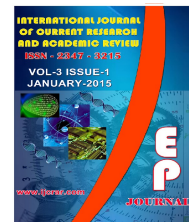




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Inhibitory Effect of Onions and Garlic Extract on the Enzymatic Browning of an Edible Yam (*Dioscorea cayenensis-rotundata* cv. Kponan) cultivated in Côte d'Ivoire

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A B S T R A C T

Polyphenol oxidase (PPO) presented different specific activities at different locations in the yam tuber (*Dioscorea cayenensis-rotundata* cv. Kponan), with the highest values in the proximal part (62.88 ± 1.87 U/mg de protein). The inhibitory effect of different variety of onion and garlic extract on PPO and enzymatic browning of yam tuber was investigated in order to elucidate the kinetics and mechanism of these extracts inhibition. The PPO from yam was strongly inhibited by various reducing agents, such as β -mercaptoethanol, L-ascorbic acid, sodium bisulphite and L-cysteine. The enzyme was also inhibited by addition of onion and garlic extract. The addition of red and yellow onion extract at 25°C for 10 min, gave a greatest inhibitory effect on yam polyphenol oxidase activity. The various sulfur compounds present in *Allium* species, such as onion might be responsible for the inhibitory action of the onion extract.

Introduction

Browning in fruits and vegetables damaged by mechanical injury during harvesting and post harvest storage or processing, is one of the main causes of quality loss (Flick *et al.*, 1977; Perez- Gilabert and Carmona, 2000). The major groups of reactions leading to browning are enzymatic browning and non-enzymatic browning (Manzocco *et al.*, 2001). Basically, enzymatic browning can be defined as the initial enzymatic oxidation of phenols into slightly colored quinones (Nicolas *et al.*, 1994).

These quinones are then subjected to further reactions leading to the formation of pigment (Ozoglu and Bavindirli, 2002; Wen and Wrolstad, 2001). Browning reactions which occur after infliction of a mechanical injury to some plant tissues, live fruits, tubers and vegetables are initiated by enzymes such as polyphenol oxidase, peroxidase and leads to the production of polyphenols and derived products (Osagie and Opoku, 1984). Polyphenol oxidases (PPO) which are able to act on phenols in

the presence of oxygen have been associated with enzymatic browning (Vamos-Vigyázó, 1981; Sapers and Miller, 1992). Polyphenol oxidase (PPO), a copper-containing enzyme with a dinuclear copper centre, catalyzes both the hydroxylation of monophenol and the oxidation of the o-diphenol respectively to their corresponding o-diphenol (cresolase activity, E.C. 1.14.18.1) and o-quinones (catecholase activity, E.C. 1.10.3.1) in the presence of molecular oxygen, which are polymerized into pigments (Gao, *et al.*, 2009).

Further condensation of quinones leads to brown melanin pigments (Martinez and Whitaker, 1995). Enzymatic and/or nonenzymatic browning reactions may adversely affect the quality, nutritional value and safety of foods (Laurila and Ahvenainen, 2002; Billaud *et al.*, 2003) and just washing with water is not effective in preventing discoloration (Mattila *et al.*, 1995). Consequently, the control of enzymatic browning in order to maintain their quality, nutritional value and safety has aroused strong interest in the food industry (Langdon, 1987).

One approach to the prevention of this phenomenon in plants and vegetables has been the use of anti-browning agents such as sulphite-containing additives. However, due to health concerns, sulphites use has been restricted (Anon, 1991). On the other hand, due to consumer's demand for natural food additives, studies have been devoted to the search for natural inhibitors of enzymatic browning (Naimiki, 1990; Nicolas *et al.*, 1994). Thus, Laurila and Ahvenainen (2002) have noted that the most attractive way to inhibit browning would be by natural methods. The polyphenol oxidase inhibitors occurring in natural resources have been studied in several plants (Jang *et al.*, 2002; Lee *et al.*, 2007; Gnangui *et al.*, 2010), but

the development of natural and efficient polyphenol oxidase inhibitors is needed.

The browning phenomenon is predominant in roots and tubers particularly in yam, a staple food in many tropical and subtropical areas of the world. Indeed, when they were peeled, the colour of the pulp ranges from creamy white to dark brown. This browning process leads to a change in flavour and a reduction in nutritional quality of yam (Golan-Goldhirsh and Whitaker, 1984). Yam must be processed to be utilized commercially, and the control of enzymatic browning, due to polyphenol oxidase, seems to be necessary. In the present work, we investigate on the distribution of polyphenol oxidase and the inhibitory effect of onion and garlic extract as a natural inhibitor of this enzyme and therefore, enzymatic browning of yam tuber (*Dioscorea cayenensis-rotundata* cv. Kponan).

Materials and Methods

Chemicals

β -mercaptoethanol, L-ascorbic acid, sodium bisulphite, citric acid and L-cysteine were purchased from Sigma Chemical (St. Louis, USA). All other chemicals used were of analytical grade.

Plant Material and polyphenol oxidase preparation

The studied cultivar yam (*Dioscorea cayenensis-rotundata*, cv.Kponan) was grown during its appropriate cropping season in June 2008 at the experimental farm of the University Nangui Abrogoua [Abidjan, Côte d'Ivoire] (5°23 latitude North, 4°00 longitude West, and 7 meters altitude). The ripened tubers were randomly harvested 6 months after planting (December 2008). After harvesting, tubers

were peeled using a stainless steel kitchen knife and the pulp was cut into slices. Then, 150 g were ground using a blender in 300mL of 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 10,000 x g for 30 min at 4°C. The supernatant filtered through cotton wool was kept refrigerated (4 °C) and used as the crude extract.

Onion and garlic extract preparation

Onion and garlic used as natural anti-browning agents were purchased from a local market in Adjamé [Abidjan, Côte d'Ivoire].

Onion or garlic (100 g) was homogenized with 100 mL of distilled water for 5 min, and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 10,000 x g for 20 min at 4 °C and the supernatant, after centrifugation, was used for this experiment.

Protein quantification

The protein content was measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Measurement of PPO activity

Under the standard test conditions, PPO activity was measured spectrophotometrically using a modification of the method of Wong *et al.* (1971). The reaction mixture (2 mL) containing 0.8 mL of 8 mM dopamine solution, 1.1 mL of a 100 mM phosphate buffer (pH 6.6) and 0.1 mL of the enzyme solution was incubated at 25°C for 10 min. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 480 nm. One unit of

enzymatic activity was defined as an increase in absorbance of 0.001 per minute (Cong *et al.*, 2005). Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein.

Inhibition of chemical reagent on PPO extract

To determine the Inhibition of chemical reagent of the PPO, each enzyme solution was preincubated with 1 mM of different chemical agents for 20 min at 25 °C. After incubation, the activity was assayed under the enzyme assay conditions using dopamine as a substrate.

The percent inhibition was calculated using the polyphenol oxidase activity values from the control and each test fraction, defining the control as zero percent inhibition (Equation 1).

The percentage of inhibition was expressed as:

$$\text{Inhibition (\%)} = [(A - A^*) / A] \times 100$$

Where, A and A* indicate the variation of absorbance in absence and presence, respectively, of the inhibitor.

Inhibition of natural reagent on PPO extract

Inhibition of yam tuber (*Dioscorea cayenensis-rotundata* cv. Kponan) PPO was made according to the method of Yoruk *et al.* (2003). Briefly, the reaction mixture containing 0.3 mL of yam polyphenol oxidase, 0.6 mL of phosphate buffer (pH 6.6, 100 mM) and 0.3 mL of onion or garlic extract as inhibitor, was preincubated for 20 min at 25 °C. To that reaction mixture was added to 0.8 mL of dopamine solution (8 mM) to initiate the enzyme reaction. As a

control, 0.3 mL of PPO extract was added to 0.8 mL of the dopamine solution to which 0.9 mL of 100 mM phosphate buffer (pH 6.6) had been added.

The PPO activity was measured spectrophotometrically (Shimadzu UV-120-02, Kyoto, Japan). The absorbance at 480 nm was recorded continuously at 25 °C for 1 min (Zauberman *et al.*, 1991). The total volume of assay for inhibition of PPO activity was 2.0 mL. The percentage of inhibition was expressed as Equation 1:

Determining the type and kinetics of inhibition

The inhibition kinetics of the enzyme was analyzed using the Lineweaver-Burk plots by taking the reciprocals of the initial velocity and the substrate concentration for (dopamine) in presence of each inhibitor (different onion and garlic extract).

Results and Discussion

Polyphenol oxidase distribution in yam tuber

Figure 1 shows the polyphenol oxidase activity distribution in different parts (proximal, median and distal) of yam tuber. Otherwise, this enzymatic activity decreases from the proximal portion (62.88 ± 1.87 U/mg of protein) to the distal portion (45.05 ± 0.67 U/mg of protein). This result suggests firstly, an unequal distribution of polyphenol oxidase activity in the studied yam tuber. This could be related to differences substrate content and/or enzyme concentration or enzymatic activity in each part (Simpson *et al.*, 1987).

The unequal distribution of polyphenol oxidase activity has already been reported in the yams *Dioscorea alata* and *Dioscorea*

cayenensis-rotundata cultivated in Nigeria (Onayemi, 1986) and in yam tuber *Dioscorea cayenensis-rotundata*, cv Longbô (Gnangui *et al.*, 2010).

Effect of anti-browning agents on polyphenol oxidase activity of yam tuber

Table 1 demonstrates the effect of various anti-browning agents on the studied yam tuber polyphenol oxidase was examined. The results summarized in table 1, showed that the enzyme activity was most inhibited by addition of various reducing agents, such as L-ascorbic acid, β -mercaptoethanol, sodium bisulphite and L-cysteine. The inhibition of polyphenol oxidase activity by reducing agents has been also reported in browning control in litchi fruit (Jiang and Fu, 1998) and fresh-cut pear wedges (Oms-Oliu *et al.*, 2006). As regards ascorbic acid, it is an effective reducing agent which is used as an antioxidant in the food industry. Indeed, the action of ascorbic acid in enzymatic browning prevention is to reduce the intermediate *o*-quinones to the original phenolic compounds before they can undergo further reaction to form pigments (Wen and Wrolstad, 2001). It may also reduce Cu^{2+} to Cu^+ in the PPO thus retarding enzymatic browning (Tan and Harris, 1995).

Effect of onion and garlic extract on polyphenol oxidase activity of yam tuber

Figure 2 shows the inhibitory effect of onions and garlic extracts on polyphenol oxidase activity of yam *Dioscorea cayenensis-rotundata*, cv. Kponan.

Red and yellow onion extracts presented a higher inhibitory effect on the yam polyphenol oxidase activity than those of other onions and garlic extracts at the studied volumes (100 at 1000 μL). Green, white and small onions and garlic extracts

were far less effective in inhibiting enzymatic browning of yam (*Dioscorea cayenensis-rotundata*, cv.Kponan) polyphenol oxidase hence considered ineffective when compared with both the red and yellow onions extracts. The inhibition percentages with all the four natural agents are lower than 50 % for the amount of onion and garlic extract used was 1000 μ L (Fig. 2).

It was reported that various volatile sulfur compounds, including thiols and peptides, were present in *Allium* species such as onion (Negishi *et al.*, 2002; Gnangui *et al.*, 2010). Since inhibition of enzymatic browning with thiol compounds such as cysteine and dithiothreitol was reported by Negishi and Ozawa (2000), the thiol compounds and peptides contained in onion might be responsible for the inhibition of browning in the studied yam tuber polyphenol oxidase activity. The results reported here are in good agreement with the result of a previous study on taro (*Colocasia antiquorum* var. esculenta) (Lee *et al.*, 2007) and on edible yam *Dioscorea cayenensis-rotundata*, cv Longbô polyphenol oxidase (Gnangui *et al.*, 2010).

Since the browning of yam was effectively inhibited by the heated onion extract, onion extract has potential as a natural inhibitor of browning in various plants and vegetables.

Effect of reversible inhibitors

The representation of Lineweaver and Burk (1934) was used to obtain affine lines. Increase of inhibitors concentration resulted in a decrease of the slopes of the lines, showing that crude extracts of onion and garlic have a reversible inhibition on the polyphenol oxidase of yam *Dioscorea rotundata cayenensis* (cultivar " kponan ") tuber.

With dopamine (substrate), the studied polyphenol oxidase activity was as predicted by the Michaelis-Menten equation in the presence of yellow onion extract and green onion extract (Fig. 3 and 4). The V_{max} value did not change with increasing of yellow and green onion extracts inhibitors concentrations, while K_M increased (Table 2). The Lineweaver-Burk plots for dopamine revealed a competitive inhibition in the presence of the two inhibitors. This would mean they set or on a site other than the enzyme active center preventing the substrate from binding to the processing area. These results are similar to those obtained by Goupy (1989).

When reaction was carried out in presence of red, white and small onion extracts, the Lineweaver-Burk plots (Fig. 5, 6 and 7) showed that both inhibitors exercise non-competitive inhibition (Table 2). Increasing the inhibitors concentration results in a family of lines with a common intercept on the $1/[S]$ axis but with different slopes and intercepts on the $1/v$ axis, indicating that K_M is unchanged by the presence of different inhibitor concentrations while V_{max} is changed.

This result suggested that the non-competitive inhibitor did not compete with substrate and the substrate concentration had no influence on the degree of inhibition of the enzyme's catalytic rate. In this case, where the noncompetitive inhibitor reacted with the enzyme at a site other than the active site, both the free enzyme (E) and the enzyme-substrate complex (E-S) reacted with inhibitor (Dogan *et al.*, 2007). Similar observation have been reported for polyphenol oxidase from apple using products of the Maillard reaction (glutathione-fructose) (Brun-Merimée *et al.*, 2004) and for jack bean urease inhibition by Hg^{2+} (Du *et al.*, 2012).

Table.1 Inhibitory effects of various anti-browning agents on polyphenol oxidase activity of an edible yam (*Dioscorea cayenensis-rotundata*, cv.Kponan)

Anti-browning agents	Percent inhibition (%)
control	0
Sodium bisulfite	94.75 ± 1.36
β-mercaptoethanol	90.26 ± 0.6
L-Cysteine	87.03 ± 1.56
L-ascorbic acid	85.51 ± 2.2
Citric acid	7.55 ± 4.32

Anti-browning agents were used at a final concentration of 1 mM. The enzyme activity was measured at 25 °C, using the spectrophotometric (480) procedure. Bars represent ± SE

Table.2 Type of inhibition and kinetic parameters of dopamine oxidation by edible yam (*Dioscorea cayenensis-rotundata* cv Kponan) polyphenol oxidase in the presence of the onion and garlic extracts inhibitor

Crude extract	Protein content of crude extract in the reaction mixture (mg)	V _{max} (U/ mg protein)	K _M (mM)	V _{max} /K _M (U/ mg protein × mM)	Type of inhibition
Red onion	0	125	4.55	27.47	Non competitive
	1.8	71.43		15.70	
	3.6	45.45		9.99	
	4.5	40.00		8.79	
Small onion (Echalote)	0	66.67	0.85	78.44	Non competitive
	1.8	52.63		61.92	
	3.6	41.67		49.02	
	4.5	38.46		45.25	
White onion	0	66.67	0.77	86.58	Non competitive
	1.8	45.45		59.03	
	3.6	35.71		46.38	
	4.5	33.33		43.28	
Green onion	0	62.5	0.63	99.21	Competitive
	1.8		2.08	30.05	
	3.6		2.56	24.41	
	4.5		2.86	21.85	
Yellow onion	0	125	5.13	24.37	Competitive
	1.8		7.25	17.24	
	3.6		10.53	11.87	
	4.5		15.63	7.99	
Garlic	0	90.91	3.57	25.46	Uncompetitive
	1.8	50.00	2.17	23.04	
	3.6	35.71	1.61	22.18	
	4.5	32.26	1.35	23.89	

Figure.1 Polyphenol oxidase activity in different portions of yam (*Dioscorea cayenensis-rotundata* cv. Kponan) tuber. Bars represent \pm SE.

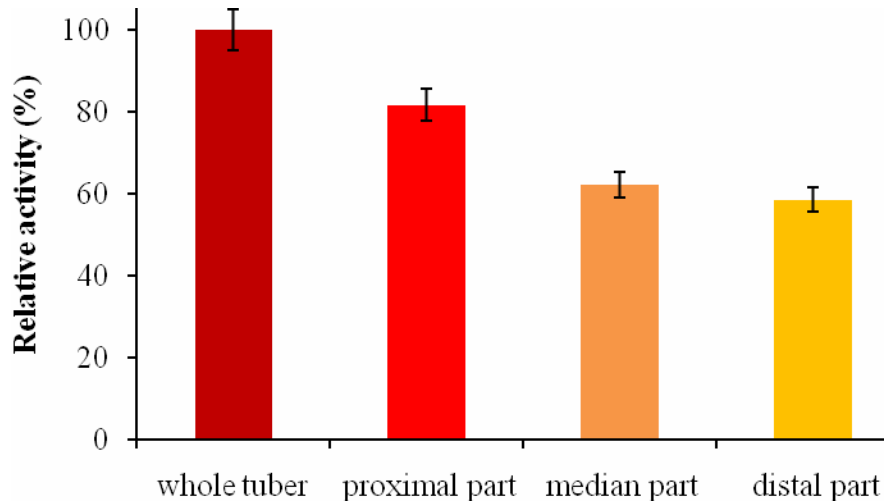


Figure.2 Influence of onions and garlic extracts on polyphenol oxidase activity of yam *Dioscorea cayenensis-rotundata*, cv. Kponan at different volumes ♦extract of red onion, ■ extract of yellow onion, ▲ extract of white onion, ● extract of garlic, □ extract of small onion (echalote), ○ extract of green onion

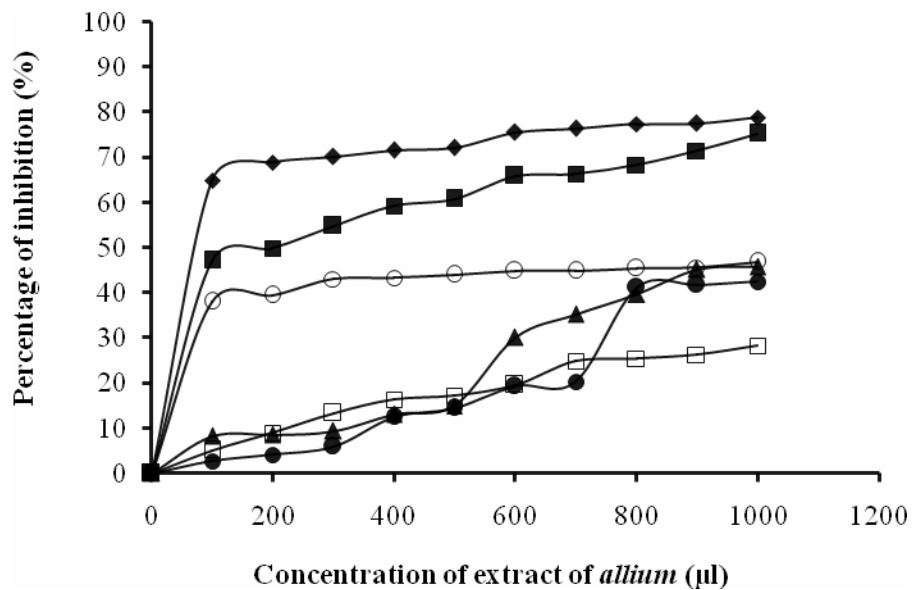


Figure.3 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of yellow onion extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins

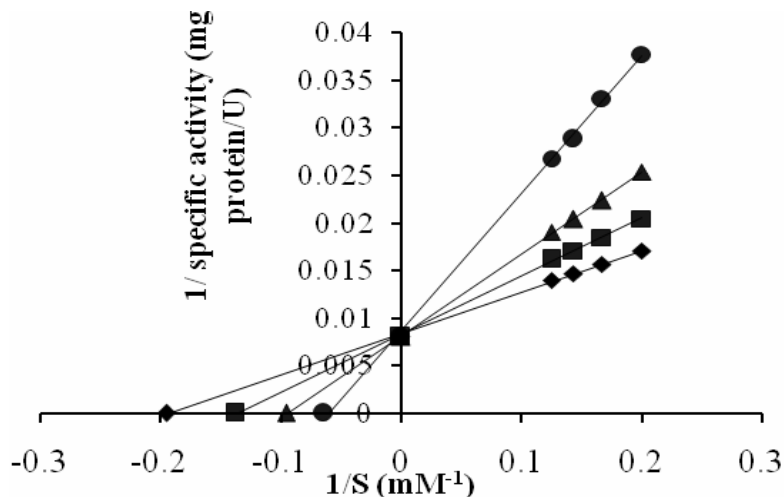


Figure.4 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of green onion extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins

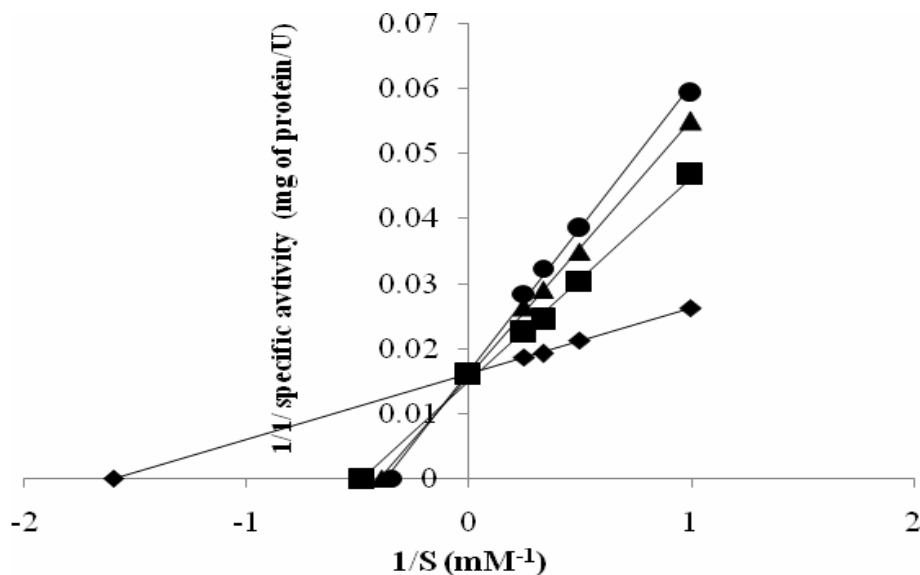


Figure.5 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of red onion extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins

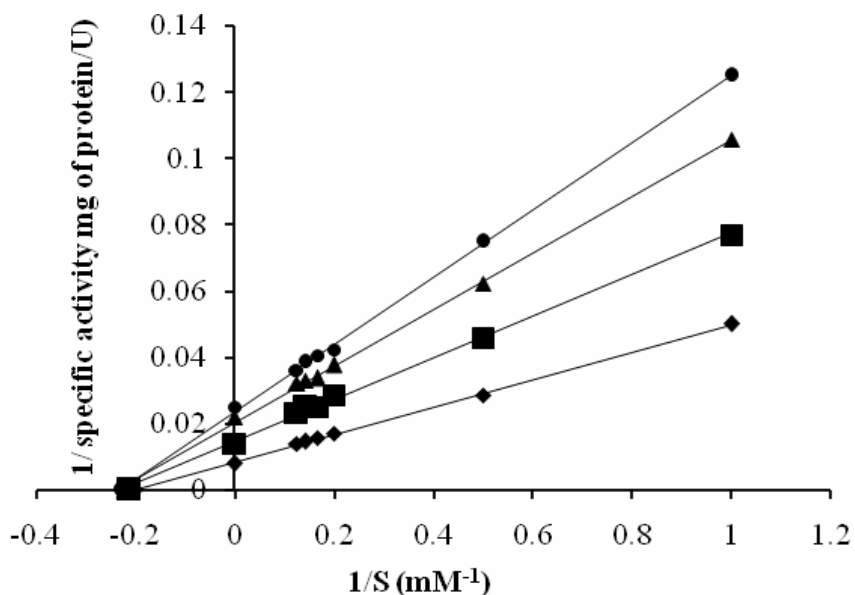


Figure.6 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of small onion (echalote) extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins

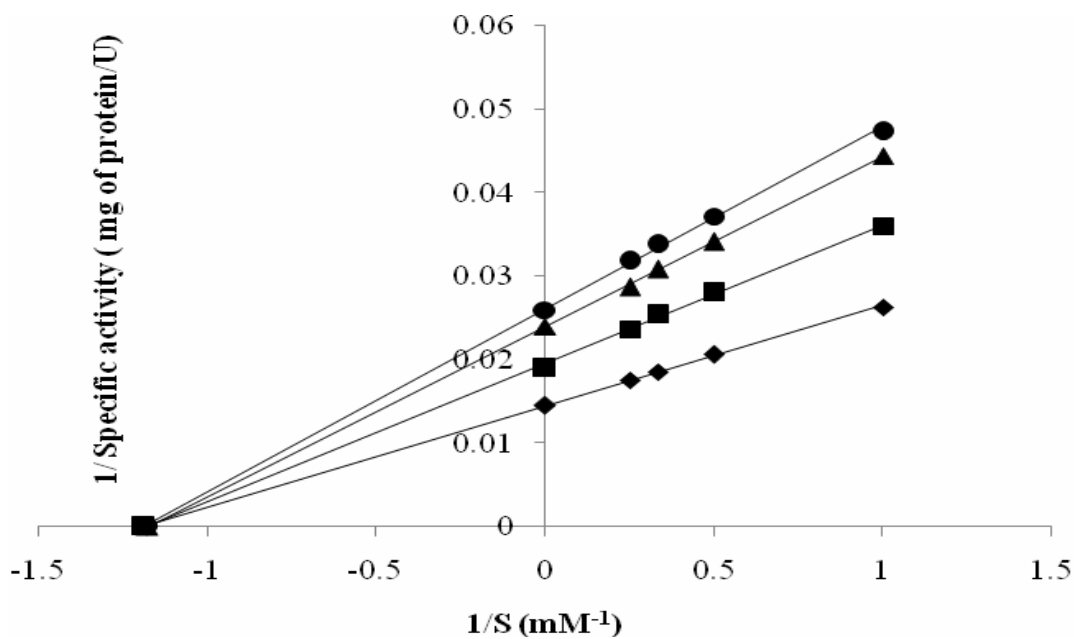


Figure.7 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of white onion extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins

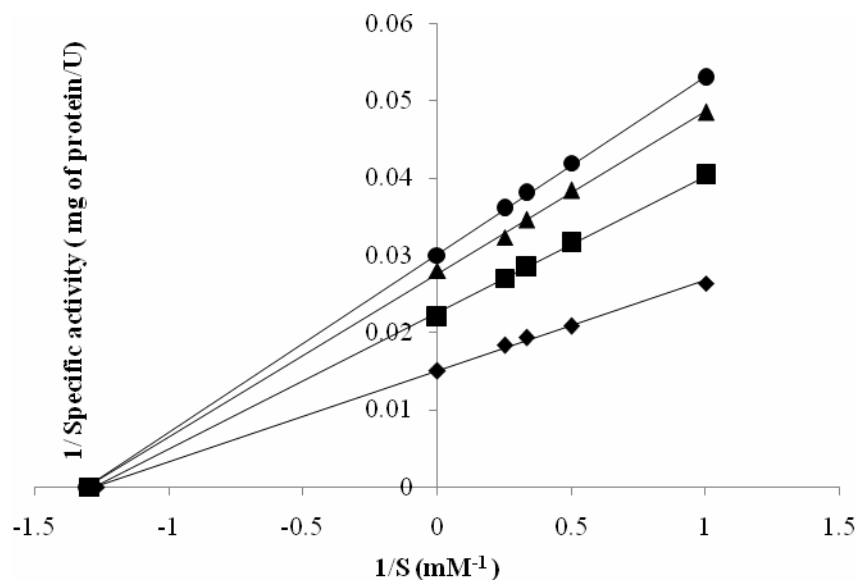
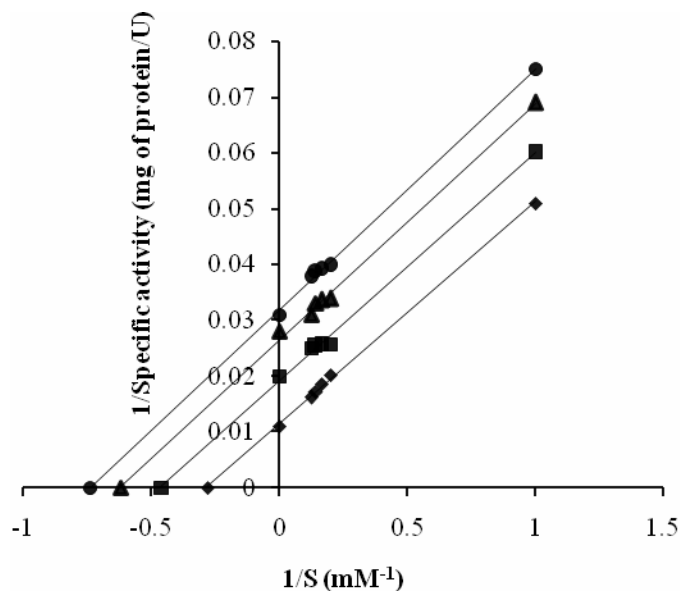


Figure.8 Lineweaver-Burk plot of the inhibition of the oxidation of dopamine by PPO from yam in the presence of garlic extract ♦ 0 mg of protein, ■ 1.8 mg of proteins, ▲ 3.6 mg de proteins ● 4.5 mg of proteins



This finding was contradictory to that reported by McEvily *et al.* (1992) who observed that proteins exerted an inhibitory effect on PPO activity by chelating the essential copper at the active site of PPO, and the substrate-similar compounds could work through competitive inhibition (McEvily *et al.*, 1992). The copper maintains an equilibrium between enzyme-Cu²⁺ and enzyme-Cu⁺ during enzymatic browning (McEvily *et al.*, 1992; Whitaker, 1972). However, this pattern seems to reflect the type of inhibition of polyphenol oxidase with onion (Longdon 1987) and honey (Ates *et al.*, 2001) extracts.

As far as concerned the garlic extract, the K_M values changed with increasing garlic extract inhibitor concentration, while V_{max} decreased (Table 2). It exercises an uncompetitive inhibition with dopamine as substrate (Fig. 8). This would mean that the inhibitor binds only to the enzyme-substrate complex (ES). The enzyme appears in this case less active because of the enzyme-substrate-inhibitor complex (ESI), which is inactive. This result is similar to that obtained by Barthet (1997). This author showed that benzoic acid and *p*-coumaric acid were uncompetitive inhibitors of polyphenol oxidase of the cassava root when D, L -dopa is used as a substrate.

The effects of inhibitors and kinetic parameters on the enzyme activity in presence of dopamine as substrates are summarized in Table 2.

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