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Effect of Anti-browning Agents on Cresolase and Catecholase activity of Polyphenol oxidase in Acanthus (Gundelia Tournefortii)

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Abstract

Polyphenol oxidase (PPO) from acanthus (Gundelia tournefortii) was extracted and partially purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. Cresolase and catecholase activity of PPO was investigated in Gundelia tournefortii. Of the substrates tested, catechol and p-cresol was the substrate for catecholase and cresolase activity of PPO with a K_m value of 15 and 11 mM, respectively. The optimum pH for catecholase and cresolase activity was found to be 6.5 and 8. The enzyme showed high activity over a broad pH range of 4 - 8. The optimal pH and temperature were found to be 6.5 and 8 and 45-55 °C, respectively. k_m value for acanthus catecholase activity in presence of ascorbic acid, citric acid and kojic acid were 13.8, 10 and 14.2 mM and for cresolase activity were 10.5, 8.5 and 13.5 mM respectively. As can be seen, affinity of PPOs for various substrates varies widely. The thermal inactivation studies showed, catecholase activity is heat resistant from 30 until 70 ^oC, but heat resistant for cresolase activity was from 30 untill 55 ^oC so after 55^oC cresolse activity gradually decreased. The enzyme showed the highest activity toward p-cresol in comparision to catechol. Of the inhibitors tested, the most potent inhibitors were kojic acid, ascorbic acid and citric acid. Ascorbic acid, citric acid and kojic acid were used to inhibit the activity of catecholase and cresolase at different concentrations (0-10 mM). Kojic acid was found to be the most potent anti-browning agent (kojic acid> ascorbic acid > citric acid).

Keywords: Inhibition, Kinetics, Purification, cresolase, catecholase, Acanthuss, Thermal inactivation.

Introduction

Acanthus with scientific name: G. tournefortii is in Chicory family. Acanthus is permanent vegetable covered with wool hair down and lots of blade its stem is thick, simple or branching with short branch and in the form of corymb [1]. G. tournefortii L. is a medicinal plant, native to the Asian temperate zones of Western Asia, namely Cyperus, Egypt, Iran, Israel, Turkey, Azarbaijan and Turkmenistan. A look at the Iranian collections results in this supposition that all collections from the Flora Iranica could, in reality, belong to G.rosea. And its common name is kanger and locally known as 'Kangar' in Iran is found as a wild herb growing during late winter and early spring in the hills in the western and southern parts of Iran as an occasional food in different forms and also as a folk remedy[2]. G. tournefortii grows well in different localities. The plant, is not able to grow in shade, but prefers sandy and loamy or acid, neutral and alkaline, drained and moist soils [3]. Many vegetables and fruits become discoloured during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) [4]. PPO (tyrosinase, EC 1.14.18.1) is a copper-containing enzyme that is widespread in plants, and synthesised early in tissue development and stored in chloroplasts [5]. The enzyme is a copper protein widely distributed in a multitude of organisms, from bacteria to mammals [6]. Enzymatic browning is the main function of PPOs in fruits and vegetables but is often undesirable and responsible for unpleasant sensory qualities as well as losses in nutrient quality [7]. When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to o-quinones in a two-step process of hydroxylation of monophenols to diphenols (monophenolase activity), followed by the oxidation of diphenols to o-quinones (diphenolase activity) [8]. These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness is reduced [9].PPO from different plant tissues shows different substrate specificities and degrees of inhibition. Therefore, characterisation of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products. Our objective was to characterise PPO from acanthus cultivated in Kurdistan, Iran under different conditions. Substrate and temperature effects were also studied.

Materias and Methods

The acanthuss used in this study were obtained from baneh in Kurdistan of Iran and frozen at -25 °C until

used. Catechol, polyvinylpyrolidone (PVPP), p-cresol, tyrosine were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, kojic acid, L-glycine, polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), cellulose membrane (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

Enzyme Extraction

1 kilo-grams of friut of acanthuss were homogenized in 1000 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 30000 g for 20 min at 4ºC. Solid ammonium sulphate $(NH_4)2SO_4$ was added to the supernatant to obtain 30 and 80% (NH4)2SO4 saturation, respectively. After 1 h, the precipitated proteins for each stage were separated by centrifugation at 30000 g for 30 min. The precipitate was redissolved in a small volume of distilled water and dialyzed at 4ºC against distilled water for 24 h with 4 changes of the water during dialysis. The dialysate was applied to a column (2.5 cm x 30 cm) filled with DEAE-cellulose, balanced with 10 mM phosphate buffer, pH 6.8. In order to remove non adsorbed fractions the column was washed with 200 mL of the same buffer at the flow rate of 0.5 mL/min. Then, a linear gradient of phosphate buffer concentration from 20 to 180 mM was applied. 5 mL fractions were collected in which the protein level and catecholase activity towards catechol as substrate were monitored. The fractions which showed catecholase activity were combined and were used as enzyme source in the following experiments [10].

Protein Determination

Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard [11].

Assay of Enzyme Activity

Catecholase and cresolase activity was determined by measuring the absorbance at 420 nm using a spectrophotometer (6305 JENWAY). To determine the best concentration of enzyme preparation corresponding to the highest enzyme activity, the activity was assayed in 3 mL of reaction mixture consisting of 2.5 mL substrate (25mM catechol and 15mM p-cresol separately) and different concentrations (0.1-0.3 mL) of the enzyme preparation (1mg/mL). This mixture was topped-up to 3.0 mL with the phosphate buffer (pH 6.8) in a 1 cm light path quartz cuvette. The blank consisted of 3.0 mL 0.1 M phosphate buffer (pH 6.8). Two controls were prepared: the cuvette of the first control contained 2.5 mL substrate and 0.5 mL buffer solution, whereas the second control cuvette contained 2.9 mL buffer and 0.1 mL enzyme preparation. Absorbance values of these controls were subtracted from that of the sample. Cresolase and catecholase activity was calculated from the linear portion of the curve. The initial rate of PPO catalyzed oxidation reaction was calculated from the slope of the absorbance–time curve. An enzyme preparation of 0.2 mL showed the highest activity using catechol as a substrate which was used in all other experiments. One unit of PPO activity was defined as the amount of enzyme that produces 1 micromole of quinone per minute. Assays were carried out at room temperature and results are the averages of at least three assays and the mean and standard deviations were plotted.

pH optimum and stability

Catecholase and cresolase activity as a function of pH was determined using catechol and p-cresol as substrates. Phosphate and phosphate-citrate buffer, ranging from pH 3.0 to 9.0 was used at the assays. The pH stability was determined by incubating the enzyme in the above buffer (pH 3.0 to 9.0) for 30 min and at the end of the incubation period, samples were taken and assayed under standard conditions as described above. All of the assays were performed in triplicate. PPO activity was calculated in the form of unit per mg protein at the optimum pH. The optimum pH value obtained from this assay was used in all the other experiments.

Substrate Specificity

Enzyme Kinetics

For determination of Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enzyme, cresolase and catecholase activities were measured with two substrates at various concentrations. In order to determine Michaelis constant (K_m) and maximum velocity (V_m), catecholase activity was measured using catechol (0-50 mM), and cresolase activity was measured using p-cresol (0-50 mM) as substrates. K_m and V_m values of the enzyme were calculated from a plot of V vs. S.

Table 1

Purification step	Volum (ml)	Total (mg. protein	Avtivity µM/min	Specific activity (µM/min mg protein	Purification fold
crude extrac	350	13.5	185	0.039	1
$(NH_4)_2SO_4$ precipitation (30%)	220	8.5	289	0.154	3.9
$(NH_4)_2SO_4$ precipitation (80 %)	110	6.8	445	0.594	15.2
DEAE-cellulose	25	3.9	98	1.01	25.8

Effect of Temperature on cresolase and catecholase activity of PPO

To determine the optimum temperature for catecholase, the activity of the enzyme was measured at different temperatures (30-80°C) using 0.2 mL enzyme, 2.7 mL of 20 mM catechol as substrate and completed to 3 mL with 0.1 M sodium phosphate buffer (pH 7). The blank consisted of 3.0 mL of 0.1 M phosphate buffer. Controls were run under the same tested temperature. The tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of a 0.2 mL aliquot of enzyme solution. The enzyme samples were removed from water bath after pre-set times and were immediately transferred to ice bath to stop thermal inactivation. After the sample was cooled in ice bath, the residual activity was determined spectrophotometrically using the standard reaction mixture. A non-heated enzyme sample was used as blank. The percentage residual activity was calculated by comparison with the unheated sample. This assay was done for cresolase activity,too.

Effects of Inhibitors

The inhibitors examined were Ascorbic acid, citric acid and kojic acid. The reaction mixture contained 2.7 mL of catechol at a final concentration of 20 mM in 0.1M phosphate buffer (pH 7), 0.1 mL inhibitor at a final concentration of 0.2, 1 or 1.5 mM and 0.2 mL enzyme solution. The change in absorbance was measured spectrophotometrically at 420 nm. Control tests for inhibitors plus substrate plus buffer were also run at the same time. Percentage inhibition as calculated using the following equation: Inhibition (%) = $[(A_O - A_i)/A_O)].100$, where, A_0 is the initial PPO activity (without inhibitor) and A_i is the PPO activity with inhibitor.

RESULTS

Extraction and Purification

PPO was purified from acanthuss using a DEAE-cellulose column. A summary of extraction and purification is given in Table 1. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to DEAE-cellulose column, yielding two peak with PPO activity (Fig. 1). A 25.8 fold purification was achieved.

Table 1. Purification of PPO from acanthus.

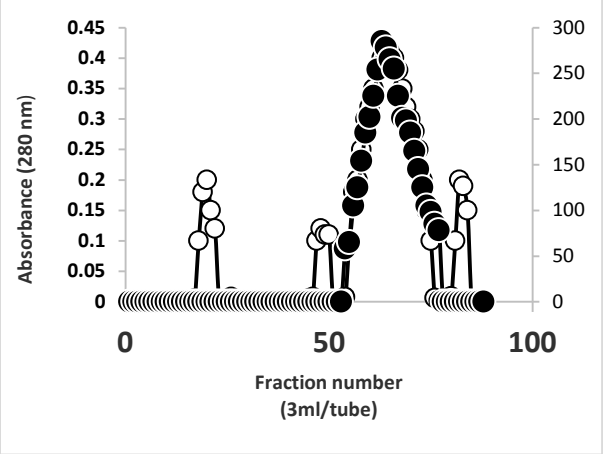

Figure 1. Elution pattern of acanthus PPO on DEAE-cellulose. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a 2.5 cm x 30 cm column, equilibrated and washed with 10 mM phosphate buffer, pH 7. Elution of adsorbed proteins was performed using a linear gradient of 10 to 200 mM phosphate buffer (pH 7) at a flow rate of 0.5 mL/min.

pH Optima

Optimum pH for PPO activity with catechol(catecholase) and p-cresol(cresolase) as substrates was 6.5 and 8 (Fig 2). As the pH increased from 4 to 9, the enzyme activity increased in two step at pH 6.5 and 8, with maximal activity occurring at pH 8, after which the activity started to decline. Differences in optimum pH for PPO with distinct substrates have been reported for the enzyme from various sources [12, 13, 14, 15 and 16]. So, pH optima for catecholase and cresolase activity in presence of catechol and p-cresol in acanthus is the same.

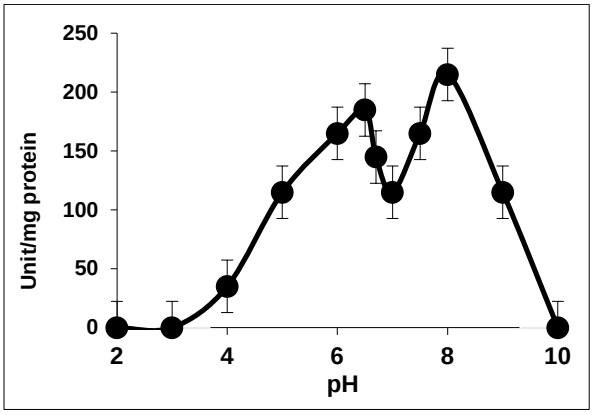

Figure 2. Activity of acanthus PPO as a function of pH. Each data point is the mean of three determinations. The vertical bars represent standard deviations.

The effect of temperature on PPO activity

Optimum temperature for cresolase activity with p-cresol was 50°C; however, when using cathecol as substrate, it was 55°C (Fig 3). This behavior of the cresolase and catecholase activity with these substrates was confirmed after several repetitions. Heating for 30 min at 40 °C for p-cresol and catechol increases the activity; however, at the higher temperatures such 55 °C, catecholase activity at first increased and after 10 minute incubation gradually decreased. For cresolase activity after 5 minute incubation showed 30% increasing in activity but after that a sever decreasing in activity happened. Optimum temperatures for PPO activity in others sources were reported to be between 20 and 40°C. The enzyme was reasonably stable at 50°C and,

as expected, the rate of inactivation was higher with increasing temperature (Fig. 3). When enzyme exposed to 70°C, a decrease in activity earned so activity reached to 50% after 13 minute for ccatecholase activity and after 7 minute for cresolase activity. Cresolase activity reached to zero after 15 minute of incubation at 70C but for catecholase activity happened after 35 minute (Fig. 4). Acanthus - PPO is a heat-stable enzyme at 40 - 55°C; so cresolase activity of acanthus is less resistant to heating than PPO from solanum lycopersicum and catecholase activity of acanthus is more resistant to heating than PPO from solanum lycopersicum [17].

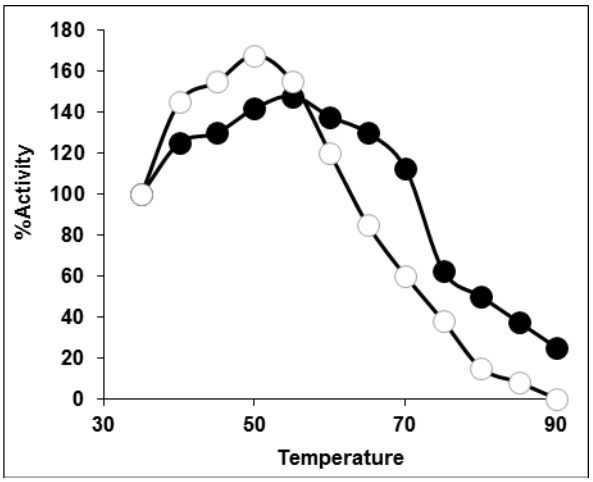

Figure 3. Activity of acanthus PPO as a function of temperature. Each data point is the mean of three determinations. The vertical bars represent standard deviations. [(■) , catechol 20 mM]and [(□), p-cresol 8 mM].

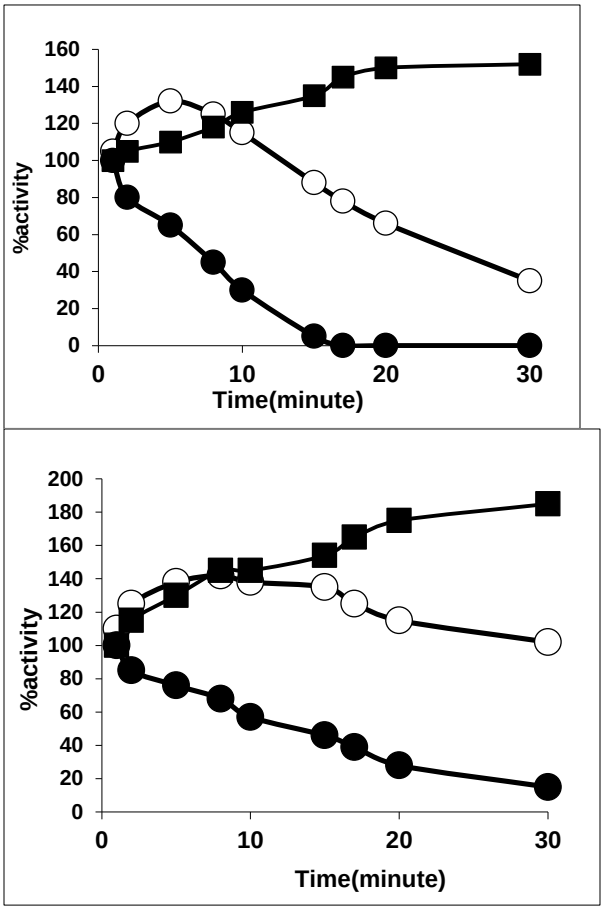

Figure 4 - Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures [27,(■); 40,(○); 50,(●) and 60(Δ) °C) and the remaining activity was determined with catechol as substrate.

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Effect of Inhibitor

Effects of ascorbic acid, citric acid and kojic acid on acanthuss cresolase and catecholase activity were studied at various concentrations using catechol and p-cresol as the substrates and the results were reported as percentage inhibition in Table 2. The inhibition degree varied in dose dependent manner. From the results, it can be concluded that the most potent inhibitors was kojic acid, because a higher degree of inhibition was achieved. Citric acid was the least potent inhibitor, so in presence of 1 mM kojic acid, cresolase activity of PPO in acanthus reached to 12% , but in the same concentration of ascorbic acid and citric acid, activity reached to 60% and 70%, respectively. In presence of 1 mM kojic acid, catecholase activity of PPO in acanthus reached to 8% , but in the same concentration of ascorbic acid and citric acid, activity reached to 55% and 74%, respectively. Catecholase activity reached to zero at 2 mM kojic acid but cresolase activity reached to zero at 3 mM kojic acid(figure 5).

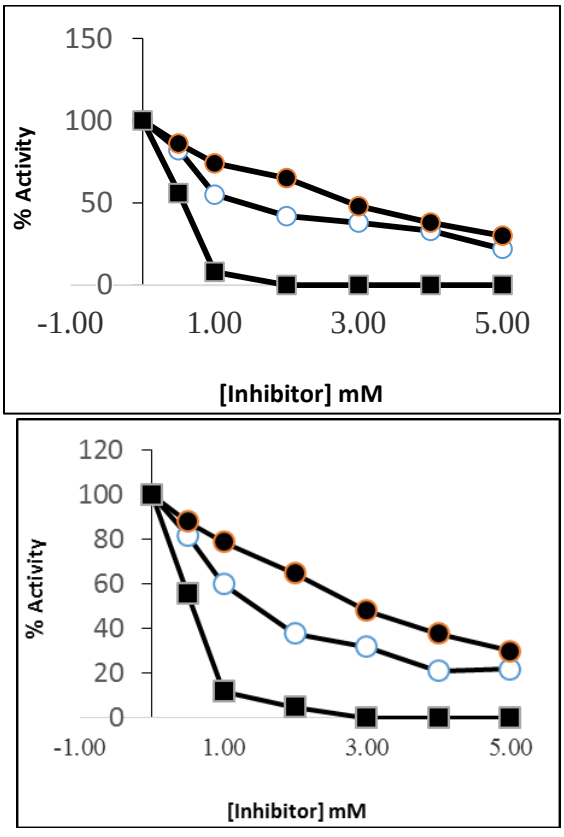

Figure 5: Effect of different concentrations of citric acid(●), ascorbic acid(○) and kojic acid (■) on cresolase (Upper figure) and catecholase activity (lower figure).

Table 2. Effect of inhibitors on acanthuss cresolase and catecholase activity
*Each value is the mean of three determinations ± standard deviations

Inhibition of **Cresolase** activity		Inhibition of **Catecholase** activity
Inhibitor Concentration (mM)	% Inhibition	% Inhibition
Ascorbic acid		
0.5	20 ± 1.5	25 ± 3
1	40 ± 2	45± 6
1.50	61 ± 1.8	75± 8
Citric acid		
0.5	10 ± 2.3	15± 4
1	21 ± 3.3	26± 6
1.50	30 ± 1.5	38± 9
Kojic acid		
0.01	15 ± 1.5	19± 3
0.20	35 ± 2.5	42± 5
1	88 ± 2.8	92 ± 7

Kinetic Parameters

Km and Vm values for acanthuss PPO for different substrates are presented in Table 3. The affinity of the enzyme varied depending on the substrate used. Acanthus PPO had a higher affinity for p-cresol, as evidenced by lower Km value in comparision to catechol oxidation. The criterion for the best substrate is the Vm/Km ratio. Of the substrates tested, the best substrate for acanthuss PPO was p-cresol. In a study carried out by Gao jia et al. (2011) Km value for PPO from sour cherry pulp was found to be 3.5 mM, using catechol. Duangmal and Apenten (1999)[18] reported the following Km values for taro PPO: 9.0 mM for 4-methylcatechol, 67.9 mM for catechol and 89.9 mM for p-cresol. The same investigators reported the following Km values for potato PPO: 1.1 mM for 4-methylcatechol, 6.8 mM for catechol and 1.5 mM for p-cresol, but km value for acanthus catecholase activity is calculated 15 mM for catechol and 11 mM for cresolase activity. As can be seen, affinity of PPOs from various sources for various substrates

varies widely. Km value for catecholase activity in presence of ascorbic acid(1mM), citric acid (1mM) and kojic acid (1mM) is calculated 13.8, 10 and 14.2 mM, respectively, and km value for cresolase activity in presence of ascorbic acid(1mM), citric acid (1mM)and kojic acid (1mM) is calculated 10.5, 8.5 and 13.5 mM, respectively Vmax for catecholase activity in presence of ascorbic acid decreased from 385 to 231unit/mg protein, so 40% of ctecholase activity lost. Vmax for cresolase activity in presence of ascorbic acid decreased from 574 to 365 unit/mg protein, so 37% of ctecholase activity lost. Maximum of Inhibition for catecholase and cresolase activity earned in presence of kojic acid (1mM) so, 25.5% and 18.6% of activity remained.

Activity	Substrate	Km (mM)	Vmax (Unit/ mg.protein)	Vmax/Km Unit/mg protein. mM-
Catecholase	**catechol**	**15**	385	23.7
	Catechol + Asc 1 mM	**13.8**	231	16.7
	Catechol + Cit 1 mM	**10**	308	30.8
	Catechol + Koj1 mM	**14.2**	98	6.9
Cresolase	**p-cresol**	**11**	574	52.1
	p-cresol + Asc 1 mM	**10.5**	365	34.7
	p-cresol + Cit 1 mM	**8.5**	457	53.7
	p-cresol + Koj1 mM	**13.5**	107	7.9

Table 3: K_m and V_m values for acanthuss PPO for different substrates

DISCUSSION

It has been reported that some plant PPOs are membrane-bound. Therefore, use of detergents is required to solubilize the enzyme. Phenol compounds interfere with purification of proteins from plants. They cross-link proteins by hydrogen bonds and covalent interactions. Furthermore, homogenization of the plant tissues initiates enzymatic browning which results in the formation of quinones. The quinones may also form covalent linkages that may not be reversible. Use of phenol-absorbing polymers, such as polyethylene glycol (PEG) or PVPP and use of reducing agents such as ascorbic acid are commonly applied in order to overcome these problems [19]. The pH optimum for catecholase and cresolase activity from acanthus was found to be 6.7 and 8. It is noteworthy to mention that the pH optimum for PPO is found to be dependent on the enzyme source, substrate and extraction methods used. Other reported values include 6.50 for banana peel PPO [20], 4.20 for grape PPO [21], 5.70 for broccoli PPO [22] and 7.5 for avocado PPO [23]. Halder et al. (1998)[24] reported an optimum pH value of 5.0 for tea PPO. In a study carried out by Dogan et al. (2002)[25] on different aubergine cultivars, the temperature optima varied between 20-30 °C using catechol and 4-methylcatechol as substrates. Ding et al. (1998)[10] reported an optimum temperature of 30°C for loquat PPO using chlorogenic acid as substrate. Other reported values include 25 °C for grape PPO [21] and 30 °C for banana PPO [26]. The optimum temperature obtained in this study is 50-55°C that is dependent on the substrate. PPO is generally considered as an enzyme of low thermostability. Heat stability was reported to differ among cultivars and multiple forms of PPO from the same source as well as between fruit tissue homogenates and their respective juices [27]. Catecholase activity from acanthuss showed high thermal stability at the temperatures studied in comparision to cresolase activity. The mode of action of inhibitors differs from each other. The mode of inhibition of kojic acid is by reducing the enzyme Cu+2 to Cu + rendering the enzyme inactive an unavailable for O2 binding and by complexing with quinone compounds to prevent melanin

formation via polymerization . In a study carried out by Gomez-Lopez (2002)[23], it was found that the most effective inhibitor for avocado PPO was cysteine. Rapeanu et al. (2006)[16] found that most potent inhibitors for grape PPO were ascorbic acid, cysteine and sodium metabisulfite. In conclusion, after the final purification step, a 25.8 fold purification.The optimal pH and temperature for enzyme activity were found to be 6.7 and 8 and 50-55 °C, respectively. The enzyme showed a broad activity over a broad pH and temperature range. The thermal inactivation studies showed that the enzyme is heat resistant. The enzyme showed the highest activity toward p-cresol. Of the inhibitors tested, the most potent inhibitors was kojic acid. The effects of Ascorbic acid, citric acid and kojic acid on partially purified PPO (cresolase and catecholase activity) activity and 19, 42 and 92% inhibition of catecholase activity, respectively. While the results obtained at 0.5, 1, and 1.5 mM of ascorbic acid were 20, 40, and 61% inhibition for cresolase and 25, 45 and 75% inhibition for catecholase activity, respectively. At the concentrations of 0.5, 1, and 1.5 mM of from acanthus were studied. The results are shown in tables 1 and 2. The percentage inhibition was compared with that of the control (0% inhibition). Kojic acid was the most effective inhibitor. At the concentrations of 0.01, 0.2, and 1 mM of kojic acid, there were 15, 35, and 88% inhibition of cresolase At low ascorbic acid concentrations 0-0.5 mM, the inhibitory effect on cresolase and catecholase activity were 20-40% inhibition, respectively, while the result obtained at a higher ascorbic acid concentration (1.5-5 mM) was nearly 100% inhibition. This is as expected provided that ascorbic acid does not act directly on the enzyme structure. Mardtinez and Whitaker [28] reported that the mechanism of ascorbic acid inhibition involves the reduction of quinone generated by PPO. The inhibitory effect increased as the concentration of citric acid increased. The results presented demonstrated that kojic acid appears to be a potent inhibitor of acanthus PPO, followed by ascorbic acid, and citric acid, respectively. These results are in agreement with the study of Ding et al. [10]. They reported the effectiveness of a series of sulfhydryl compounds in inhibiting PPO activity in a model system (chlorogenic acid) and in loquat (Eriobotrya japonica Lindl.) juice. The concentration of kojic acid for 90-100% browning inhibition depended on concentration of kojic acid and ranged from 0.5 mM to 2.0 mM. Similarly, Yaar and Sairolu [29] reported that L-cysteine and ascorbic acid (at the concentration of 2 mM) showed good inhibition of quince (Cydonia oblonga) PPO (99 and 98% inhibition, respectively). They also studied the influence of citric acid on the quince enzyme. Formerly, the effect of citric acid on controlling browning of litchi fruit was studied by Jiang and Fu [30]. However, restrictions of sulfite usage in foods associated with consumer concern about its safety generate the need for substitutes. Therefore, alternative chemicals without toxic effects are needed, such as sulfhydryl (SH or thiol), ascorbic acid, and citric acid. These compounds have potential to be used commercially, to substitute sulfite as anti-browning agent, to prevent enzymatic browning in processed fruit products.

CONCLUSION

The effectiveness of some anti-browning agents including kojic acid, ascorbic acid, and citric acid on partially purified acanthus PPO was evaluated. Kojic acid was the most effective browning inhibitor, followed by ascorbic acid, and citric acid, respectively. Kinetics data confirmed that polyphenol oxidase has atleat two isoforms so that these isoforms showed different cresoalse and catecholase activities.

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