

Purification, Characterization, and Inhibition of Tyrosinase from Jerusalem Artichoke (*Helianthus Tuberosus* L.) Tuber

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Abstract

Background: Because it tends to cause deterioration in the quality of food and appearance, food browning is unacceptable. Tyrosinase, which catalyzes the transformation of mono phenolic compounds into o-quinones, has been associated with this phenomenon. Natural anti-browning agents were used to help avoid the enzymatic browning that occurs in many foods.

Methods: Tyrosinase of Jerusalem Artichoke tubers was purified through (NH₄)₂SO₄ sedimentation, dialysis, chromatography, and finally gel electrophoresis. The purified enzyme was characterized and inhibited by rosemary extracts.

Results: Purification of tyrosinase from Jerusalem Artichoke tuber were accomplished. The specific activity at the final step of purification increased to 14115.76 U/mg protein with purification fold 32.89 using CM-Cellulose chromatography. The molecular mass was evaluated by electrophoresis and found to be 62 KDa. Maximum tyrosinase activity was found at 30 °C, pH 7.2, and higher affinity towards L-tyrosine. Inhibition percentage of heated extracts for leaves and flowers on tyrosinase activity was better than nonheated with 29.65% and 23.75%, respectively. The kinetic analysis exposed uncompetitive inhibition by leaves and flowers heated extracts.

Conclusions: In this study, we concluded the usage of natural anti-browning inhibitors like rosemary extract be able to be castoff to substitute the chemical agents which might be dangerous to social healthiness. Natural anti-browning agents can be used to prevent the browning of many foods.

Keywords: Jerusalem artichoke, Rosemary, Tyrosinase.

Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) tuber is a perpetual herb, straight and rhizomatous. It is grown-up mostly as a once-yearly even though it is perennial (1). It is originated from North America, and extensively cultivated between 40 °C and 55 °C (2). The tubers have abundant content of carbohydrate especially inulin, nonetheless relatively little contents of protein and fiber (3). The protein content of tubers ranges from 5 to 12%, and sulfur amino acids make up the bulk

of this content (4). The mineral content in the flowering of the Jerusalem artichoke stage appears to be sufficient for K, Ca, Mg, but insufficient for P (5). In comparison to traditional crops, Jerusalem artichoke has several advantageous features, such as high growth rate, strong frost, drought and soil type tolerance, strong resistance to pathogens, and plant diseases (6). Tyrosinase (EC 1.14.18.1) is classified as an oxidoreductase enzyme which distributed widely in the kingdom of

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Received: 20 Mar, 2021; Accepted: 8 May, 2021

plant that has been discovered in many other vegetables and fruits. It catalyzes monophenol hydroxylation to form diphenol that then oxidized into quinone compound, which then polymerizes and forms black melanin pigment (7).

It's a plastid-localized enzyme that is usually separated by cell compartments from its substrate, phenolic compounds. The browning reaction happens when interaction between enzyme and phenolic compounds is caused by mechanical injury and tissue damage, such as slicing, peeling, and cutting on plant cells (8). To maintain food quality and ingratiate consumer needs, control of enzymatic browning was essential. Because of the attentiveness of the side special effects that may result from chemicals, the food productiveness is at present demanding to exclude the use of chemicals in foodstuff. In several plants, such as potato (9), and quince (10), tyrosinase inhibitors taking place in natural means have been considered. The inhibition of ginger tyrosinase by honey as a natural enzymatic anti-browning was reported (11). The enzymatic browning inhibition was achieved in potato puree by rice bran extract, and the reason perhaps due to the extract involves cysteine that has shown chelating activity (12). An onion extract was used to suppress the activity in the juices of apples (13). Limited evidence is available for exactly how natural inhibitors may impact the activity of Jerusalem artichoke tyrosinase.

Rosemary (*Rosmarinus officinalis* L.), a perennial aromatic woody shrub, its leaves resemble pine. The leaves are used as a flavor in foods. It grows in warm regions, so the Mediterranean region and Asia were their original habitat. It contains many phytochemicals as camphor, rosmarinic acid, caffeic acid, and ursolic. Many antioxidants were determined in this plant such as carnosic acid and carnosol (14-15), as well as polyphenolic compounds like quercetin, apigenin, luteolin, and catechin (16). The research aims to separate and estimate the molecular weight of tyrosinase from Jerusalem

artichoke tuber, then inhibit it using some rosemary extracts and reduce the enzymatic browning that is negative for many foods.

Materials and Methods

Chemical and Plant material

Jerusalem artichoke originated from Nineveh province; Iraq was used in this work. It was bought from a Mosul local market. Rosemary was harvested from the herbarium of the forest department of agriculture and forest College, Mosul University. All chemicals used were of analytical grade which was obtained from Sigma and Molecule Chemical Company.

Tyrosinase activity

According to the Worthington Manual Decker (1977) method (17), the activity of tyrosinase was determined spectrophotometrically using 2mM tyrosine as a substrate. The method is based on the oxidation of tyrosine to L-DOPA. The enzymatic activity was measured by following an increase in absorption at 280 nm for 5 minutes per 15 seconds. Each measurement was made in duplicate. One unit of tyrosinase activity is described as an enzyme quantity that caused an increase in absorption of 0.001/ min.

Protein Determination

The protein conc. was estimated at 650 nm by using the standard curve of bovine serum albumin solution ranging from 0-240 µg/ ml (18).

Preparation of crude tyrosinase extraction

Extraction was improved by taking 250g from Jerusalem artichoke. After washing and chopping, the homogenization was carried out using 5 g of Polyethylene glycol, 0.25 g of ascorbic acid (AA), and 0.1 mL of detergent, Triton X-100 in 300 mL 100 mM phosphate buffer (pH 6.8) at 4 °C for 2 min using a blender. After filtration, the homogenate was centrifuged at 2016 g at 4 °C for 20 min. The supernatant extract acquired was considered crude tyrosinase.

Purification of tyrosinase by Ammonium sulfate precipitation

To give a saturation of 80%, ammonium sulfate salt was slowly added to the crude tyrosinase extract at 4 °C. Precipitation was permitted overnight, followed by centrifugation for 20 min at 2016 g. In a minimum of 100 mM phosphate buffer (pH 6.8), the precipitate was dissolved and dialyzed via a 10 kDa cut-off membrane for 24 hours, with four changes in the same buffer at 4 °C and then centrifuged at 2744 g for 5 minutes and the clear supernatant was used in the following procedure (19).

DEAE-Cellulose chromatography

Dialyzed enzyme was applied to DEAE-Cellulose column (2.5×40 cm) before equilibrated with the 100 mM phosphate buffer (pH 6.8). After washing with double bed volumes of the original buffer, elution was completed at a flow rate of 1 ml/ min. Fractions showing tyrosinase activity were collected after monitoring at intervals of the individual fraction (5 ml), then dialyzed and lyophilized.

CM-cellulose chromatography

The lyophilized peak attained from the previous step was dissolved in 5 ml. of the same buffer. The enzyme solution was placed onto CM-cellulose column (2×25 cm) before equilibrated with buffer. The purification was accomplished at a flow rate of 1ml/ min. Fractions showing tyrosinase activity were collected after monitoring at intervals of an individual fraction (3ml) and then lyophilized (19).

Determination of Molecular weight

The molecular mass of purified tyrosinase was estimated by using Laemmli (1970) method (20). The protein solution was heated for 5 minutes at 100 °C. The stacking gel was run at a voltage supply of 70 V for about 45 minutes, and the separating gel was run at a voltage supply of 100 V for about 90 minutes. The Coomassie Blue staining technique was used to visualize the protein bands, and the

molecular weight was calculated using proteins of known molecular weight ranged between 14-116 KDa.

Determination of optimum pH, temperature, and Substrate specificity

The optimum pH for the purified tyrosinase was investigated by incubating the enzyme at a various pH range of 100 mM phosphate buffer solutions between 5.6-8. The optimum temperature was investigated by incubating the enzyme at temperatures from 10 to 70 °C. Enzyme specificity toward several substrates was studied by using 2 mM of phenol, tyrosine, resorcinol, catechol, pyrocatechol, L-Dopa, guaiacol, and gallic acid.

Inhibitors effects

The effects of (L-glycin, L- cysteine, ascorbic acid, cinnamic acid, citric acid, EDTA, and SDS) as inhibitors on Jerusalem artichoke purified tyrosinase activity were examined with presence two conc. (1 and 5 mM), after incubating 0.1 ml of each inhibitor with 0.2 ml of an enzyme for 30 min. Inhibition percentage was evaluated by the equation: Inhibition percentage= ((Acont. – Ainh.)/Acontrol) × 100.

Carbohydrates Effect

The effects of some carbohydrates (sucrose, galactose, fructose, glucose ribose) on Jerusalem artichoke purified tyrosinase activity were examined at two constant carbohydrate conc. (1 and 2 mM), after incubating 0.1 ml of each carbohydrate with 0.2 ml of an enzyme for 30 min.

Effect of natural inhibitors

Rosemary leaves and flower extracts were freshly used as natural inhibitors with a concentration of 10 mg/mL as a stock solution (21). Extraction was done by homogenizing individually one hundred grams with 100 mL of distilled water for 1 minute at a maximum speed at 22400 g. They filtered the homogenates via cloth. At 2016 g for 25 min at 4 °C, the filtrate has been centrifuged. By Buchner funnel, the supernatant obtained

was filtered to use as a new inhibitor. To make heated inhibitors, the inhibitor extracts were incubated at 90 °C for 20 minutes. The inhibitors were centrifuged for 1 minute at 8064 g. To 9.9 mL distilled water, 100 µL of heated inhibitors were added for preparing 10 mg/mL before use.

Tyrosinase inhibition assay

Inhibition of tyrosinase activity was assayed after incubating 0.1 ml of each inhibitor with 0.2 ml of an enzyme for 30 min. The conc. ranges of extracts used to calculate IC50 values was 1-10 mg/mL. Tyrosine concentration was prepared between (0.25- 10 mM) to investigate inhibition mode.

Results

Isolation and Purification

After precipitating with 80% (NH4)2SO4, the enzyme-specific activity has resulted from 293.49 U/mg protein with purification fold 0.68 compared to crude sample. The results shows that tyrosinase from Jerusalem artichoke was successfully purified to 32.89 purification fold with 22.86 total recovery. As can be noticed, from a primary value of 429.102 U/mg protein, specific activity increased to 14115.76 U/mg protein, however, the protein conc. decreased afterward successive purification steps (Table 1). The elution profile of tyrosinase from both DEAE-Cellulose and CM-Cellulose chromatography revealed a single peak (Figs. 1 and 2).

Table 1. tyrosinase purification steps from Jerusalem artichoke (*Helianthus tuberosus* L.).

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Specific activity (U/mg protein)	Yield %	Purification Fold
Crude extract	118	34.676	14879.55	429.102	100	1
Ammonium sulfate	21	22.620	6638.87	293.49	44.617	0.68
Dialysis	15	8.013	4214.22	525.92	28.322	1.22
DEAE-Cellulose	67	0.833	3970.23	4766.18	26.68	11.10
CM-Cellulose	43	0.241	3401.9	14115.76	22.86	32.89

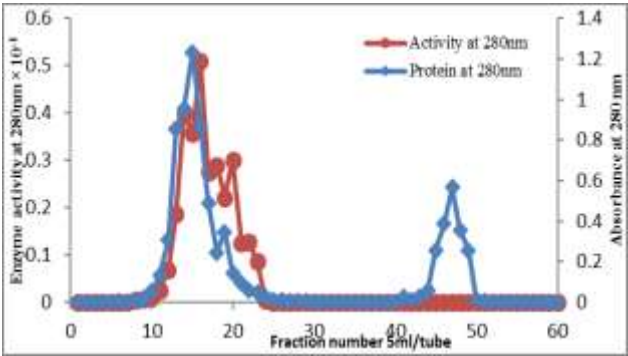


Fig. 1. Elution profile of tyrosinase from Jerusalem Artichoke on DEAE-cellulose column.

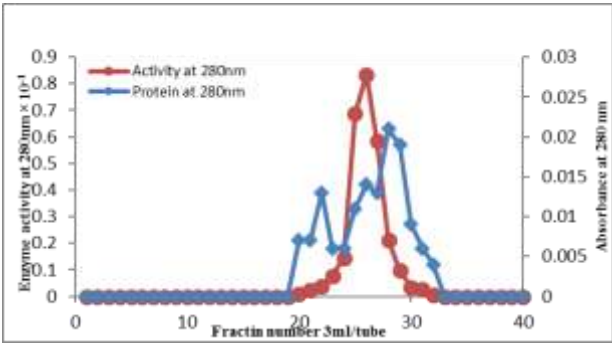


Fig. 2. Elution profile of tyrosinase from Jerusalem Artichoke on CM-cellulose column.

Molecular weight

The molecular weight of the main band of partially purified tyrosinase from Jerusalem

artichoke was approximately calculated to be 62 kDa (Fig. 3).

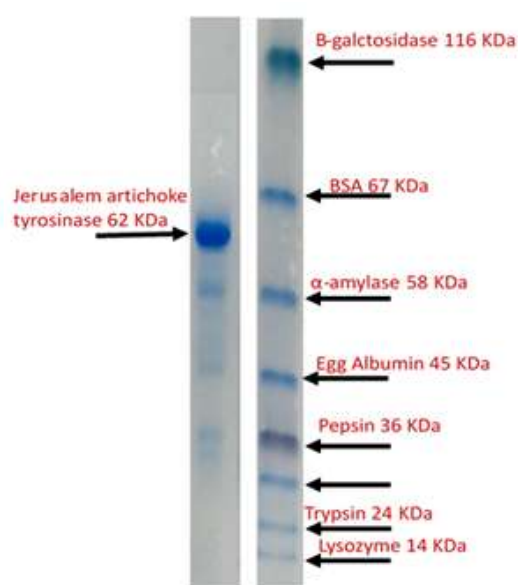


Fig. 3. SDS-PAGE of tyrosinase obtained from Jerusalem artichoke. Lane 1: standards molecular weight, lane 2: Jerusalem artichoke tyrosinase.

Optimal pH

Enzymes are highly sensitive to pH variation, which would affect tyrosinase's external charge, solubility, binding capability, and

conformation. The optimal pH for the activity of the Jerusalem Artichoke tyrosinase was found to be 7.2 (Fig. 4).

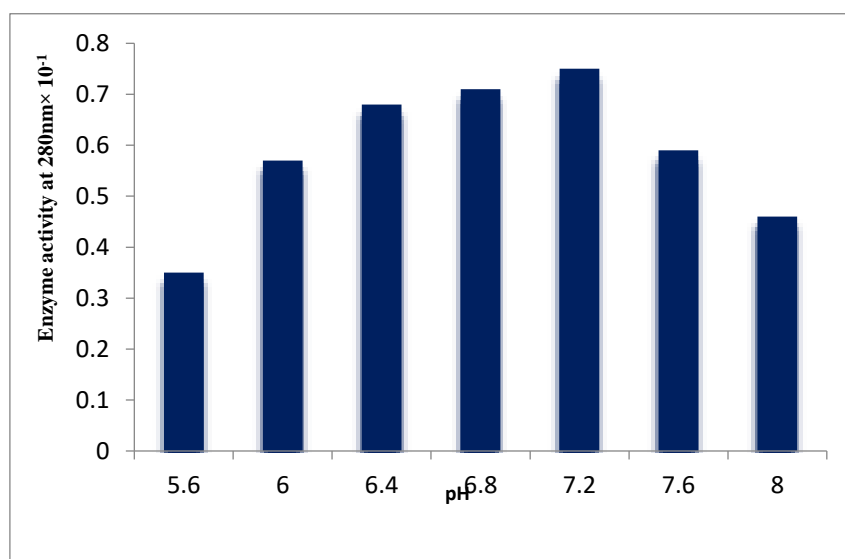


Fig. 4. pH optima of Jerusalem Artichoke tyrosinase.

Optimal Temperature

The influence of temperature is varies based on numerous variables, very high temperatures can change an enzyme-substrate complex's rupture speed. Figure 4

demonstrates that the Jerusalem Artichoke's tyrosinase is extremely active at 30 °C. After this temperature, the activity declined gradually.

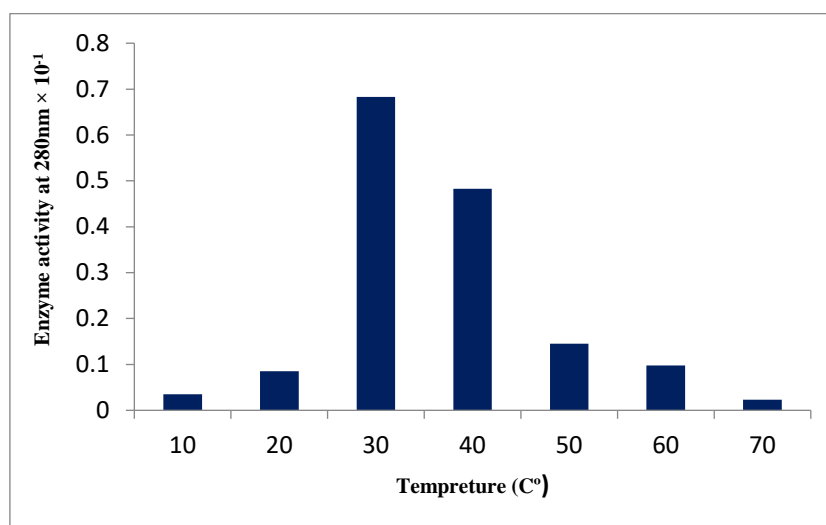


Fig. 5. Temperature optima of Jerusalem Artichoke tyrosinase.

Substrate specificity

The substrate specificity of an enzyme differs widely depending on the sorts of substrate and enzyme purity. Tyrosinase oxidized all substrates significantly including the study

except gallic acid (Table 2). The substrate with the maximum activity was established to be L-tyrosine, but guaiacol revealed the lowest activity.

Table 2. Substrate's specificity of purified tyrosinase.

Substrate	Conc.(mM)	Relative activity (%)
Phenol	2	74.8
Tyrosine	2	100
resorcinol	2	74.8
Catechol	2	91.5
Pyrocatechol	2	89.4
L- Dopa	2	46.0
Guaiacol	2	22.4
Gallic acid	2	0

Inhibitor study

In a recent study, the influence of the inhibitor on Jerusalem Artichoke tyrosinase is presented in Table 3. The most effective

inhibitors were citric acid and ascorbic acid respectively, however, the weak inhibitor was SDS.

Table 3. Effect of different inhibitors on purified tyrosinase activity.

Inhibitors	Conc. mM)	% Inhibition
L- cysteine	1	26.9
	5	56.10
L- glycyl	1	12.30
	5	36.30
Ascorbic acid	1	57.75
	5	77.11
Citric acid	1	65
	5	87.50
Cinnamic acid	1	34.60
	5	58.45
EDTA	1	24
	5	46. 20
DS	1	8.50
	5	19.6

Carbohydrates Effects

The effects of many carbohydrates on the activity of purified tyrosinase have been

investigated and the enzyme is activated by all carbohydrates except galactose.

Table 4. Carbohydrates effect on purified tyrosinase activity.

Carbohydrates	Conc. (mM)	% Activation
Sucrose	1	7.5
	2	9.1
Galactose	1	---
	2	---
Fructose	1	12.5
	2	16.1
Glucose	1	8.5
	2	11.2
Ribose	1	4
	2	6

Effect of rosemary extracts

Table 5 illustrates the inhibitory effect of rosemary leaves and flower extracts on purified Jerusalem Artichoke tyrosinase

activity. The heated extracts demonstrated a stronger inhibition percentage than nonheated.

Table 5. Effect of rosemary extracts on tyrosinase activity.

Plant Part	Extract (2 mg/ml)	%Inhibition
Leaves	Nonheated	17.55
	Heated	29.65
Flowers	Nonheated	15.45
	Heated	23.75

When using the heated extract of rosemary leaves and flowers as natural inhibitors at a concentration (IC₅₀) of 4.2 and 5.7 mg/ml respectively, the results indicated that the inhibition of the purified enzyme was

uncompetitive as explaining in Figures 5 and 6 respectively. Through Lineweaver – Burk plot, the values of K_m and V_{max} decreased compared to the enzyme without inhibitors (Table 6).

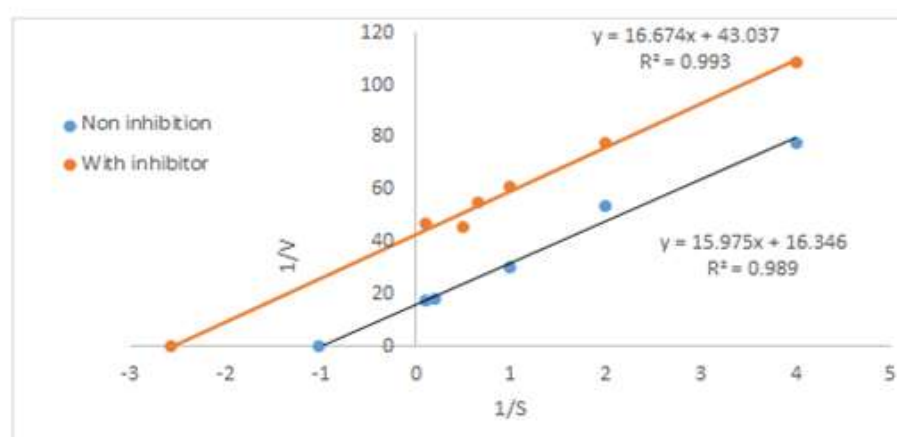


Fig. 6. Inhibition type of tyrosinase by heated extract of rosemary leaves.

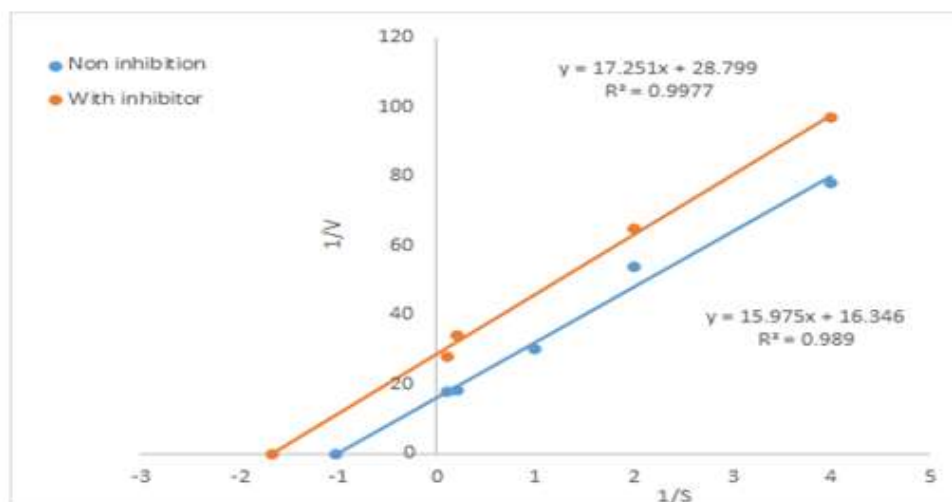


Fig. 7. Inhibition type of tyrosinase by heated extract of rosemary flowers.

Table 6. The kinetic parameters of purified Jerusalem Artichoke tyrosinase.

Inhibitors	IC50 (mg/ml)	Inhibition type	Km (mM)	Vmax U/ml.min
Control	---	---	0.98	349.55
Leaves heated extract	4.2	Uncompetitive	0.38	131.8
flowers heated extract	5.7	Uncompetitive	0.6	194.83

Discussion

The extraction of tyrosinase was accomplished in presence of polyethylene glycol and AA, which have been used to combine phenolic compounds to prevent inhibiting enzymes and reduce quinones. Cortez *et al* (2013) (23) observed after ammonium sulfate saturation of 80%, for sapodilla plum tyrosinase, the specific activity was 762.7 U/mg protein.

By ammonium sulfate precipitation and DEAE- Cellulose column, tyrosinase from jackfruit waste was purified, the folds of purity increase from 1.19 to 22.9 respectively (24). Tyrosinase in skin and flesh Turkish Jerusalem artichoke were extracted. The purification degrees were 18.0 and 12.1-fold after gel filtration, respectively (25). Artichoke (*Cynara scolymus* L.) head tyrosinase has been purified by several steps. A 43-fold purifying was obtained at the end of the purification (26). From the information, it could be noted that the process of extraction, the type of raw materials, and the technique of purification are associated with the purification fold and the yield. The

determined molecular mass in this study was alike to that molecular mass of 63 kDa of jackfruit waste and 65 kDa of jackfruit bulb (24, 27). However higher than that of purified enzyme from, beetroot 54 kDa (28) and artichoke be 57 kDa (26). These findings confirmed that tyrosinase was a single polypeptide chain (monomer) for Jerusalem artichoke.

Generally, the best pH of tyrosinase from different plants is typically 4.0 - 8.0, according to earlier reports (29). It was informed that the greater pH of skin Turkish Jerusalem Artichoke was 7.5 (25). The optimum pH of Artichoke tyrosinase was found to be 7.0 (26). Moreover, the optimal pH of water yam and tea leaves enzyme was found to be 6.0 and 8.0 respectively (30-31). Alike results were also reported where enzyme revealed a maximum activity at 30 °C for flesh of Turkish Jerusalem Artichoke (25), and eggplants (32). On the other hand, optimum temperatures of tyrosinase either lower or higher than 30 °C were observed

from various sources such as artichoke heads (25 °C) (33), pear (35 °C) (34), and quince (40 °C) (35). This result proposed that a temperature over 60 °C was useful for enzymatic browning inhibition of Jerusalem Artichoke during storage and processing.

The results of substrate specificity according to the nettle tyrosinase which has the best activity toward L-tyrosine (36). Also, it was an agreement to tea leaves enzyme which had not an activity with gallic acid (37). In contrast, artichoke tyrosinase did not use the monophenols compounds, but did use di- and tri-phenol compounds (33). The skin and flesh of Turkish Jerusalem Artichoke tyrosinase exhibited activity toward the diphenols, but low activity toward gallic acid (25). The best inhibitors for Rape Flower and jackfruit tyrosinase were determined as citric acid and ascorbic acid (27). The influences of amino acid, cysteine, and glycine on the activity of tomatoes enzyme were evaluated and the results were mentioned that cysteine had been the least potent inhibitor (38). Ginseng tyrosinase activity inhibited by AA and kojic acid. In general, certain carboxylic acids play a key role either in reducing quinones or in the active site by chelating the copper ion. In food products, AA used to prevent the browning reaction, is widely used as a reducing agent (39). The best activation was using fructose and glucose, with a proportion of 16.1% and 11.2% respectively (Table 4). The corn tassel tyrosinase was activated after using glucose and fructose with 17% and 16%, respectively (40). The tyrosinase activity of Jack fruit Bulbs was activated by Triton X-100 (27). The results were observed by

using a natural inhibitor, rosemary extracts in agreement with heated and nonheated pineapple extracts which showed the inhibition percentage (17.76–27.03%) on the ginger enzyme (11).

Similar results have been found that the tyrosinase activity of peach juice inhibited markedly by heated onion extracts (41). Mushroom tyrosinase activity was inhibited by adding 2.5 mg/ml of ethanolic and hexane extracts of green and roasted coffee (42). The natural antibrowning activity was showed when cinnamon bark oil had 49.51 inhibitory activity on enzyme activity in *Musa acuminata*, while the aqueous extract of ginger had a 60.90 % inhibitory activity on enzyme activity in *Annona muricata* (43).

These can be due to that heated extract increased flavonoids and phenolics contents along with increased antioxidant ability, which would also increase inhibition percentage. This can be deduced that the ability to bind enzymes to substrates larger than the fresh extracts might have been reduced by heated extracts. Ginger tyrosinase inhibited uncompetitively by chili pepper extract for individually non-heated and heated extracts (11). Conversely, onion revealed competitive inhibition for yam tyrosinase (22). Rosemary herb may possess some organic acids which bind copper at the active site of an enzyme and help to control browning reaction.

Acknowledgements

The researchers are truly thankful to University of Mosul which has provided enormous facilities and support to accomplish this work.

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