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Inhibitory effect of onion extract on cassava leaf (*Manihot esculenta* Crantz) polyphenol oxidase

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Abstract

The inhibitory effect of onion extract on cassava leaf polyphenol oxidase was investigated. The polyphenol oxidase from cassava leaves was strongly inhibited by various anti-browning agents such as L-ascorbic acid and L-cysteine. The percentage of inhibition increased with the increased of anti-browning agents concentrations. The addition of heated onion extract exhibited a stronger inhibitory effect on cassava leaf polyphenol oxidase than the fresh onion extract. The highest percentage of inhibition was exhibited with heated onion extract in the presence of glucose and glycine, which was 87.18%. The onion extract inhibited the cassava leaf polyphenol oxidase non-competitively.

Introduction

Polyphenol oxidase (PPO) is a copper-containing enzyme that is widely distributed in the plant kingdom. It is also known as monooxygenase, catechol oxidase, and tyrosinase. The enzyme catalyses two distinct reactions: the o-hydroxylation of monophenol to o-diphenols and oxidation of o-diphenols to o-quinones in the presence of oxygen, which lead to the formation of brown, red or black pigments (Dogan and Dogan, 2004; Gawlik-Dziki *et al.*, 2007).

Enzymatic browning of fruit and vegetables during handling, processing and storage is one of the most important causes deterioration of nutritional quality and affects appearance. According to Vámos-Vigyázó (1995), the enzymatic browning prevention is classified into two types, which are inhibition or inactivation of the enzyme. However, inactivation of PPO, thermal processing has limits like loss of sensory and nutritional quality of food products (Sun *et al.*, 2002). Although browning can be prevented by the addition of sulfites containing anti-browning agents, they could be a problem to human health (Sapers, 1993). Therefore, development of natural inhibitors is needed to substitute the synthetic compounds as food ingredients.

Cassava (*Manihot esculenta* Crantz) is perennial shrub of the family *Euphorbiaceae.* It is a staple crop for over 500 million people living throughout the tropics. It is a crop with great economic importance worldwide (Olsen and Schaal, 1999). Cassava leaves are a good source of proteins, calcium, iron and vitamins. Cassava leaves have the ability to provide a valuable supplement to the predominantly starchy diets (Lancaster and Brooks, 1983). Young green cassava leaves are consumed as vegetables in some Asian country such as Malaysia, Philippines and Indonesia (Alves, 2002). However, the fresh cassava leaves are unable to store long as the browning started after harvested from the plant due to the PPO.

The PPO inhibitors occurring in natural resources have been studied in several plants such as potato (Lee *et al.*, 2002), pear (Kim *et al.*, 2005), banana (Lee, 2007) and taro (Lee *et al.*, 2007). However, no research has been conducted on cassava leaves. Onions are rich in natural sulfur compounds of low molecular weight, these are the flavonoids and the alk(en)nyl cysteine sulfoxides. Compounds from onion have been reported to have a range of health benefits which include anticarcinogenic properties, antithrombotic activity, antioxidative, antiasthmatic and antibiotic effects (Griffiths *et al.*, 2002; Kim and Kim, 2006). Onion extract provides not only beneficial effects to human health but it is effective inhibitor on browning against PPO. Thus, we have investigated the inhibitory effect of onion extract as natural inhibitor of PPO from cassava leaves in the present work.

Materials and Methods

Plant material and chemicals

Cassava (*Manihot esculenta* Crantz) leaves were picked from a yard located in Kajang, Selangor, Malaysia. The green leafy plant can be easily obtained from wide area in Malaysia as it undergoes vegetative reproduction just by cutting its stem and planted to a

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wet soil. All chemicals were of analytical grade and used as obtained.

Enzyme extraction

Cassava leaves (60 g) were washed and chopped into small pieces using knife. These leaves samples were then homogenized in 400 mL of prechilled (4°C) 0.1 M phosphate buffer, pH 7.5 using Waring blender for 1 minute at maximum speed. The slurry was poured into 50 mL Falcon tubes and centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was collected and filtered under vaccum by using Buchner funnel containing filter paper. The collected filtrate was pipette drop by drop into 200 mL of cold acetone (-20°C) for the formation of precipitates. The precipitates obtained were centrifuged at 4000 rpm for 30 minutes at 4°C. The pellet was the resultant acetone powder, was dried overnight at room temperature and stored at -20°C. In order to obtain the enzyme extract, 0.4 g of acetone powder was resuspended into 60 mL of prechilled 0.1 M phosphate buffer, pH 7.5 and stirred it for 1 hour at 4°C. The suspension was then centrifuged at 4000 rpm for 40 minutes at 4°C. The supernatant was used as the crude PPO extract from the cassava leaves.

Effect of anti-browning agents

L-ascorbic acid, L-cysteine and citric acid were used as anti-browning agents for PPO. The PPO activity was determined without and in the presence of anti-browning agents at three different concentrations (1, 3 and 5 mM by using 10 mM to 100 mM catechol as substrate). Lineweaver-Burk plots of were used to determine the Michaelis-Menten constant (K_m), maximum velocity (V_{max}), dissociation constant (K_i) and inhibition type for each inhibitor.

Onion extract preparation

Onion extract was prepared according to the method of Lee *et al.* (2007) with minor modifications. 100 g of red onion was homogenized with 200 mL of 0.1 M phosphate buffer, pH 7.5 for 2 minutes using Waring blender at maximum speed. The homogenized onion was centrifuged at 4000 rpm for 20 minutes at 4°C. After centrifugation, the supernatant was filtered through Buchner funnel with filter paper and filtrate was collected. The filtrate was used for this study. Heated onion extract was prepared by incubating the fresh onion extract at 100°C for 10 minutes.

Assay of PPO activity

PPO activity was determined with a spectrophotometer (Secomam, France). The reaction mixture contained of 0.1 mL of cassava PPO, 0.9 mL of 0.1 M phosphate buffer, pH 7.5 and 1.0 mL of onion extract as inhibitor, incubated for 5 minutes at 20ºC. Then, 1.0 ml of catechol (10 mM to 100 mM) was added to the reaction mixture. The control contained of 0.1 mL PPO and 1.0 mL substrate solution in 1.9 mL of 0.1 M phosphate buffer. The total volume of assay was 3 mL. The increase in absorbance at 410 nm with 15 seconds intervals up to 360 seconds were measured. The linear graph of the absorbance versus time(s) curve was plotted to determine the initial rate of PPO activity. The PPO activity obtained was used to calculate the percentage inhibition, as compared to initial PPO (A_0) activity without inhibitor (Lee *et al*., 2007).

Statistical analysis

Statistical analysis of all the experimental data was performed using SPSS (Version.18.0 software, Chicago, USA). Data were statistically analyzed using one-way ANOVA. The data were presented as mean values ± standard deviations and also relative activity in percentage (%) of triplicates. The confidence limits used in this study was based on 95% ($p \leq 0.05$).

Results and Discussion

PPO extraction

Cold acetone played an important role in the process of enzyme precipitation. The stability of the enzyme was improved and greater storage was achieved. Centrifugation was necessary to obtain the pellet of the precipitation, and supernatant was decanted. Evaporation of the acetone was relative fast and dried enzyme acetone powers were obtained (Unen *et al*., 2011). The enzyme acetone powders were stored at -20°C. 2.47g ± 0.21 of acetone powder was obtained from 60 g of cassava leaves.

Effect of anti-browning agents on cassava leaves PPO

Table 1 demonstrates the effect of various anti-browning agents with different concentrations on cassava leaves PPO with catechol as substrate. It can be seen that the percentage of inhibition increased with the increased of inhibitor concentrations (1.0 – 5.0 mM). The enzyme was strongly inhibited by L-ascorbic acid and L-cysteine, which able to inhibit the PPO activity from 60.69% to 81.03%. Furthermore, L-ascorbic acid has the lowest I_{50} and K_i values among the other anti-browning agents, which were 0.45 mM and 0.20 mM that further proofs that L-ascorbic acid has greater efficiency in inhibiting cassava leaves PPO. However, citric acid in this study was the least efficient inhibitor towards the cassava leaves PPO, which exhibited the lowest percentage of inhibition.

Table 1. The inhibitory effect of various anti-browning agents on cassava leaves PPO

Anti-browning agents	[I] (mM)	I_{50} (mM)	Inhibition (%)	V_{max} (EU/min/ml)	K_m (mM)	K_i (mM)	Type of inhibition
L-ascorbic acid	1.0		70.51	333.33	125.00	0.20	Competitive inhibitor
	3.0	0.45	78.21	400.00	167.00	0.43	
	5.0		79.48	400.00	250.00	0.43	
L-cysteine	1.0		60.69	200.00	20.83	1.00	Non-competitive inhibitor
	3.0	0.65	76.92	111.11	21.74	1.15	
	5.0		81.03	64.52	20.83	0.96	
Citric acid	1.0		14.62	333.33	21.74	4.99	Non-competitive inhibitor
	3.0	5.25	29.92	333.33	20.00	15.00	
	5.0		36.74	250.00	22.00	8.33	

Table 2. The inhibitory effect of onion extract, heated onion extract with added glucose and glycine on cassava leaves PPO

Compound	Relative activity (%)	
	Fresh	Heat-treated
Onion	38.46±1.2^{c}	47.00±1.4^{d}
Glucose	17.95±0.8^{f}	48.72±2.1^{d}
Glycine	25.64±1.4^{e}	56.92±1.2^{c}
Glucose + glycine	33.33±0.7^{d}	60.26±0.5^{e}
Onion + glucose	71.79±2.0^{b}	80.77±1.8^{b}
Onion + glycine	73.08±1.5^{b}	85.90±2.2^{a}
Onion + glucose + glycine	84.62±1.1^{a}	87.18±1.7^{a}

The amount of the onion extract was 3 mg/ml.
Means with different superscripts within a column are significantly different at $p \leq 0.05$.

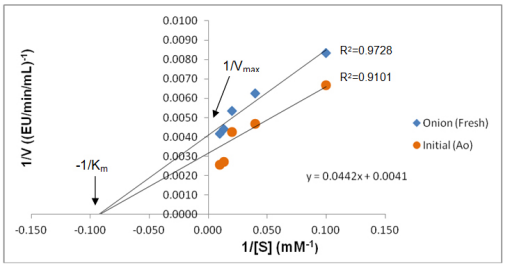

Figure 1. Lineweaver-Burk plot of cassava leaves PPO in the presence of fresh onion extract.

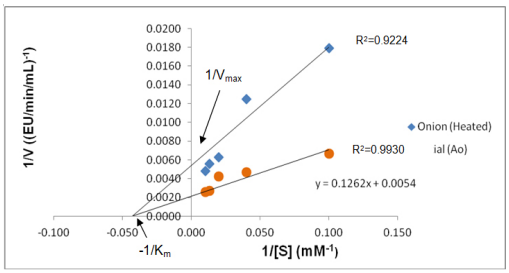

Figure 2. Lineweaver-Burk plot of cassava leaves PPO in the presence of heated onion extract.

L-ascorbic acid was a competitive inhibitor and able to diminish the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate. However, competitive inhibition can be relieved by the increased of substrate concentration, thus exhibited higher V_{max}. L-cysteine and citric acid were non-competitive inhibitors, which cannot be overcome by increasing of the substrate concentration.

The application of anti-browning agents has been reported for inhibiting the enzymatic browning in the post-harvest or processed fruits and vegetables, such as litchi fruit (Jiang and Fu, 1998) and fresh-cut pear (Oms-Oliu *et al*., 2006). Ascorbic acid is an effective reducing agent and has been used as an antioxidant in the food industry. Several factors such as cost, treatment method, efficacy and any effects on flavour, texture, taste or colour will determined the choice of used of particular anti-browning agent (Mc Evily *et al*., 1992).

Effect of onion extract on cassava leaves PPO

The percentage of inhibition by onion extract was shown in Table 2. The addition of onion extract that had been heated at 100°C for 10 minutes exhibited a stronger inhibitory effect on the cassava leaves PPO than did the fresh onion extracts. Similar results were reported by Lee *et al.* (2002) and Lee *et al.* (2007) when potato PPO and taro PPO were treated with heated onion extract. The increase in inhibitory effect by heating the onion extract might be due to a synergy effect with Maillard reaction products produced during heating as the products were inhibitors of PPO (Lee and Park, 2005).

The strong inhibitory effect of the heated onion extract was further studied by the addition of glucose and glycine. From Table 2, the highest percentage of inhibition was exhibited with heated onion extract in the presence of glucose and glycine, which was 87.18%. This was probably due to the non-enzymatic browning products produced (Nicoli *et al*., 1991).

Figures 1 and 2 shows the Lineweaver-Burk plots of cassava leaves PPO in the presence of fresh onion extract and heated onion extract, respectively. It was found that both Figures exhibit similar type of inhibition, which was non-competitive and irreversible. The K_m value for PPO with fresh onion was lower (1.08 mM) than the heated onion (23.36 mM). It can be concluded that heated onion has a stronger inhibitory effect on the cassava leaves PPO, where it reduced the capability of the binding of enzyme to substrate greater than the fresh onion. V_{max} values for the PPO were 243.90 EU/min/mL and 185.18 EU/min/mL with fresh onion and heated onion as inhibitor, respectively. The V_{max} for PPO with heated onion was lower than the fresh onion, indicated that heated onion has greater inhibition to the activity of enzyme.

It was reported that various volatile compounds, including thiols, were present in *Allium* species, such

as onion (Negishi *et al.*, 2002) and these compounds are reported to inhibit PPO. Thiol compounds that contained in the onion might be the active component responsible for the inhibitory effect of onion extract. Thus, onion extracts could be used as a natural food ingredient for the prevention of the browning caused by cassava leaves PPO.

Conclusions

This study concluded that onion extract has potential to be used commercially as a natural inhibitor to prevent the browning of cassava leaves as well as others plants and vegetables. Onion extract can be used to replace the sulfite-containing anti-browning agents which could be a problem for human health since there is an increasing demand by consumers for substituting synthetic compounds with natural substances as food ingredients. Future studies on the inhibitory effect of thiol compounds that are contained in onion will help to increase knowledge of the natural inhibitor of PPO.

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