 1.14.18.1), a copper-containing enzyme, is distributed widely in the plant kingdom. Being widely distributed in nature it has been studied in fruits such as banana (*Musa cavendishii*) [7], cocoyam (*Colocasia antiquorum* var *esculenta*) [8], grape [9], lettuce (*Luctuca sativa* var. *capitata* L.) [10] and pepper (*Capsicum annuum* L.) [11]. It catalyses two different reactions using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity) [12]. The *o*-quinones take part in a series of chemical reactions and are ultimately polymerized into brown pigments. They are responsible for the quality deterioration of many fruits and vegetables during post-harvest processing. Prevention of undesirable enzymatic browning is a primary challenge for food scientists, and extensive research has been performed to characterize PPO in many fruits and vegetables [13]. This paper describes the purification and characterization of two polyphenol oxidases from an edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) that is known to show the most intense browning phenomenon on cutting or peeling. Improved understanding of the characteristics of PPO from yam will lead to the prevention of browning during the processing of this tuber.

2. MATERIALS AND METHODS

2.1 Plant Material and Enzyme Extraction

The cultivar of yam (*Dioscorea cayenensis-rotundata*, cv.Kponan) used for the study was grown during its appropriate cropping season in June 2008 at the experimental farm of the University Nangui Abrogoua (5º23 latitude North, 4º00 longitude West, and 7m altitude), Côte d'Ivoire. The matured tubers were randomly harvested 6 months after planting (December 2008). After harvesting, fruits were split using a stainless steel kitchen knife and were treated for the crude extract preparation.

One hundred and fifty grammes of the tubers were ground using a blender in 200mL NaCl solution 0.9% (w/v). The homogenate was subjected to sonication (4ºC) at 50-60 Hz

frequency using a TRANSSONIC T420 for 10 min and then centrifuged at 10 000g for 30 min at 4ºC. The supernatant filtered through cotton wool was refrigerated and used as the crude extract.

2.2 Chemicals

Phenolic compound substrates such as pyrocatechol, resorcinol, catechin, L(-)-tyrosine, pyrogallol, tannic acid, gallic acid, 1-Naphtol, dopamine-chloro-hydrate, vanilin, cafein, coumarine, *para* coumaric acid, syringic acid, ferulic acid, 4-hydroxybenzoïc acid, phenylalanine, 4-hydroxyphenylacetyl acid, 1-4-tyrosol, 4-hydroxyanisol, et phloroglycinol were purchased from Sigma-Aldrich. DEAE-Sepharose Fast Flow, CM-Sepharose CL-6B, Sephacryl S-100 HR and Phenyl-Sepharose 6 Fast Flow were obtained from Pharmacia Biotech, bovine serum albumin (BSA) from Fluka Biochemika and standard molecular weight proteins from Bio Rad. All the other reagents used were of analytical grade.

2.3 Zymogram

To reveal iso-forms of polyphenol oxidase from yam tuber *Dioscorea rotundata-cayenensis* (cv kponan), a zymogram was made according to the method of [14]. It consisted of making a native electrophoresis of crude extract of yam tuber on plates of 1.5mm thick gel (7×8 cm) containing 7.5% acrylamide at 10ºC under a current of 20 mA according to the method of [15]. After migration, he protein bands were revealed by immersion in 100mM sodium phosphate buffer pH 6.6 containing 8mM substrate dopamine for 5 min of incubation.

2.4 Purification of Enzymes

The purification procedure was carried out in the cold room (4°C). The crude extract of yam tuber *Dioscorea cayenensis-rotundata* (cv kponan) seeds was loaded onto an anion-exchange chromatography using a DEAE-Sepharose Fast Flow column (2.5cmx4.5cm), equilibrated with 20mM sodium phosphate buffer (pH 6.6). The column was washed at a flow rate of 3mL/min with two bed volumes of equilibration buffer to remove unbound proteins. Bound proteins were then eluted with a stepwise salt gradient (0.3, 0.5, 0.7 and 1M) of NaCl in 20mM sodium phosphate buffer (pH 6.6), and fractions of 3 mL were collected. Two peaks of polyphenol oxidase activity were obtained.

The unbound polyphenol oxidase activity (Peak 1) was loaded onto a cation-exchange chromatography using a CM-Sepharose CL-6B column (2.6cmx4.0cm), equilibrated with 20mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer at a flow rate of 1mL/min. Polyphenol oxidase activity was eluted with a stepwise salt gradient (0.3, 0.5 and 1M) NaCl in 20mM sodium phosphate buffer (pH 6.0). Fractions of 2mL were collected and, to the pooled active fractions, solid ammonium sulphate was slowly added to give a final concentration of 1.7M and the resulting enzyme solution was subsequently applied on a Phenyl Sepharose 6 Fast Flow column (1.5cm×3.2cm) previously equilibrated with 20mM sodium phosphate buffer (pH 6.0) containing 1.7M of ammonium sulphate salt. The column was washed with a reverse stepwise gradient of ammonium sulphate concentrations (from 0-1.7M) dissolved in the same sodium phosphate buffer at a flow rate of 1mL/min and fractions of 1mL were collected. The pooled active fractions were dialyzed overnight during 12 hours against 20mM sodium phosphate buffer (pH 6.0) and constituted the purified enzyme solution.

On the other hand, the bound polyphenol oxidase activity (Peak 2) eluted from DEAE-Sepharose Fast Flow at the first step was also subjected to 80 % saturation with ammonium sulphate. The precipitate obtained after centrifugation (10 000g) was dissolved in 1mL of 20 mM sodium phosphate buffer and loaded onto the same Sephacryl S-100 HR column in the same experimental conditions as described above. Polyphenol oxidase activity peak obtained was saturated to a final concentration of 1.7M ammonium sulphate and loaded onto the Phenyl-Sepharose 6 Fast Flow column in the same procedure as above. Finally, the pooled active fractions were also dialyzed against 20mM phosphate buffer (pH 6.6) and kept refrigerated at 4ºC for assays.

2.5 Enzyme Assay

Under the standard test conditions, the activity of two PPO was determined with dopamine as a substrate using the method of [16]. An assay mixture (2mL) consisting of a 100mM phosphate buffer pH 6.0 for PPO1 or pH 6.6 for PPO2, 8mM dopamine and enzyme solution was incubated at 25ºC for 10 min. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 480nm. Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per min [17].

2.6 Protein Estimation

Protein concentrations and elution profiles from chromatographic columns were determined by the Folin method [18]. Bovine serum albumin (BSA) was used as the standard protein.

2.7 Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was carried out by using the [15] method on 10% (w/v) acrylamide gels under denaturing and non-denaturing conditions. Under denaturing conditions, samples were incubated for 5min at 100ºC with SDS-PAGE sample-buffer containing β-mercaptoethanol. Under non-denaturing conditions, samples were mixed just before running in sample-buffer without β-mercaptoethanol and SDS. Silver staining was used to localize protein bands [19]. The standard molecular weights (Bio-Rad) comprising myosin (209kDa), β-galactosidase (124kDa), carbonic anhydrase (34.8 kDa), BSA (80 kDa) and inhibiteur trypsique de soja (45.0kDa) were used.

2.8 Native Molecular Weight Determination

The purified enzyme was applied to gel filtration on a Sephacryl S-200 HR column (0.8cmx35cm) equilibrated with 20mM sodium phosphate buffer (pH 6.6) to estimate the native molecular weight. Elution was done at a flow rate of 0.2mL/min and fractions of 0.5 mL were collected. Standard molecular weights (SIGMA) used for calibration were β-amylase (206kDa), cellulase (26kDa), bovine serum albumin (66.2kDa), ovalbumine (45kDa) and amyloglucosidase (63kDa).

2.9 pH optimum and stability

The PPO activity was determined in a pH range of 3.6– 5.6 in 100mM acetate buffer in a pH range of 3.0-7.0 in citrate buffer 100mM and in a pH range of 5.6–8.0 in 100mM phosphate buffer. Dopamine (8mM) was used as substrate. PPO activity was assayed, using the

standard reaction mixture but changing the buffer. PPO activity was calculated in the form of per cent residual PPO activity at the optimum pH. The optimum pH obtained for these enzymes was used in all other studies. The pH stability of each polyphenol oxidase was studied at a pH range of 3.0-8.0 with 100mM buffers. Buffers used were the same as in pH study. After 2 hours preincubation at 25ºC (room temperature), residual polyphenol oxidase activities were measured at 25ºC for 10 min by adding substrate dopamine. Experiments were performed in triplicate, and the results expressed as percentage activity of zero-time control of untreated enzyme.

2.10 Temperature Optimum and Thermostability

The effect of temperature on polyphenol oxidases activities was performed in 100mM sodium phosphate buffer (pH 6.0 and 6.6) over a temperature range of 10-90ºC by using 8mM dopamine as substrate under the standard test conditions. The thermal inactivation was determined at 37ºC and at each enzyme optimum temperature (pH 6.0 for PPO1 and pH 6.6 for PPO2). Enzymes in appropriate buffers were exposed to each temperature for up to 120 min. Then, aliquots were withdrawn at intervals (10min) and immediately cooled. In the thermal denaturation tests, aliquots of each enzyme solution were preheated at different temperatures at a range of 10-90ºC for 15min. Residual activities, determined at 25ºC under the enzyme assay conditions were expressed as percentage of activity of zero-time control of untreated enzymes.

The effect of temperature and the rate constant in a activation process was related according to the Arrhenius equation [20]:

$$k = Ae(-Ea/RT)$$

Where;

k is the reaction rate constant value,
A is the Arrhenius constant,
Ea is the activation energy (energy required for the activation to occur),
R is the gas constant (8.31 $Jmol^{-1}K^{-1}$),
T is the absolute temperature in Kelvin.

The Q10 temperature coefficient is a measure of the reaction rate of temperature increase of 10ºC. The Q10 is calculated as:

$$Q_{10} = (X_2 / X_1)$$

Where;

X1 represents the lower absorbance (D.O at 10ºC);
X2 represents the higher absorbance (D.O at 20ºC)

2.11 Substrate Specificity and Kinetic Parameters

Substrate specificity of the two polyphenol oxidases was determined by incubating the enzymes with various phenolic substrates (10mM) at 25ºC in 100mM sodium phosphate buffer (pH 6.0 and pH 6.6) for 10min. The results were expressed as the percentage of

maximum PPO activity. Kinetic parameters (KM, Vmax and Vmax/KM) of the two polyphenol oxidases were determined in 100mM sodium phosphate buffer (pH 6.0 and pH 6.6) at 25ºC. KM and Vmax were determined from a [21] plot by using different concentrations (0.1-15mM) of pyrocatechol and dopamine substrates at (pH 6.0 and 6.6) and 25ºC.

2.12 Effect of Metal Ions and Chemical Agents on Enzyme Activity

To determine the effect of various compounds as possible activators or inhibitors of the purified polyphenol oxidases, each enzyme solution was preincubated at 25ºC for 20 min with the compounds and the activity was assayed under the enzyme assay conditions. Residual activities were expressed as percentage referred to control without chemical agents.

2.13 Statistical Analysis

The Statistical Analysis System (SAS) for the personal computer program (SAS Inst., 1988) was used for the ANOVA; LSD means separation, single, Pearson and stepwise regression analyses. P≤0.05 was selected as the decision for significant differences.

3. RESULTS

3.1 Identification of Polyphenol Oxidase

Zymogram analysis of the enzyme activity showed a two isoforms of polyphenol oxidase from yam tuber *Dioscorea cayenensis rotundata* (cv kponan) (Fig. 1).

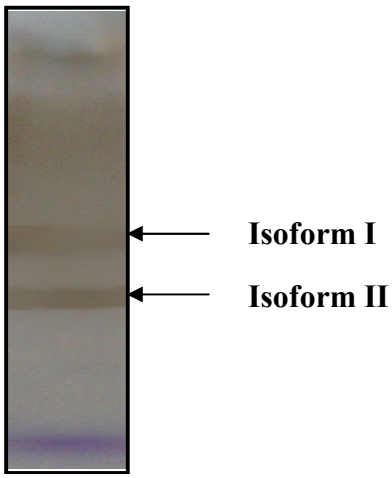

Fig. 1. Zymogram of the active fraction using dopamine as substrate Polyacrylamide gel electrophoresis in native

3.2 Enzyme Purification

The purification procedure of the two polyphenol oxidases from crude yam tuber involved three steps including ion-exchange, size exclusion and hydrophobic interaction

chromatographies; the results are summarized in Table 1. Dopamine was used as the substrate to monitor this enzymatic activity.

Two peaks of polyphenol oxidases activity named PPO1 and PPO2 were resolved on the anion-exchange (DEAE-Sepharose Fast Flow) chromatography used as the first step of purification. Active proteins were eluted respectively at 0 (PPO1) and 0.3M (PPO2) of NaCl (data not shown). PPO1 activity was in addition subjected to a cation-exchange chromatography on a CM-Sepharose CL-6B column, and a single peak of bound polyphenol oxidase activity was eluted with 0M of NaCl (data not shown). The polyphenol oxidase (PPO2) exit of the first step of purification (DEAE-Sepharose Fast Flow) was loaded onto a gel filtration chromatography using a Sephacryl S-100 HR column. One peak showing polyphenol oxidase activity was resolved in this step. After these steps the two polyphenol oxidases (PPO1 and PPO2) activities were subsequently purified by using an ultimate hydrophobic chromatography on a phenyl-Sepharose 6 Fast Flow column. The active proteins were eluted with 0.3 and 0.5M of ammonium sulphate, respectively (data not shown). Finally, PPO1 and PPO2 were purified with overall yields of 12.17 and 3.39% and enriched about 55.64 and 18.61 fold, respectively (Table 1). The specific activity was 4783.87±2.14 and 1355.77±2.86U mg^{-1} proteins, respectively.

Each isoenzyme showed a single protein band by silver staining on native polyacrylamide gel electrophoresis (Fig. 2A).

3.3 Molecular Weight Estimation

SDS-PAGE exhibited two closed subunits for the purified polyphenol oxidases PPO2 with molecular weights estimated to be 115.65kDa (68.77±1.25 and 46.88±0.53kDa) (Fig. 2B). By gel filtration, the native enzyme had a molecular weight of 178.65kDa (Table 2). However, the molecular weight of polyphenol oxidase PPO1 by gel filtration (122 kDa) was somewhat larger than that obtained by SDS-PAGE analysis (113.71±0.34kDa) (Fig. 2B; Table 2).

The activities of two PPO was measured at different pHs using dopamine as substrates. As seen in Fig. 3, the optimum pH of both enzymes was found to be pH 6.0 for PPO1 and pH 6.6 of PPO2 in sodium phosphate buffer (Table 2). The purified enzymes showed best stability over pH values ranging from 5.6 to 7.0, for 120min at 25ºC, conserving at least more than 80% of total activities (Table 2).

3.4 Effect of Temperature

The optimum temperature of the enzyme with the dopamine were found to be 35 and 25ºC respectively, for PPO1 and PPO2 (Fig. 4 and Table 2). The temperature coefficient (Q10) for PPO1 activity as calculated between 15 and 30°C was around 1.27±0.06 from the Arrhenius plot, the activation energy was found to be 54.2±2.04kJ/mol (Table 2). Concerning PPO2 activity, the value of temperature coefficient (Q10) calculated between 10 and 20ºC was 1.38±0.02 and the activation energy was found to be 48.5±1.12kJ/mol (Table 2).

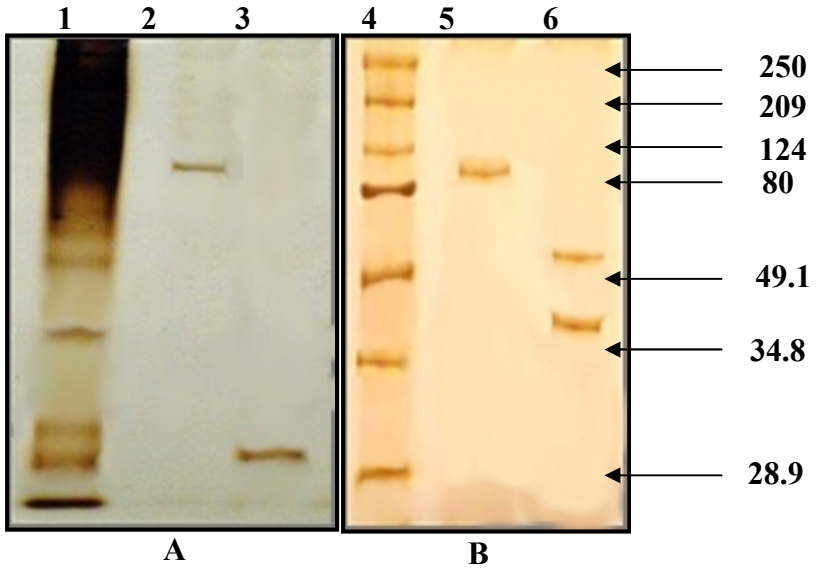

Fig. 2. PAGE analysis of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan): (A) purified enzymes PAGE. (B) purified enzymes SDS-PAGE. The samples were loaded onto a 10% gel. Lane 1, crude extract; Lane 2, PPO2; Lane 3, PPO1; Lane 4, Markers; Lane 5, PPO1; Lane 6, PPO2. Numbers on the right indicate the molecular weights (kDa) of protein markers

The thermal denaturation shows that the two enzymes were fairly stable at temperatures up to 35ºC and 25ºC respectively, for PPO1 and PPO2. At higher temperatures, thermostability decreased progressively and the enzymes were completely inactivated at 90ºC (Fig. 5).

The two polyphenol oxidases were fully active for more than 2 hours at their optimal temperature in sodium phosphate buffer pH 6.0 and pH 6.6 respectively for PPO1 and PPO2, indicating a thermal stability at their optimal temperature (Fig. 6). However, their catalytic activities were abruptly affected after 30 min incubation at 37ºC (Fig. 6).

3.5 Substrate Specificity and Kinetic Properties

A variety of phenolic substrates were tested for their suitability to serve as substrates. PPO1 and PPO2 did not attack the following substrates: tyrosin, naphtol, vanilin, gallic acid, resorcinol, cafein, coumarin, *para* coumarin acid, syringic acid, ferulic acid, 4-hydroxybenzoïc acid, phenylalanine 4-hydroxyanisol, phloroglycinol, 4-hydroxyphenylacetyl and 1-4-tyrosol (Table 3). Although, the enzymes attacked dopamine, pyrocatechol, pyrogallol, catechin and tannic acid (Table 3).

Kinetic parameters of the two polyphenol oxidases were studied by using dopamine and pyrocatechol as substrates. With the two substrates, the enzyme obeyed the Michaelis-Menten equation (Table 2). The Km, Vmax and Vmax/Km values are reported in Table 4. The catalytic efficiency of polyphenol oxidases, given by the Vmax/Km ratio was much higher for the dopamine than the pyrocatechol (Table 4).

Table 1. Purification of the polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire

Purification Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U mg-1)	Yield (%)	Purification Fold
Crude extract	285.46±2.3	20803±9.14	72.87±4.52	100	1
DEAE-Sepharose Fast-flow					
PPO1	43.12±1.40	16260±8.23	377.08±3.45	79.16±4.12	5.17±0.69
PPO2	56.27±1.76	15700±7.35	279.01±3.26	87.50±3.58	3.83±0.87
CM-Sepharose CL-6B					
PPO1 (NH4)2SO4 80%	20.70±0.98	10310±6.47	498.06±2.10	49.56±2.73	6.93±1.18
PPO2	12.54±0.49	6780±5.13	540.67±2.63	32.59±0.94	7.42±3.25
Sephacryl-S 100 HR					
PPO2	3.87±0.08	4860±4.12	1255.82±3.49	23.36±1.58	17.23±2.37
Phenyl-Sepharose 6 Fast Flow					
PPO1	0.62±0.03	2532±3.69	4783.87±2.14	12.17±0.65	55.64±0.66
PPO2	0.52±0.02	705±3.48	1355.77±2.86	3.39±0.05	18.61±0.75

Values given are the averages of three replicates ± SE

Table 2. Some physicochemical characteristics of the polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire

Oxidation	Values	
	PPO1	PPO2
Optimum Ph	6.0	6.6
pH stability range	5.6-7.0	5.6-7.0
Optimum temperature (ºC)	35ºC	25ºC
Temperature coefficient (Q10)	1.27±0.06	1.38±0.02
Michaelis-Menten equation	Obeyed	obeyed
Molecular weight (kDa)		
Mobility in SDS-PAGE	113.71±0.34[a]	115.65±1.78[a]
Gel filtration	122±1.3[b]	178.65±0.5[b]

Table 3. Substrate specificity of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire

Substrate	Wavelength (nm)	Relative rate of oxidation (%)	
		PPO1	PPO2
Dopamine	480	100.00±0.0[a]	100.00±0.0[g]
Pyrocatechol	420	84.53±0.15[b]	54.16±0.27[h]
Catechin	420	41.6±0.13[c]	14.85±0.20[i]
Pyrogallol	420	37.6±0.09[d]	9.14±0.22[j]
Gallic acid	420	2.8 ±0.09[e]	1.70±0.13[k]
Tannic acid	420	14.13±0.09[f]	10.57±0.19[j]
1-Naphtol	420	0	0
L-tyrosin	420	0	0
Resorcinol	420	0	0
Cafein	420	0	0
Coumarin	420	0	0
para coumarique acid	420	0	0
Vanillin	420	0	0
Syringic acid	420	0	0
ferulic acid	420	0	0
4-hydroxybenzoïque acid	420	0	0
Phenylalanin	420	0	0
4-hydroxyanisol	420	0	0
phloroglycinol	420	0	0
Hydroxyphenylacetyl acid	420	0	0
1-4-tyrosol	420	0	0

Means not sharing a similar letter in a same column are significantly different at the P≤0.05 as assessed by the test of Duncan. Values given are the averages of at least three experiments ± SE

3.6 Effect of Metal Ions and Chemical Agents on Enzyme Activity

The effect of some chemicals on the purified polyphenol oxidases activity was examined (Table 5). The common activator of the two polyphenol oxidases activity was Mn^{2+}, Fe^{2+}, Na^{+} and Cu^{2+} while K^{+}, $Pb^{2+}$and Sr^{2+} showed an inhibitory effect on the polyphenol oxidases activity at concentration of 5 mM. Also, ions Ca^{2+}, Ba^{2+} and Zn^{2+} had almost no effect on the two enzymes. The PPO1 and PPO2 were inhibited by β-mercaptoethanol, SDS, ascorbic acid, sodium bisulphite and cysteine. Ethylenediaminetetraacetic acid (EDTA), citric acid and *p*CMB had no effect on the same enzymes activity (Table 6).

Table 4. Kinetic parameters of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) toward different substrates

Substrate	Polyphenol oxidases					
	PPO1			PPO2		
	K_M (mM)	V_{max} (Umg^{-1})	V_{max}/K_M (U/mM×mg)	K_M (mM)	V_{max} (Umg^{-1})	V_{max}/K_M (U/mM×mg)
Dopamine	0.34±0.01[a]	2000±16.23[a]	5985.25±21.43[a]	5.31±0.4[a]	1000±2.50[a]	188.32±1.88[a]
pyrocatechol	9.79±0.04[b]	10000±24.52[b]	1021.45±18.01[b]	27.02±1.46[b]	1428.57±3.07[b]	52.87±1.84[b]

Table 5. Activity of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) preincubated with some cations

Reagent	Concentration (mM)	Relative Activity (%)	
		PPO1	PPO2
control	0	100 ± 0.0	100 ± 0.0
K^+	1	97.05 ± 3.1^{f}	92.14 ± 4.5^{d}
	5	53.64 ± 1.6^{c}	42.80 ± 4.1^{a}
Na^+	1	105.08 ± 4.5^{k}	109.23 ± 3.9^{l}
	5	109.50 ± 2.9^{m}	105.60 ± 4.2^{i}
Mn^{2+}	1	101.81 ± 2.1^{i,j}	106.51 ± 4.5^{j}
	5	113.44 ± 1.5^{m}	132.39 ± 5.1^{q}
Sr^{2+}	1	88.72 ± 1.1^{e}	96.41 ± 4.7^{e}
	5	48.16 ± 2.9^{b}	43.00 ± 2.5^{a}
Ca^{2+}	1	100.43 ± 1.2^{h}	99.81 ± 3.1^{h}
	5	99.10 ± 1.1^{g}	98.10 ± 2.8^{f}
Ba^{2}	1	102.70 ± 2.5^{j}	92.87 ± 3.1^{d}
	5	107.30 ± 3.1^{l}	96.29 ± 2.8^{e}
Cu^{2+}	1	100.87 ± 1.4^{h,i}	114.01 ± 1.1^{m}
	5	102.13 ± 2.1^{J}	122.33 ± 5.6^{o}
Zn^{2+}	1	123.40 ± 3.9^{o}	98.80 ± 2.3^{g}
	5	136.20 ± 2.3^{p}	107.60 ± 2.3^{k}
Pb^{2+}	1	76.90 ± 1.2^{d}	69.54 ± 1.1^{c}
	5	44.60 ± 1.1^{a}	54.09 ± 1.4^{b}
Fe^{2+}	1	101.02 ± 2.7^{h,i}	117.19 ± 5.6^{n}
	5	121.60 ± 4.3^{n}	129.70 ± 6.1^{p}

Values given are the averages of at least three experiments ± SE. Means in a same column followed by the same letter are not significantly different at the P≤0.05 as assessed by the test of Duncan

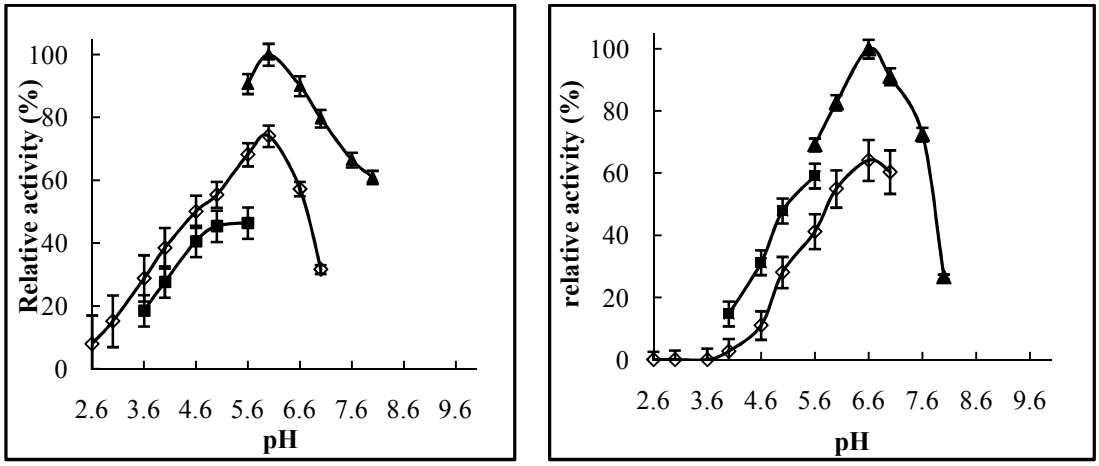

Fig. 3. Activity of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) at varying pH. Bars represent ± SE. citrate buffer (◊), acetate buffer (■), phosphate buffer (▲)

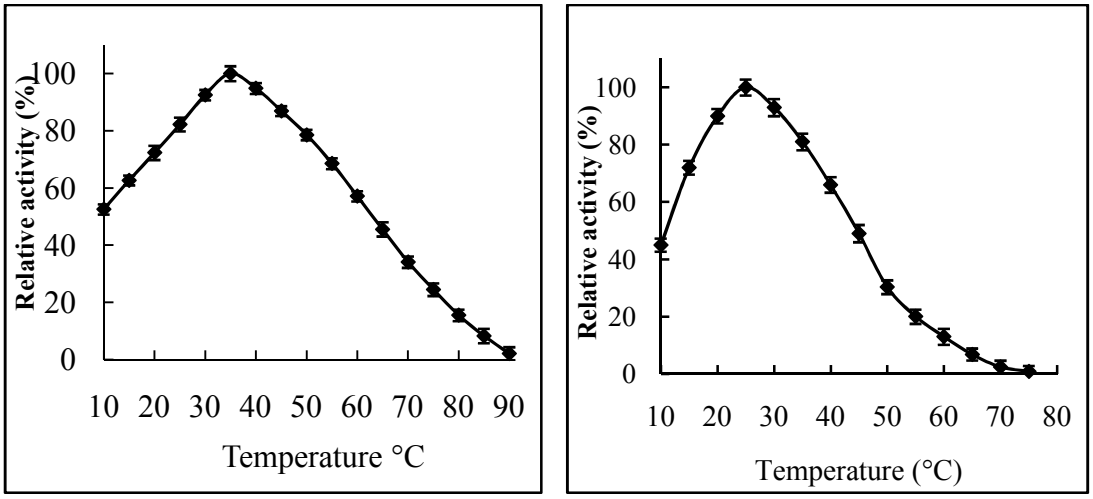

Fig. 4. Activity of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) at varying temperature. Bars represent ± SE

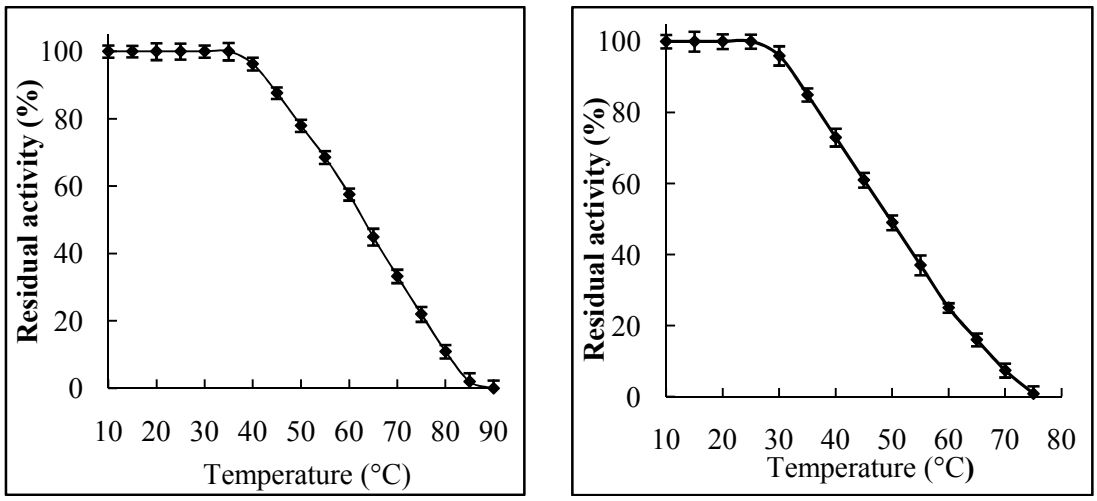

Fig. 5. Thermal denaturation of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan). Experiments were carried out at 10-90ºC. Enzymes were preincubated at each temperature for 15 min in 100 mM sodium phosphate buffer (pH 6.0 and pH 6.6) and residual activities were measured at 25ºC under enzyme assay conditions. Bars represent ± SE

Table 6. Activity of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) preincubated with chemical agents

Chemicals agents	Concentration (mM)	Relative Activity (%)	
		PPO1	PPO2
Control	0	100±0.0[a]	100 ±0.0[j]
EDTA	1	99.76±1.20[b]	98.12±2.21[k]
citric acid	1	91.54±3.43[c]	82.69± 4.23[l]
β-mercapto-ethanol	1	4.32±1.02[d]	2.77±0.6[m]
Sodium bisulphite	1	10.18±1.1[e]	4.52±1.36[n]
ascorbic acid	1	6.29±2.2[f]	5.42±2.05[o]
L-Cysteine	1	5.97±1.56[g]	6.85±1.42[p]
*p*CMB (%, w/v)	1	95.49±1.15[h]	97.36±4.20[q]
SDS (%, w/v)	1	63.40±2.76[i]	59.44±1.89[r]

pCMB, p-chloromercuribenzoate; SDS, sodium dodecyl sulphate. Means in a same column followed by the same letter are not significantly different at the P≤0.05 as assessed by the test of Duncan. Values given are the averages of at least three experiments ± SE

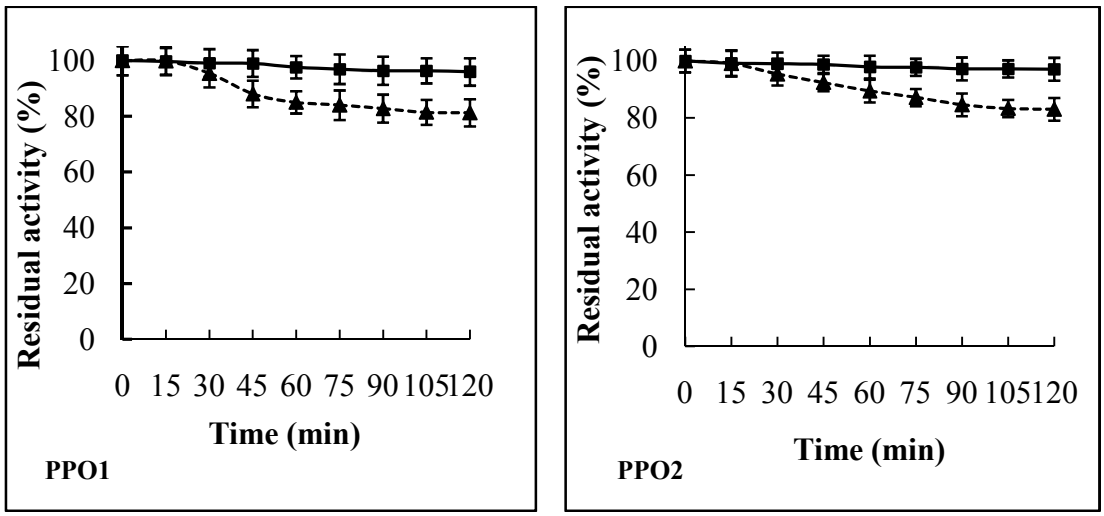

Fig. 6. Thermal inactivation of purified polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan). Bars represent ± SE. (■) 25°C; (▲) 37ºC

4. DISCUSSION

The zymographic study using crude extract as enzyme source showed two bands of protein, indicating that the crude extract of yam tuber (*Dioscorea cayenensis-rotundata* cv. Kponan) has two isoforms activities. This result was confirmed by the first step of purification that allowed one hand to separate these two isoforms and also eliminate some of the pigments in the crude enzyme extract.

Two polyphenol oxidases (PPO1 and PPO2) were purified to homogeneity from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire. The first and the last chromatographies were crucial to purify the polyphenol oxidase activity of the yam extract. Indeed, the anion exchange chromatography on a DEAE-Sepharose Fast Flow

column enabled the enzyme activity to be separated into two different isoforms (PPO1 and PPO2), while hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow led to separate each polyphenol oxidase from the other proteins and impurities. Similar results concerning Phenyl-Sepharose gel has been reported for the purification of polyphenol oxidase of yam tuber *Dioscorea cayenensis-rotundata,* cv Longbô [22] and polyphenol oxidase of apple (*Malus domestica* borkh. cv Bramley's seedling) [23].

PPO1 and PPO2 were purified with overall yields of 12.17 and 3.39% and enriched about 55.64 and 18.61 fold, respectively. This low yield for PPO2 could be due to several fractionation steps used. However, it is noteworthy that specific activities and purification folds are higher than those reported for other polyphenol oxidases already described [24,25]. Each isoenzyme showed a single protein band by silver staining on native polyacrylamide gel electrophoresis.

SDS-PAGE exhibited two close subunits for the purified polyphenol oxidase PPO2 with molecular weights estimated to 115.65kDa (68.77±1.25 and 46.88±0.53kDa). By gel filtration, the native enzyme had a molecular weight of 178.65kDa. These results indicated that the native functional enzyme is a heterodimer. However, the molecular weight of polyphenol oxidase PPO1 by gel filtration (122 kDa) was somewhat larger than that obtained by SDS-PAGE analysis (113.71±0.34 kDa), suggesting that this enzyme functions as a monomeric structure. In comparison with other molecular weights of plant purified polyphenol oxidases, those from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire are lower with regard to polyphenol oxidases from the crab *Charybdis japonica* (*Charybdis japonica*) (225 kDa) [26], mango (*Mangifera indica*) (136 kDa) [27] and deepwater pink shrimp (*Parapenaeus longirostris*) (500 kDa) [28]. Nevertheless, they had higher molecular weights than the purified polyphenol oxidases from yam tuber (*Dioscorea opposita Thunb.*) (44 kDa) [29] and coffee (*Coffea arabica*) (46-50 kDa) [30] and the leaf of *Musa paradisiacal L.* (40 kDa) [31].

PPO1 and PPO2 oxidation activities were maximal at 35ºC in sodium phosphate buffer pH 6.0 and 25ºC at pH 6.6 respectively. At 25ºC, the purified enzymes showed best stability over pH values ranging from 5.6 to 8.0, conserving at least more than 80% of total activities. Therefore, pH 6.0 and 6.6 is a good compromise between the activity and stability of these enzymes to perform oxidation of phenolic substrates over a long time. The proximate neutrality pH value determined for the two polyphenols was largely consistent with those reported for other polyphenol oxidases of banana pulp (6.5) [32], medlar fruits (*Mespilus germanica* L. *Rosaceae*) [33] and yam Longbô (6.6) [22]. From Arrhenius plot, values of 54.2±2.04 and 48.5±1.12kJ/mol were obtained as the activation energy of PPO1 and PPO2, respectively. These activation energies are lower than that obtained (82.8kJ/mol) for the polyphenol oxidases purified from pineapple puree [34]. Values of activation energy indicate the relative tendency of a failure mechanism to be accelerated by temperature. In this respect, the studied polyphenol oxidases should be top-grade tools for various catalyzing reactions since it is well known that enzymes are biocatalysts that speed up chemical reactions by lowering the required activation energy [35].

The two polyphenol oxidases were fully active for more than 2 hours at their optimal temperature in sodium phosphate buffer, indicating a thermal stability at this temperature. The purified enzymes held these properties in common with the majority of plant polyphenol oxidases [36,22]. In this context, running biotechnological processes at moderate temperatures would be advantageous for application of these enzymes.

PPO1 and PPO2 catalyzed a broad range of mono and polyphenols substrates. Similar observations have been reported for polyphenol oxidase from ripe and unripe plantain (*Musa paradisiaca* cultivars – Agbagba, Cardaba and plantain hybrid) [37], indicating that the activity of each enzyme was not specifically restricted to a single substrate. The polyphenol oxidases from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) were inactive on monophenols such as naphtol, vanillin and L-tyrosine, suggesting the absence of monophenolase (cresolase) activity. The natural substrates that were oxidized by the enzymes were dopamine, pyrocatechol, catechin, pyrogallol and tannnic acid. This result suggested that the PPO1 and PPO2 were an *o*-diphenolase, possess activities tri and polyphenolase. The catalytics efficiency given by the Vmax/KM ratio was highest for the natural substrate dopamine among the substrates tested. Therefore, the purified enzymes could be considered as a dopamine oxidase. The purified enzymes were very similar to PPO from edible yam (*Dioscorea opposita* Thunb.) [29] and to the edible yam (*Dioscorea cayenensis-rotundata* cv. Longbô) [22], but different to the PPO from plantain (*Musa paradisiacal L*.) [37], which quickly oxidised catechol.

The activators of the two polyphenol oxidases activity was Mn^{2+}, Fe^{2+}, Na^{+} and Cu^{2+} . The activator effect of polyphenol oxidase activities by Cu^{2+}, Mn^{2+} and Fe^{2+} had also been reported for other plant species such as *Musa paradisiaca* Leaf [31]. The two polyphenol oxidases are inhibited by the ions K^{+}, $Pb^{2+}$and Sr^{2}. These cations should not be included in the two enzymes preparations. The inhibitory study showed that the most potent inhibitors for edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) PPO were beta-mercaptoethanol, SDS, ascorbic acid, sodium bisulphite and L-cysteine, since these compounds induced a high degree of inhibition, even at the lowest concentration used. [38] and [39] have also reported similar effect of these chemical agents on inhibition of polyphenol oxidase and the browning control of fresh-cut pear wedges and litchi fruit, respectively.

5. CONCLUSION

Based on the present study, it appears that the two polyphenol oxidases (PPO1 and PPO2) purified from the yam tuber (*Dioscorea cayenensis-rotundata* cv. Kponan) are acidic and mesophilic. The substrate specificity and kinetic properties showed that the two isoenzymes possess diphenolase activities having greatest activity toward dopamine. Moreover, the PPO1 and PPO2 activity are sensitive to some of general PPO inhibitors, especially to sodium bisulphite, ascorbic acid and L-cysteine. This work may help in côte d'ivoire studies for the food industries and processed food using yam.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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