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Isolation of a Latent Polyphenol Oxidase from Edible Yam (*Dioscorea cayenensis-rotundata* cv. *Zrèzrou*) 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 SD performed the statistical analysis, managed the literature searches and authors SNG and JK wrote the protocol, wrote the first draft of the manuscript and managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

A polyphenol oxidase was purified to homogeneity from edible yam (*Dioscorea cayenensis-rotundata* cv. *zrèzrou*) cultivated in Côte d'Ivoire. The purification procedure consisted of ion-exchange, ammonium sulphate fractionation, size exclusion and hydrophobic interaction chromatography. Dopamine was used as substrate. The enzyme designated PPO_Z had native molecular weight of approximately 64.56±0.03 and 64.08±0.23 kDa and functioned as monomer structure. Maximal PPO_Z activity occurred at 30°C and pH 6.0. The enzyme was stable at 30°C and its pH stability was in the range of 5.6–7.0. Substrate specificity revealed that the purified enzyme oxidized preferentially dopamine and then, catechol and catechin. PPO_Z showed no activity toward the monophenols and was completely inhibited by beta-mercaptoethanol, sodium thiosulphate, L-ascorbic acid, sodium bisulphite and L-cysteine reagents and it was strongly activated by SDS. In

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this study; the latent PPO was kinetically characterized in parallel with an active PPO present in the soluble fraction.

Keywords: Polyphenol oxidase activity; SDS; activation; Dioscorea cayenensis-rotundata cv. zrèzrou; dopamine; edible yam.

1. INTRODUCTION

Yams are monocotyledonous plants, belonging to the genus Dioscorea in the family of *Dioscoreaceae*. It is an important source of carbohydrate for many people of the sub-Sahara region, especially in the yam zone of West Africa [1]. But, when they were peeled, the colour of the pulp changes from creamy white to dark brown. This browning process leads to a change in flavour and a reduction in nutritional quality [2]. The discolouration phenomenon has been studied on fresh tubers and has mainly been associated with enzymatic browning, due to the action of polyphenol oxidase (PPO), peroxidase and to the production of polyphenols and derived products [3]. Polyphenol oxidases (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18. I), which is known by various names such as tyrosinase, phenolase, catechol oxidase, catecholase, monophenol oxidase, *o* and *p*-diphenol oxidase, orthophenolase, urushiol oxidase and laccase based on its substrate specificity, are widely distributed in higher plants, fungi and also in microorganisms [4]. In intact plant cells, PPO and its phenolic substrates are physically separated in chloroplasts and vacuoles respectively, precluding the oxidation of phenolics and eventually the enzymatic browning. PPO or tyrosinases are enzymes with a dinuclear copper center, which are able to insert oxygen in ortho position to a hydroxyl group existing in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. One unusual characteristic of this enzyme is its ability to exist in an inactive or latent state [5,6]. PPO can be released from the latency, or activated, by a variety of treatments or agents, including acid and base shock [7], urea [8], anionic detergents such as SDS [9], proteases [10], and fatty acids [11].

In vegetables, enzymatic browning is caused predominantly by PPO, which uses molecular oxygen to catalyze the *o*-hydroxylation of monophenols to *o*-diphenols and their further oxidation to coloured and highly reactive *o*-quinones [5]. These *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments.

Furthermore, peroxidases are also responsible for enzymatic browning but to a lesser degree. The oxidation of phenolic substrates by polyphenol oxidase is thought to be the major cause of the browning discoloration of many fruits and vegetables during handling, storage and processing [5,12,13]. This problem is of considerable importance to the food industry as it affects nutritional quality and appearance, reduces consumer acceptability and therefore, causes significant economic impact, both to primary food producers and to food processing industry [14]. Thus, inhibition of enzymatic browning by control of PPO physico-chemical characteristics could be a viable option. The objective of this study therefore was to achieve purification and a better understanding of the properties of PPO from the edible yam *Dioscorea cayenensis- rotundata* cv. *Zrèzrou* that catalyses the browning reaction.

2. MATERIALS AND METHODS

2.1 Plant Material and Enzyme Extraction

Yam (*Dioscorea cayenensis-rotundata*, cv. *zrèzrou*) tubers were harvested fresh at a Biological Garden of Nangui Abrogoua University [Abidjan (N6.401°, W5.091°), Côte d'Ivoire]. After harvesting, yams were immediately transported to the laboratory and stored at -20°C until used for experiment.

Yam tubers were cleaned with distilled water, peeled and the pulp was cut into slices. Then, 150 g were ground using a blender in 300 mL NaCl solution 0.9% (w/v). The homogenate was subjected to sonication (4°C) at 50–60 Hz frequency using a TRANSSONIC T420 for 10 min and then centrifuged at 20,000 g for 10 min at 4°C. The supernatant filtered through cotton wool was kept refrigerated and used as the crude extract.

2.2 Chemicals

Phenolic substrates such as dopamine, catechin, *p*-cresol, tyrosine, chlorogenic acid, tannic acid,

catechol, tyrosine, pyrogallol, gallic acid, naphtol, resorcinol, vanillin, syringic acid, 4-hydroxybenzoïc acid, 4-hydroxyphenyl acetic acid, phloroglucinol, 1,4-tyrosol, 4-hydroxyanisol,4-méthoxyphenol were purchased from Sigma-Aldrich. DEAE-Sepharose CL-6B, Sephacryl S-100 HR and Phenyl-Sepharose CL-6B 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 PPO Assay

PPO activity was determined by measuring the increase in absorbance at 480 nm. The reaction mixture contained 0.1 mL of enzyme solution, 0.8 mL of 10 mM dopamine solution and 1.1 mL of 0.1 M phosphate buffer, pH 6.0, at 25°C for 10 min in the absence of SDS (or 1.1 mL of phosphate buffer containing 0.4% of SDS pH 7). The choice of 0.4% of SDS in the buffer is obtained according to a study to determine the percentage of SDS for optimal activation of the enzyme [9]. The blank contained only 0.8 mL of substrate solution and 1.2 mL phosphate buffer 0.1 M, pH 6.0 in the absence of SDS or pH 7 in the presence of SDS. Experiments were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 min^{-1} under the assay conditions [15].

2.4 Protein Estimation

Protein concentration was determined by the Folin Ciocalteu method [16] using bovine serum albumin as the standard or by measuring the absorbance at 280 nm for the protein profiles in column chromatography.

2.5 Zymogram of the Crude Enzyme Extract

Electrophoresis (zymogram) was carried out by using the Laemmli [17] method on 10% (w/v) acrylamide gels under non-denaturing conditions, for searching PPO isoforms in the crude enzyme. After proteins migration, the disclosure of isoforms was made by immersing the gel in a solution containing 10 mM of dopamine substrate in the presence SDS (0.4 %).

2.6 Purification Procedures

All the purification procedure was carried out in the cold room. The crude extract (5 mL) was loaded onto a DEAE-Sepharose CL-6B gel (2.4 cm x 6.5 cm), equilibrated previously with 100 mM phosphate buffer pH 6.0. The unbound proteins were removed from the column by washing with two column volumes of the same buffer pH 6.0. Proteins were eluted using a stepwise gradient (0.3 M, 0.5 M, 0.7 M and 1 M) of NaCl in 100 mM phosphate buffer pH 6.0. Fractions (3 mL each) were collected at a flow rate of 180 mL h^{-1} and assayed for enzyme activity. The active fractions were pooled and saturated by 80 % ammonium sulphate in a cold room. The mixture was stirred for at least 8 h and centrifuged at 20,000 g for 30 min. The pellet was suspended in 100 mM phosphate buffer pH 6.0 and loaded onto a Sephacryl S-100 HR column (1.5 cm × 67 cm), a gel filtration chromatography, equilibrated with the same buffer. Fractions of 1 mL were collected at a flow rate of 20 mL h^{-1}, and those containing the PPO activity were pooled. The pooled fraction from the previous step was saturated to a final concentration of 1.7 M ammonium sulphate and applied on a Phenyl-Sepharose CL-6B column (1.4 cm x 7.5 cm) previously equilibrated with 100 mM phosphate buffer pH 6.0 containing 1.7 M ammonium sulphate. The column was washed with a reverse stepwise gradient of ammonium sulphate concentrations (1.7, 1, 0.5, 0.3, 0.2, 0.1 and 0) dissolved in the same phosphate buffer at a flow rate of 15 mL h^{-1} and fractions of 1 mL were collected. The pooled active fractions were dialyzed overnight against 100 mM phosphate buffer pH 6.0 and constituted the purified enzyme.

2.7 Homogeneity and Molecular Weight Determination

To check purity, the purified enzyme was analysed using polyacrylamide gel electrophoresis on a 7.5 % separating gel and a 4% stacking gel (Hoefer mini-gel system; Hoefer Pharmacia Biotech, San Francisco, CA, USA), according to the procedure of Laemmli [17] at 10°C and constant current 20 mM Electrophoretic buffers did not contain sodium dodecyl sulphate (SDS) and beta mercaptoethanol.

Molecular weight was determined electrophoretically with silver staining and sample denatured by a 5 min treatment at 100°C. The native molecular weight of the enzyme was determined using gel filtration on Sephacryl S-100 HR. The column Sephacryl S-100 HR (1.2 cm x 48 cm) equilibrated and eluted

in 100 mM phosphate buffer (pH 6.6) was calibrated with beta-amylase (206 kDa), cellulose (26 kDa), bovine serum albumin (66.2 kDa), ovalbumine (45 kDa) and amyloglucosidase (63 kDa). Fractions of 0.5 mL were collected at a flow rate of 30 mL h^{-1}.

2.8 Optimum pH and Stability

The effect of pH on the activity of the enzyme was determined by performing the oxidation of L-dopamine (10 mM) in a series of buffers (100 mM) at various pH values (2.6–8.0). Buffers used were phosphate citrate (pH 2.6–7), phosphate (pH 5.6–8) and sodium acetate (pH 3.6–5.6). pH values of each buffer were determined at 25°C. Experiments were performed in triplicate, and the results expressed as the percentage of maximum PPO activity.

To examine the pH stability, the enzyme solution (0.15 unit/mL) was pre-incubated for 2 h at various pHs at 25°C, and the residual activity was measured under the standard assay conditions. Experiments were performed in triplicates, and the results expressed as percentage activity of zero-time control of untreated enzyme.

2.9 Optimum Temperature and Thermostability

The effect of temperature on the polyphenol oxidase activity was examined under the standard assay conditions at temperatures ranging from 10 to 80°C using 10 mM dopamine as substrate. The thermal stability of the enzyme was determined at 25 and 30°C after exposure to each temperature for a period from 10 to 120 min. The enzyme was incubated in 100 mM phosphate buffer pH 6.6. Aliquots were drawn at intervals and immediately cooled in ice-cold water. Experiments were performed in triplicate. The residual enzymatic activity, determined in both cases at 25°C under the standard test conditions, was expressed as percentage activity of zero-time control of untreated enzyme.

2.10 Substrate Specificity and Kinetic Parameters

Heighten different commercial grade substrates (dopamine, catechol, p-cresol, tyrosine, chlorogenic acid, tannic acid, catechol, tyrosine, pyrogallol, gallic acid, naphthol, resorcinol, vanillin, syringic acid, acide4-hydroxybenzoic acid 4-hydroxyphenyl acetic, phloroglucinol, 1,4-tyrosol , 4-methoxyphenol, 4-hydroxyanisole) were used for investigation of the substrate specificity of enzyme. All substrates were used in 10 mM concentrations. The results were expressed as the percentage of maximum PPO activity.

The enzyme kinetic parameters [18,19], Michaelis-Menten constant (Km) and maximum reaction velocity (Vmax) for yam PPO were determined at 25°C when using catechin, L-dopamine and catechol as substrates, respectively. They were assayed in different concentrations, and at the optimum pH and wavelength for each substrate: 0.3, 0.6, 1.2 and 2.4 mM for catechin and catechol; 0.6, 1.2, 2.4 and 4.8 mM for dopamine. The assay cuvette (2 mL) contained 0.8 mL of catechin, L- dopamine or catechol (prepared in 10 mM sodium phosphate buffer, pH 6.0) solution with gradient concentration and 0.1 mL of the enzyme solution, respectively. Data were plotted as 1/activity and 1/ substrate concentration according to the method of Lineweaver et Burk [20]. Substrate specificity (Vmax/Km) was calculated by using the data obtained on a Lineweaver-Burk plot.

2.11 Effect of Chemical Agents on Enzyme Activity

The enzyme was incubated with 1 mM of different chemical agents for 20 min at 25°C. After incubation, the residual activity was determined by the standard enzyme assay using dopamine as a substrate. The activity of enzyme assayed in the absence of the chemical agents was taken as 100%.

2.12 Statistical Analysis

Results were expressed by means of ± SD. Statistical significance was established using Analysis of Variance (ANOVA) models to estimate the different between method or product using. Means were separated according to Duncan's multiple range analysis ($p \leq 0.05$), with statistical software Statistica (Stat Soft Inc, Tulsa USA Headquarters).

3. RESULTS

3.1 Zymogram

Zymogram showed a single protein band indicating the presence of one form of polyphenol

oxidase in the crude extract of the studied edible yam (Fig. 1B) and this form will appear when the enzyme is activated by SDS. In the case of peroxidase, no form appears (Fig. 1A).

3.2 Purification of Polyphenol Oxidase

The purification protocol of the polyphenol oxidase from the edible yam (*Dioscorea cayenensis-rotundata* cv. *Zrèzrou*) cultivated in Côte d'Ivoire involved three steps including anion-exchange, size exclusion and hydrophobic interaction chromatographies (Table 1; Fig. 2). The polyphenol oxidase resolved a single peak on the anion-exchange (DEAE-Sepharose CL-6B column) at 0.3 M NaCl concentration in 20 mM phosphate buffer (pH 6.0) (Fig. 2A). Active fractions (35–47) providing of this chromatography were pooled. The peak of polyphenol oxidase activity that resolved on the preceding chromatography was precipitated in 80% ammonium sulphate and applied to a Sephacryl S-100 HR gel. One peak showing polyphenol oxidase activity was eluted (Fig. 2B). The pigments which are very abundant in the crude extract were almost completely removed during the gel filtration step. This polyphenol oxidase activity (pooled fractions 93–107) was ultimately purified using hydrophobic interaction chromatography on phenyl-Sepharose CL-6B gel and the enzyme eluted at 0.7 M ammonium sulphate (Fig. 2C). After purification, the polyphenol oxidase (pooled fractions 41-46) was enriched about 76.25±0.72-fold and the yield was 23.39±2.35 (Table 1). The specific activity was 4680.56±3.52U mg^{-1} proteins. The enzyme showed a single protein band on native polyacrylamide gel electrophoresis (Fig. 3A).

After SDS-PAGE analysis under reducing conditions, the polyphenol oxidase from the edible yam (*Dioscorea cayenensis-rotundata* cv. *zrèzrou*) cultivated in Côte d'Ivoire showed a single protein band and its relative molecular weight was estimated to be 64.56±0.03 kDa , (Fig. 3B). The relative molecular weight of the native enzyme determined by gel filtration chromatography on Sephacryl S-200 HR column was approximately 65.08±0.23 kDa (Table 2).

3.3 Optimum pH and Stability

The purified polyphenol oxidase exhibited high activity at pH 6.0 in absence of SDS and pH 7.0 in the presence of SDS (Fig. 4A). This enzyme was more active in phosphate buffer than other buffers used (Fig. 4A). At 25°C for 120 min, the polyphenol oxidase showed best stability over pH values ranging from 5.6 to 7.0, conserving at least more than 90 % of total activity. The results indicate that the polyphenol has the same pH stability area after two hours of preincubation in that either all the buffers in the presence or absence of SDS. It reached its maximum activity in phosphate sodium buffer 100 mM pH 6.0 (Fig. 4B).

Fig. 1. Zymogram of crude extract from edible yam *Dioscorea cayenensis-rotundata* cv."zrèzrou". (A) peroxidase; (B) polyphenol oxidase

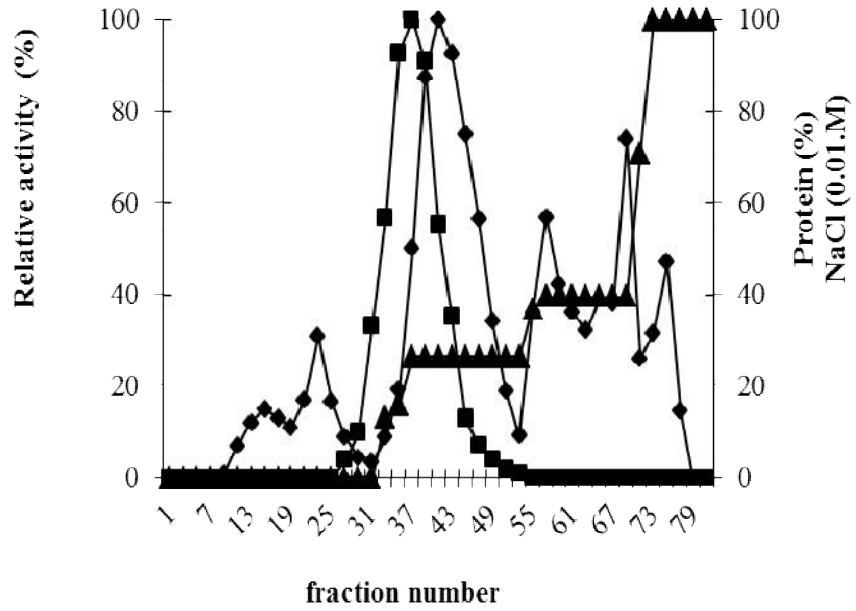

Fig. 2A. Purification steps of PPO from edible yam (*Dioscorea cayenensis rotundata* cv. "zrèzrou" by ion exchange chromatography on DEAE-sepharose CL-6B showing enzyme activity (■), chloride sodium (▲) and protein contents (♦). The enzyme activity was measured in phosphate buffer (pH 6.0) at 37°C using dopamine as substrate

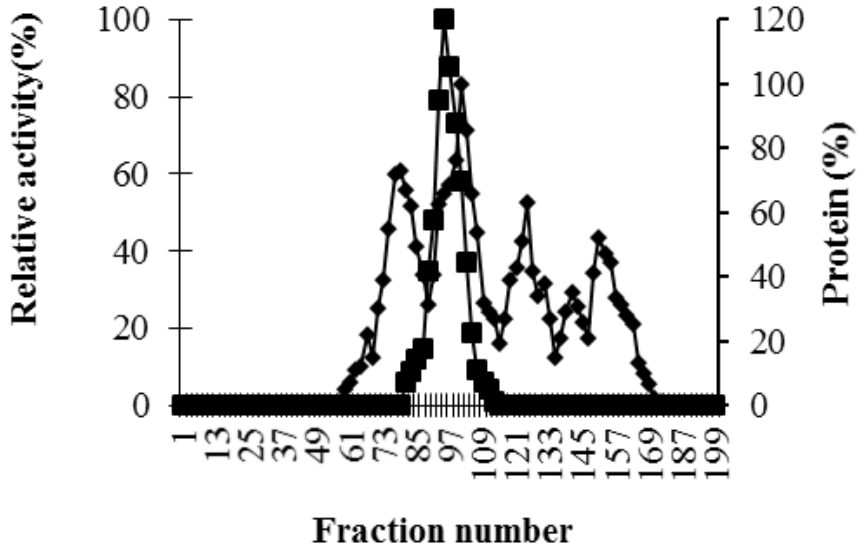

Fig. 2B. Purification steps of PPO from edible yam (*Dioscorea cayenensis rotundata* cv. "zrèzrou" by gel filtration chromatography on sephacryl S-100 HR showing enzyme activity (■) and protein content (♦). The enzyme activity was measured in phosphate buffer (pH 6.0) at 37°C using dopamine as substrate

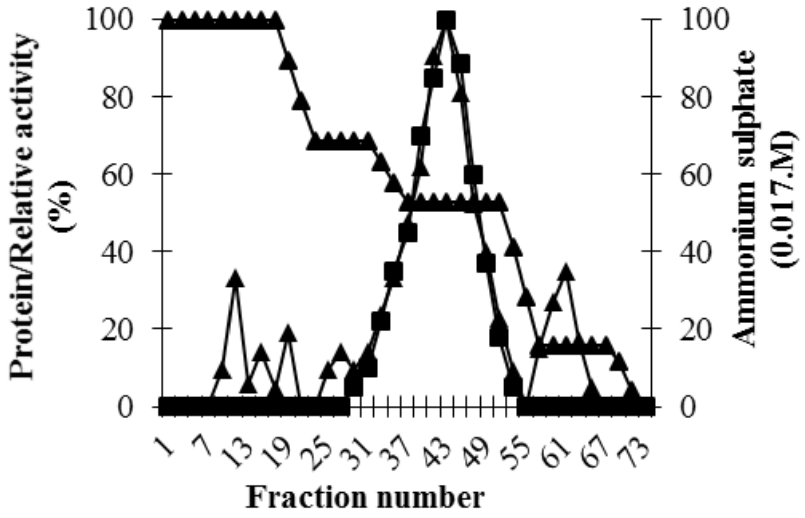

Fig. 2C. Purification steps of PPO from edible yam (*Dioscorea cayenensis rotundata* cv. "zrèzrou" by hydrophobic interaction chromatography on phenyl-sepharose CL-6B showing enzyme activity (■), ammonium sulphate (▲) and protein (♦). The enzyme activity was measured in phosphate buffer (pH 6.0) at 37°C using dopamine as substrate

Table 1. Purification procedure of polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou"

Purification steps	Total activity (U)	Total Protein (mg)	Specific activity (U mg^{-1})	Purification factor	Yield (%)	Elution concentration and pooled fractions
Crude extract	18012.50±9.18	146.85±0.58	122.66±1.80	1.00	100.00	-
DEAE-Sepharose CL-6B	11675.00±4.81	62.48±0.03	186.87±3	1.52±0.27	64.82±3.54	0.3 M NaCl fractions 35-4 7
Ammonium sulphate Precipitation	9415.13±5.25	8.38±0.02	1123.52±2.5	18.30±0.03	52.27±2.27	-
Sephacryl-S100 HR	5851.83±2.23	1.91±0.02	3063.78±5.2	49.92±0.16	32.49±1.85	fractions 93-107
Phenyl-Sepharose CL-6B	4212.50±3.08	0.9±0.01	4680.56±3.52	76.25±0.72	23.39±2.35	0.7 M AS* fractions 41-46

Table 2. Some physicochemical characteristics of the polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" cultivated in Côte d'Ivoire

Physicochemical properties	Values	
	In the absence of SDS	In the presence of SDS
Optimum pH	6.0[a]	7.0[b]
pH stability range	5.6-7.0	5.6-7.0
Optimum temperature	30°C	30°C
Activation energy (KJ mol^{-1})	21.78±2.19[a]	34.43±1.83[b]
Temperature coefficient (Q10)	1.21±0.17[a]	1.81±0.22[b]
Michaelis–Menten equation	Obeyed	Obeyed
Molecular weight (kDa)		
Mobility in SDS–PAGE	64.56±0.05[a]	
Gel filtration	65.08±0.04[b]	

SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis. means not sharing a similar letter in a same line are significantly different p≤0.05 as assessed by the test of duncan. values given are the averages of three experiments

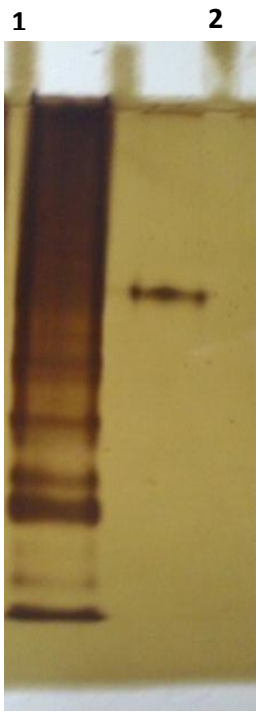

Fig. 3A. PAGE analysis of purified polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou". The sample was loaded onto a 10 % gel. lane 1: crude extract; lane 2: polyphenol oxidase purified

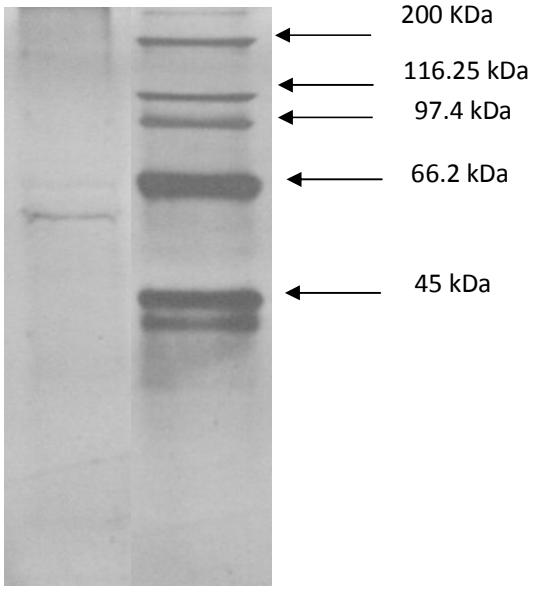

Fig. 3B. SDS-PAGE analysis of purified polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou". The sample was loaded onto a 10 % gel. lane 1: purified polyphenol oxidase; lane 2: molecular weight markers

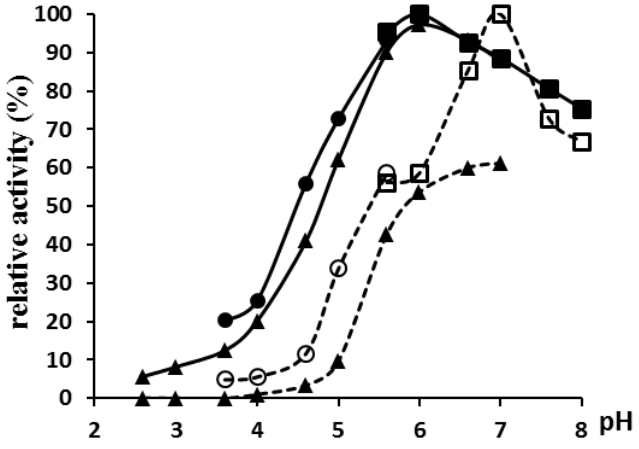

Fig. 4A. Determination of optimum pH from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" PPO activity measured with dopamine substrate. Symbols: Acetate buffer In the absence of SDS (●), phosphate buffer in the absence (■), citrate phosphate buffer in the absence (▲), acetate buffer in the presence of SDS (○), phosphate buffer In the absence of SDS (□), citrate phosphate buffer in the presence of SDS (∆)

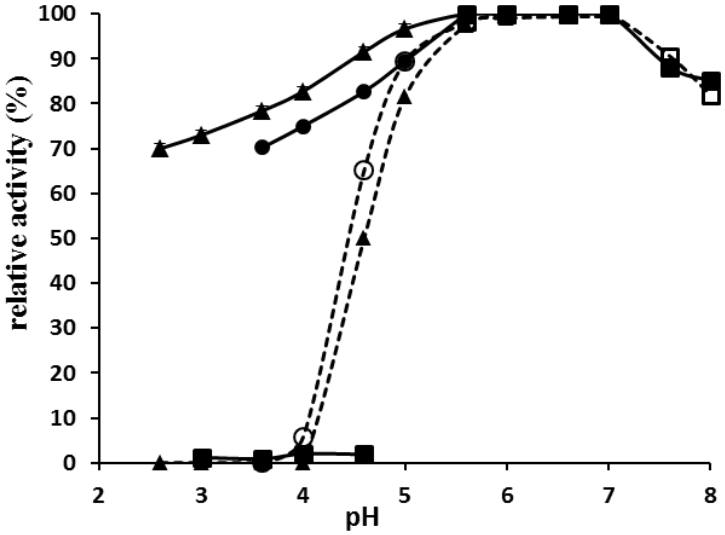

Fig. 4B. pH-stability profiles of edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" polyphenoloxidase. Symbols: (●) acetate buffer in the absence of SDS, (■) phosphate buffer in the absence of SDS, (▲)citrate phosphate buffer in the absence of SDS, (○)acetate buffer in the presence of SDS, (□) phosphate buffer in the presence of SDS, (∆) citrate phosphate buffer in the presence of SDS

3.4 Temperature Optimum and Thermostability

The optimum temperature of polyphenoloxidase of yam tuber *Dioscorea* cayenensis *rotundata* cultivarzrèzrou in the absence of SDS is identical to that obtained in the presence of SDS. This optimum temperature is 30°C (Table 2). The enzyme activity remains constant up to its optimum temperature. Beyond this temperature, the polyphenoloxidase gradually loses its activity up to 45°C, it retains more than 50 % of its activity.

The temperature coefficient (Q_{10}) for the enzyme determined in the thermal zone of increasing

reactivity between 10 and 25°C differ in the presence or absence of SDS. They are 1.21±0.17 in the absence of SDS and 1.81±0.22 (Table 2).

From Arrhenius plot, values of 21.78±2.19 kJ/mol in the absence of SDS and 34.43 kJ/mol in the absence presence of SDS were obtained for the activation energy of the purified PPO. The thermal denaturation study indicated that the Polyphenol oxidase was fairly stable at temperature up to its optima (30°C). Above this temperature, the activity declined rapidly, as the temperature increased, though the enzyme was completely inactivated at 60°C in the presence of SDS and 80°C in the absence of SDS (Fig. 5A). As concerns thermal inactivation the purified enzyme remained fully stable for 105 min in absence of SDS and 60 min in presence of SDS at 30°C, while at 37°C it was less stable and lost its activity after 15 min of preincubation, (Fig. 5B).

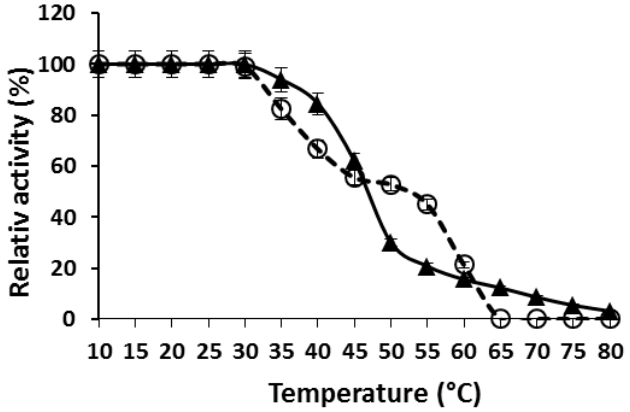

Fig. 5A. Thermal denaturation of polyphenol oxidase from edible yam (*Dioscorea cayenensis-rotundata* cv. *zrèzrou*). symbol: (▲) in the absence of SDS, (○) in the presence of SDS. The enzyme was preincubated at each temperature for 15 min and the remaining activity measured at 25°C under standard test conditions. Values are averages of at least three experiments

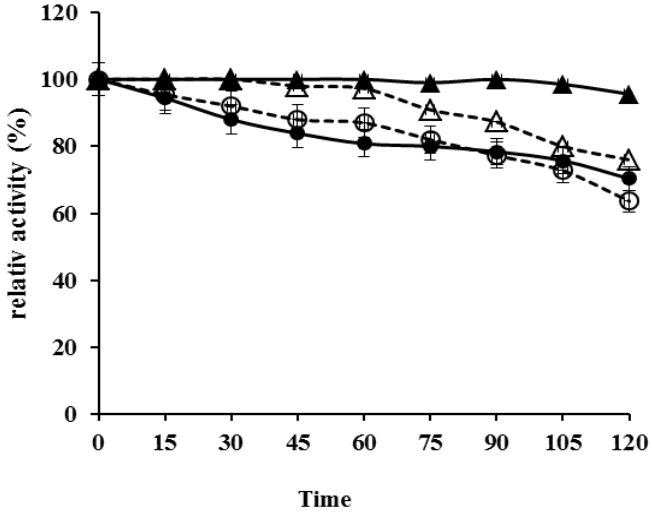

Fig. 5B. Thermal stability of polyphenol oxidase from edible yam (*Dioscorea cayenensis-rotundata* cv*. zrèzrou*ˆ) showing denaturation of the enzyme at 37°C after 15 min of preincubation. The enzyme was preincubated at 30°C and 37°C in 100 mM phosphate buffer (pH 6.0). symbol (▲) 30°C in the absence of SDS; (●) 37°C in the absence of SDS; (∆) 30°C in the presence of SDS; (○) 37°C in presence the of SDS

3.5 Substrate Specificity and Kinetic Parameters

The purified enzyme was inactive towards *p*-cresol, tyrosine, chlorogenic acid, tannic acid, tyrosine, pyrogallol, gallic acid, naphtol, resorcinol, vanilline, syringic acid, 4-hydroxybenzoïc acid, 4-hydroxyphenyl acetic acid, phloroglucinol, 1,4-tyrosol and 4-hydroxyanisol and 4-methoxyphenol in the presence as in the absence of SDS. Although, the enzyme attacked dopamine, pyrocatechol and catechin. The highest activity was observed with dopamine (Table 3). Michaelis constant (K_M) and maximum reaction velocity (Vmax) values were calculated from the Lineweaver-Burk plot for dopamine, catechin and catechol. K_M values were 26.21 ± 0.6, 4.65 ± 0.07 and 6.25 ± 0.01 mM respectively for catechol, catechin and dopamine. With the three substrates, the enzyme obeyed the Michaelis–Menten equation (Fig. 6). The catalytic efficiency of polyphenol oxidase, given by the V_{max}/K_M ratio was much higher for the dopamine than the pyrocatechol (Table 4). For the same substrate, in the presence or absence of SDS, the values of maximum velocities (Vmax) differ. K_M values in the absence or in the presence of SDS are all identical.

Table 3. Substrate specificity of the purified polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" cultivated in Côte d'Ivoire

Substrate	Wavelength (nm)	Specific activity (U mg^{-1})		Relative rate of oxidation (%)	
		In the absence of SDS	In the presence of SDS	In the absence of SDS	In the presence of SDS
Dopamine	480	2392.86±0.33^{a}	4276.78±2.73^{d}	100±0.01^{g}	100^{j}
Catechol	420	1089.28±1.72^{b}	1819.10±1.55^{e}	45.52±0.07^{h}	42.53^{k}
Catechin	420	291.07±1.72^{c}	461.54±1.00^{f}	7.46±0.07^{i}	10.79^{l}
Chlorogenic acid	420	0	0	0	0
Resorcinol	420	0	0	0	0
Pyrogallol	420	0	0	0	0
Gallic acid	420	0	0	0	0
Tyrosine	420	0	0	0	0
Naphtol	420	0	0	0	0
Vanillin	420	0	0	0	0
p-Cresol	420	0	0	0	0
Syringic acid	420	0	0	0	0
4-hydroxybenzoïc acid	420	0	0	0	0
4-hydroxyphenyl acetic acid	420	0	0	0	0
phloroglucinol	420	0	0	0	0
1,4-tyrosol	420	0	0	0	0
4-hydroxyanisol, 4-methoxyphenol	420	0	0	0	0
tannic acid	420	0	0	0	0

Means not sharing a similar letter in a same column and a same line are significantly different p≤0.05 as assessed by the test of duncan. values given are the averages of three experiments

Table 4. Kinetic parameters of the purified polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" cultivated in Côte d'Ivoire

Substrates	K_M (mM)		V_{max} (U/mg)		V_{max}/K_M (U/mM.mg)	
	In absence of SDS	In presence of SDS	In absence of SDS	In presence of SDS	In absence of SDS	In presence of SDS
Dopamine	6.25±0.03^{a}	6.20±0.42^{a}	10416.67±2.66^{d}	5813.95±8.8^{g}	1666.67±9.5^{j}	933.71±2.26^{m}
Catechol	26.21±0.02^{b}	26.32±0.28^{b}	5952.38±0.60^{e}	3289.47±1.4^{h}	227.10±0.21^{k}	124.98±1.83^{n}
Catechin	4.65 ± 0.02^{c}	4.65±1.07^{c}	617.28±1.78^{f}	453.72±2.02^{i}	132.75±1.00^{l}	97.66±0.58^{o}

Means not sharing a similar letter in a same column and a same line are significantly different p≤0.05 as assessed by the test of duncan. values given are the averages of three experiments

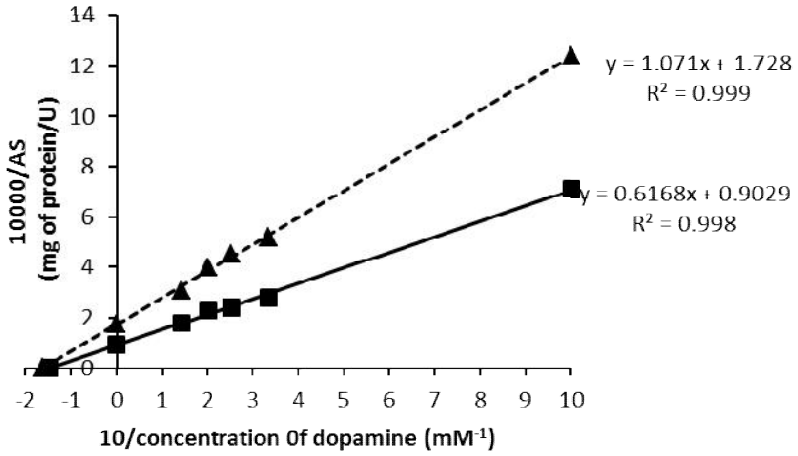

Fig. 6A. Lineweaver-burk polyphenoloxidase for dopamine. Symbol in the absence of SDS (■); in the presence of SDS (▲)

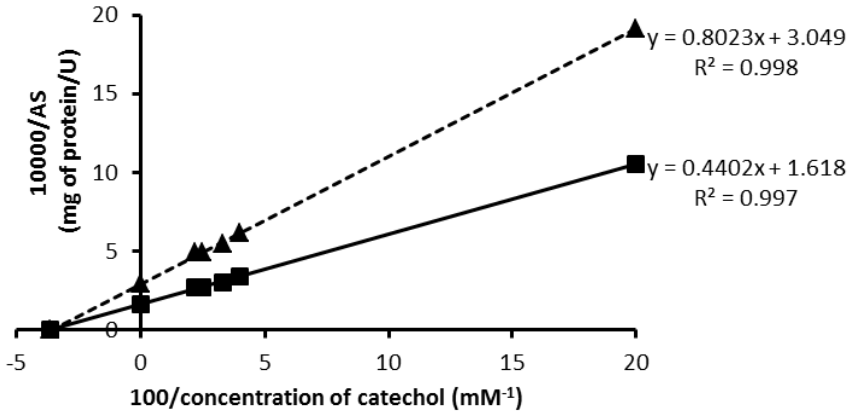

Fig. 6B. Lineweaver-burk polyphenoloxidase for catechol. symbol in the absence of SDS (■); in the presence of SDS (▲)

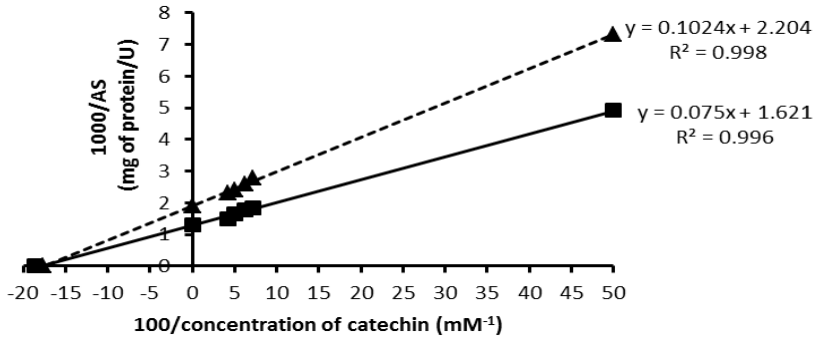

Fig. 6C. Lineweaver-burk polyphenoloxidase for catechin. Symbol in the absence of SDS (■); in the presence of SDS (▲)

3.6 Effect of Chemical Agents on Enzyme Activity

The result showed that most of cations tested did not affect significantly the polyphenol oxidase activity (Table 5). The enzyme was activated by cations such as Zn^{2+}, Cu^{2+} and Mn^{2+} and by SDS. However, this PPO was inhibited by beta-mercaptoethanol, sodium thiosulphate, ascorbic acid, sodium bisulphate, DL-dithiothreitol, DTNB, EDTA and cysteine. This inhibition was total with DL-dithiothreitol, sodium bisulphate and beta-mercaptoethanol.

4. DISCUSSION

Zymogram of crude extract from edible yam *Dioscorea cayenensis-rotundata* cv. "Zrèzrou" revealed one form of polyphenol oxidase and absence of peroxidases. This form of polyphenol oxidase appears when the substrate was mixed with SDS (an activator). This result suggests that polyphenol oxidase would be only responsible of enzymatic browning of yam tuber *Dioscorea cayenensis-rotundata* cv. "Zrèzrou". Thus, a polyphenol oxidase was purified to homogeneity from the studied yam, confirming Zymogram result. This enzyme was successfully purified in three steps including ion exchange (DEAE Sepharose CL-6B), size exclusion (Sephacryl S-100 HR) and hydrophobic interaction (Phenyl Sepharose CL-6B) chromatography. The hydrophobic interaction chromatography on Phenyl Sepharose CL-6B gel was crucial to separate the PPO from the other proteins and impurities [21,22]. The purification yield (23.39 %) was higher than those of PPO from Dioscorea bulbifera (10 %) [23] but it was lower than that of PPO from Dioscorea cayenensis-rotundata cv. longbô (34 %) [22]. But, the specific activity (4680.56 U / mg proteins) was higher compared to PPO from *Dioscorea cayenensis-rotundata* cv. Longbô (3575,72 U / mg of proteins) [22].

The molecular weight of the purified PPO_Z was estimated to be 64,56 kDa and 65.08 kDa respectively, by SDS-PAGE and gel filtration on Sephacryl S-100 chromatography. These results suggested that the enzyme was a monomer as those from pear [24] and Chinese cabbage [25].

Table 5. Effect of some chemical agents on the polyphenol oxidase from edible yam *Dioscorea cayenensis-rotundata* cv. "zrèzrou" cultivated in Côte d'Ivoire

Chemical agents	Concentration in assay (mM)	Residual activity (%)	
		In absence of SDS	In presence of SDS
control	0	100	100
Na^+	1	97.27±0.08	90.73±0.38
K^+	1	104.91±0.91	108.74±1.58
Mg^{2+}	1	98.18±1.21	97.65±3.15
Ca^{2+}	1	97.29±2.01	92.92±0.88
Ba^{2+}	1	90.27±1.34	81.09±1.25
Zn^{2+}	1	107.10±0.90	113.11±0.91
Cu^{2+}	1	119.28±2.23	110.62±1.63
Mn^{2+}	1	116.39±1.73	111.53±1.45
EDTA[a]	1	88.12±0.93	85.76±1.01
Sodium thiosulphate	1	2.18±0.02	0
Sodium azide	1	80.33±2.27	72.53±1.84
Vitamin C (ascorbic acid)	1	1.38±0.01	0
DL-dithiothreitol (%, w/v)	1	0	0
*p*CMB [b] (%, w/v)	1	83.35±1.39	87.04±0.92
DTNB [c] (%, w/v)	1	63.41±1.15	65.81±1.24
Sodium bisulphite	1	0	0
L-cystein	1	2.18±0.12	1.72±0.05
β-mercapto ethanol (%, w/v)	1	1.71±0.02	0
SDS (%, w/v)	1	165. 3±3.04	
Tween 80 (%, w/v)	1	73.08±1.21	75.26±0.16

EDTA: ethylene diamine tetra-acetic acid; DTNB: 5, 5-dithio-bis (2-nitrobenzoate); pCMB: p-chloromercuribenzoate; SDS: sodium dodecyl sulphate. (values given are the averages of three experiments given are the averages of three experiments)

The purified enzyme showed a single but broad pH optimum curve between pH 5.0 and 8.0 with maximum activity at pH 6.0 in and 7.0 when SDS was used in the reaction medium. The activity increased at acidic pH because of the acid-shocking effect [26] in the absence of SDS. However, in the presence of SDS, the optimum pH shifted to values greater than pH 6.0. This SDS-induced change in optimal pH is a common feature with other latent PPOs [26,27]. This pH in the absence of SDS was similar to that of mango PPO [28]. But it result differ from those reported for the soluble PPO of loquat fruits of the Mogi variety [29] who showed that PPO activity was optimal at pH 4.5. So, it is clear that the polyphenol oxidase optimum pHs were highly dependent on the enzyme source, the substrate nature and the conditions of reaction medium [30]. The pH stability profile for the enzyme showed that 100 % of the purified PPO activity was retained between pH 5.6 and pH 7.0 in the presence and absence of SDS as PPO from yam *Dioscorea cayenensis-rotundata* cv. Longbô [22]. SDS had almost no effect on the pH stability. The PPO system in yams was shown to be most active at a near neutral pH value [31]. According to Lee et al. [32], the rapid inactivation of the enzyme above pH 8.0 may be due to conformational changes in the enzyme under the alkaline conditions and/or the enzyme may react rapidly with quinones through Maillard reactions. According to the pH stability results, in the presence or absence of SDS the PPO would only catalyze the oxidation of phenolics in pH range of 5.6 - 7.0.

The purified enzyme showed maximum activity at 30°C and was stable at a same temperature in the absence and presence of SDS. It retained 100 % of its original activity when incubated at this temperature for 105 min in the absence of SDS and 60 min in the presence of SDS. Thermal inactivation of PPO is a fast phenomenon at temperatures above 37°C, the same as for those of PPOs of another yam tuber species [33,34]. It has been reported that pear PPO was stable at 60°C and plum PPO at 70°C [24, 35]. The activation energy (21.78±2.19 kcal/mol) is in agreement with results from PPOs of other vegetable sources (18.4–84.8 kcal/mol) [36,37].

PPO from yam *Dioscorea cayenensis-rotundata* cv. *zrèzrou* was active toward *o*-diphenol substrates such as catechol, catechin and dopamine. But, no activity was detected toward monophenols such as *p*-cresol and L-tyrosine, suggesting absence of monophenolase activity. In terms of physiological efficiency (V_{max}/K_M), dopamine appeared to be the best substrate among the substrates tested. Therefore, the PPO from yam *Dioscorea cayenensis-rotundata* cv. *zrèzrou* could be considered as a dopamine oxidase. This result was similar to PPO from edible yams *Dioscorea opposite* Thunb. [31], *Dioscorea cayenensis-rotundata* cv. *Longbô* [22] and from banana *Musa sapientum* pulp [38], but different to the PPO from white yam (*Dioscorea rotundata*), which quickly oxidised chlorogenic acid and catechol [33]. The study of the kinetic parameters (Vm and K_M) was carried out at pH 6.0 in the absence of SDS and pH 7 in the presence of SDS with dopamine, catechol and catechin as substrates. This study showed that the K_M value for PPO_Z toward dopamine, catechin and catechol in the presence of SDS was the same effect in the absence of SDS. The Vmax values are different against. This result suggests that the SDS acts on the enzyme by changing the reaction rate by increasing the number or enzymatic entity by activation of latent forms of polyphenoloxidase present in the reaction medium [39]. This result of K_M values differs from those of Wuyts et al. [40], who screened various substrates for their suitability to be oxidized by banana (*Musa acuminate* Grande naine) roots PP0. These authors reported a low K_M value of 0.6 mM toward dopamine.

It has been reported that the nature of the inhibitor may affect its action on PPO [30,41,42]. The effect of general inhibitors of PPO, namely ascorbic acid, sodium azide, EDTA and sodium bisulphite, on purified polyphenol oxidase of *Dioscorea cayenensis-rotundata* from the cultivar *zrèzrou* was examined. DL-dithiothreitol, sodium bisulphate and beta-mercaptoethanol were the most effective inhibitors. The inhibition by sodium thiosulphate, ascorbic acid, and L-cystein was also very strong. Cations Zn^{2+}, Cu^{2+} and Mn^{2+} and SDS were found to be strong activator of the purified PPO from edible yam (*Dioscorea cayenensis-rotundata* cv. *zrèzrou*. This activation process was saturable, reaching its maximum activation at 0.4% of SDS. This result indicate that the latent form exist in the reaction medium. Later, Moore & Flurkey [43] determined the effect of SDS on broad bean PPO, and their results also indicated that a limited conformational change due to the binding of small amounts of SDS may induce or initiate the activation of the latent enzyme.

The use of SDS as an activating agent is particularly interesting because few enzymes are known to be activated by SDS while many are inactivated by this detergent. Indeed, in vitro studies have shown that the addition of anionic detergents such as sodium dodecyl sulfate (SDS) caused significant increase of the activity of certain PPO extracts, suggesting the presence of latent forms of the enzyme in these extracts [44]. Swain et al. [8] showed that this enzymatic activation process is related to a limited conformational change in the latent enzyme. According to many authors, activation of PPO may be by two completely independent mechanisms from each other. This is on the one hand, the activation by partial proteolysis and activation of the other hand in the presence of detergents. The first mechanism used to move from a so-called immature to mature form of PPO, while the second mechanism can switch from an inactive form to an active form [45]. According to Moore & Flurkey [43], the interactions between the PPO and the SDS alter the three-dimensional conformation of the enzyme and thus make the active site accessible to substrates. On the other hand, there is at least one mechanism for enabling the said latent forms of polyphenol.

The mechanism of inhibition by ascorbic acid may involve reduction of quinonoid compounds produced by the enzyme [41,42,46]. Two other mechanisms of inhibition involving direct interaction with the enzyme have been reported: chelation of the copper ion Cu^{2+} in the active site [46] and its reduction in Cu^{+} [47]. Ascorbic acid was used by Makower et Schwimmer [48] to delay browning of avocado slices. Dorantes [49] showed that the inhibitory effect of ascorbic acid on avocado browning depends on the variety.

The inhibition by bisulphite and L-cysteine would suggest that the thiol compounds are potent inhibitors of PPOs [50,30,51,38]. The effect of L-cysteine consisted to trapping *o*-quinones as a colourless cysteine-quinone addition compound stable [52]. The mechanism of browning inhibition by L-cysteine has been controversial for a long time. There are two main opinions which exist: firstly, inhibition results from the formation of a conjugate between quinone and cysteine [52] and secondly, cysteine may directly inhibit the enzyme by combining irreversibly with copper at the active site [53]. This latter opinion is true in so far as cysteine affects the PPO directly. Indeed, the SH groups of cysteine have a strong affinity for copper and displace histidine residues liganded to the copper of the active site of PPO and/or completely remove the copper from the enzyme [54].

As regard EDTA, It seems that it was not a good inhibitor for this enzyme. This observation could result of the acidic pH 6.0 of the reaction mixture which may prevent binding of EDTA to the metal center at the active site of the enzyme [55]. Inhibition by suggest the existence of thiol group in active site of enzyme and that these thiol group are essential in catalysis. Indeed, it is well know that DTNB and PCMB are thiol group reducer agents.

5. CONCLUSION

The results from the present study showed that the polyphenol oxidase purified from edible yam (*Dioscorea cayenensis-rotundata* cv. *Zrèzrou*) was a monomer enzyme. The substrate specificity and kinetic properties indicated that dopamine was a potential physiological substrate of the enzyme. It was sensitive to some of general PPO inhibitors. This work suggest that the major PPO form for the oxidation of dopamine, leading to enzymatic browning under physiological conditions, is the latent one.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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