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Original Article

Inhibitory effects of ethanolic, methanolic and hexanolic extracts of propolis on the activity and structure of tyrosinase

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Abstract

Introduction: Tyrosinase is a key enzyme in the biosynthesis of melanin, which plays a crucial role in determining mammal's skin and hair color. In this experimental study, the inhibitory effect of different extracts of propolis were investigated on tyrosinase activity.

Methods: Tyrosinase activity was measured in the presence of ethanolic, methanolic and hexanolic extracts of propolis by using thermal denaturation (Catecholase and cresolase reaction). Also the tyrosinase stability was examined in the presence of the extracts with the chemical (urea) denaturation method. Data were analyzed with SPSS software using ANOVA and Tokey post hoc test.

Results: Tm was 56.1 °C for tyrosinase in the absence of the extract and 47.4, 53.4 and 40.2 °C in the presence of ethanolic, methanolic and hexanolic extracts of propolis, respectively. Also ΔG 25 °C values were obtained 16.03 in the absence of the extracts and 13.5 ($p<0.05$), 15.3 and 13.3 ($p<0.05$) kJ/mol, respectively in the presence of ethanolic, methanolic and hexanolic extracts of propolis. In chemical denaturation, Cm was obtained 3.11 in the absence of the extracts and 15.8 ($p<0.01$), 11.9 ($p<0.05$), and 12.5($p<0.05$) mM, respectively in the presence of ethanolic, methanolic and hexanolic extracts of propolis.

Conclusion: Ethanolic, methanolic and hexanaloic extracts of propolis are appropriate inhibitors for the tyrosinase. These reduce the thermal and chemical tyrosinase stability.

Introduction

Tyrosinase is a copper-containing enzyme present in all microorganisms, animals, and plants. This enzyme has a key role in the synthesis of melanin and thus is very important in the determination of hair and skin color in mammals. Tyrosinase is also responsible for unwanted brown marks on edible plants (1, 2). Tyrosinase inhibitors are used as anti-browning agents for fruits and vegetables in the food industry, and for the treatment of hyperpigmentation in the health industry (3). Although many drugs have been proposed for this purpose, current drugs have side effects that often have long-term complications. For example, hydroquinone has the risk of mutagenesis and can enter the blood through the mucosa or skin and cause toxicity; kojic acid is less toxic than hydroquinone, but more carcinogenic, and drugs containing arbutin are toxic and produce hydroquinone (4-6). Therefore, studies on finding safe natural ingredients to inhibit tyrosinase in food and cosmetics industries are necessary and worthwhile.

Propolis is a bee product used in traditional medicine for a long time. Propolis is a resinous, hard and brown substance collected by bees from resin, buds and other parts of plants. The collected raw material is hydrolyzed by beta-glucosidase from the hypopharyngeal glands of honeybees. Today, propolis has diverse medical applications, for example, it is recommended for the treatment of various inflammatory diseases, heart diseases, diabetes mellitus, liver toxicity caused by drugs and toxins, and some cancers (8, 7). More than 300 different compounds have been identified in propolis so far. Although its chemical composition might vary slightly depending on the quality and quantity of vegetation of each region (10), propolis is naturally made up from 50% resin (mostly flavonoids), 30% wax, 10% essential oils, and 10% different types of organic compounds. Extracts can be used to inhibit enzymes. Polyphenols have received special attention as they are active ingredients of propolis due to their ability to inhibit several enzymes (9, 11). Considering the available compounds in propolis, it appears that its extracts might affect the tyrosinase kinetics and activity.

The present study examined the inhibition or activation effects of different propolis extracts on the activity and structure of tyrosinase.

Materials and Methods

Extraction:

In this experimental study, propolis was first gathered from hives in Dasht-e Qazvin. The extracts were prepared by the cold extraction method such that 30 g of propolis was cut into small pieces of about 2 mm, mixed with 300 ml of 96% ethanol, methanol or hexane, incubated at 37 °C for 20 days, and stirred with a shaker every day. After 20 days, the resulting suspensions were filtered using Whatman® 41 filters and condensed in a rotary machine at 45 °C.

Enzyme activity examination:

During two consecutive hydroxylation activities, tyrosinase catalyzes monophenols to diphenols and oxidase them to relevant quinone. Kinetic measurements of these two activities were carried out in the presence of monophenolic and diphenolic substrates. Kinetic measurement of cresolase and catecholase reactions in inhibition experiments were performed using Cary 100 Bio Spectrophotometer. Fresh substrates and enzyme concentrations were used in the experiments. The enzyme activity was measured using caffeic acid substrate in the catecholase reaction, and p-Coumaric acid in cresolase reactions (12). Enzyme denaturation by urea was studied to draw the relevant curves. Thermodynamic stability of the enzyme was examined from denaturation curves in the presence and absence of ethanol, methanol and hexane extracts of propolis. The magnitudes of free energy as the stability index of the enzyme was obtained in the presence of these compounds (13).

Fluorescence Studies

Cary 100 Bio Spectrophotometer was used to examine the tertiary structure of mushroom tyrosinase through intrinsic fluorescence method at 280 nm excitation wavelength and 300 to 450 nm emission wavelengths (12). All tests were repeated three times and the mean was calculated. The results were analyzed by SPSS Version 18 using one-way ANOVA and Tukey's post hoc statistical tests at a significance level of 0.05.

Results

Tyrosinase activity was non-classical in both catecholase and cresolase reactions in the presence of ethanol, methanol and hexane extracts of propolis. Using dose-response curves, the half maximal inhibitory concentration (IC50) for these compounds was 22.5, 3.5 and 42.5 mcg/l in catecholase reaction and 19.8, 28.6, and 40.6 mcg/l in cresolase reaction, respectively. (Table 1)

Stability of the tyrosinase in the presence of ethanol, methanol and hexane extracts of propolis decreased during chemical and thermal denaturation reactions. Tm was 56.1 °C in thermal denaturation without extract and 47.4 °C, 53.4 °C, 40.2 °C in the presence of ethanol, methanol and hexane extracts, respectively. The hexane extract was statistically significant ($p<0.01$). The amounts of ΔG 25c was 16.03 KJ/mol in the absence of extracts, and 13.5, 15.3, 13.3 KJ/mol in the presence of ethanol, methanol and hexane extracts, which were significant compared to when the inhibitor was not used (both $p<0.05$). Stability of the enzyme was studied using the chemical denaturation method (urea denaturation) with the IC50 and ΔG H2O in the presence and absence of the extracts. The results confirmed the destabilizing effect of extracts. The values of ΔG H2O decreased and IC50 increased in the presence of the extracts. The amount of IC50 was 3.11 mM for the enzyme in the absence of the extract, and 15.8 ($p<0.01$), 11.9 ($p<0.05$), and 12.5 ($p<0.05$) mM in the presence of ethanol, methanol and hexane extracts, respectively (Table 2). The examination of the tertiary structure of tyrosinase in the presence of different concentrations of ethanol, methanol and hexane extracts of propolis with intrinsic fluorescence technique revealed the opening and disruption of the enzyme structure in a concentration-dependent manner.

Table 1. Mean and standard deviation of tyrosinase kinetic parameters in catecholase and cresolase reactions in the presence of different extracts of propolis

Kinetic parameters of tyrosinase in the presence of propolis extracts	Non-classical inhibitory concentration (µgr/ml)(IC_{50})	
	Cresolase reaction	Catecholase reaction
Ethanol	19.8±0.9	22.5±2.1
Methanol	28.6±2.28	30.5±3.33
Hexane	40.6±2.5	42.9±2.92

Table 2. Mean and standard deviation of thermodynamic parameters (thermal and chemical denaturation of tyrosinase) without inhibitor and in the presence of various extracts of propolis

Thermodynamic parameters	Thermal denaturation		Chemical denaturation	
	ΔG°(25°c) (kJ/mol)	Tm (°C)	ΔG°(H2O) (kJ/mol)	Cm (mM)
In the present of tyrosinase without inhibitor	16 16.03±1.1	56.1±1.4	13.3±1.5	3.11±0.5
Tyrosinase and ethanol extract	13.5±0.6 *	47.4±1.3	7.5±0.7 *	15.8±1.8 **
Tyrosinase and methanol extract	15.3±1.5	53.4±1.6	9.5±0.8	11.9±1.1 *
Tyrosinase and hexane extract	13.3±0.9*	40.2±1.1*	5.6±0.4**	12.5±1.2*

* $P<0.05$ and ** $P<0.01$ Extracts' inhibition compared to the presence time of the enzyme without inhibitors

Discussion

The results suggested the inhibitory effect of different extracts of propolis on tyrosinase activity. Tyrosinase is a copper-containing monooxygenase that has an important role in the biosynthesis of melanin pigments and polyphenolic compounds. Tyrosinase catalyzes cresolase (monophenolase) and catecholase (diphenolase) reactions. Orthohydroxylation of monophenols happens in the cresolase reaction, and the oxidation of diphenols to ortho-quinone happens in the catecholase reaction. Many studies have been conducted to identify natural inhibitors and the relationship between their activity and inhibition capability. Some plant flavonoids have been introduced as natural inhibitors of tyrosinase so far (14). The results of the study on catecholase activity of tyrosinase showed that all extracts significantly reduced the enzyme activity. In this regard, kinetic inhibition studies showed that these materials bind to the active site of the enzyme. According to the competitive inhibition pattern, the role of the carboxyl group in binding to the copper ions of the active site of the enzyme is important. This effect is possibly created by EI complex and copper chelation in the active site of the enzyme. Copper ions chelation in the active site of the tyrosinase is an important strategy in the inhibitory mechanism (15-17). Another possible mechanism is enzyme interactions and competitive inhibition of the extracts. That is the materials can have hydrogen interactions with amino acids in the enzyme's active site. These amino acids somehow participate in the binding of substrates to the active site of the enzyme's tyrosine amino acids and thus the extracts make the tyrosine amino acid (Tyr98) stable and prevents the participation of this amino acid in the binding of the substrate to the active site and thereby the enzyme is competitively inhibited (18). Tyrosinase has a dinuclear copper active site, whose structure can follow Oxy, Deoxy and Met forms (19). Monophenolic and diphenolic substrates bind to the enzyme's active site with a different pattern. Monophenoles bind with a copper through their phenolic oxygen, while the diphenoles coordinate with the active site through their both phenolic oxygen atoms and build a bridge-like structure (20). The crystallographic data of hemocyanin and recently catechol oxidase show that in the three-dimensional structures of the tyrosinase family, the copper-containing catalytic position is in a center of four group helix in an hydrophobic core near the surface (21). As noted before, both catecholase and cresolase reactions of tyrosinase inhibition in the presence of ethanolic, methanolic and hexanolic extracts of propolis were non-classical. The non-classical inhibition is due to the multi-compound and mix nature of some of these extracts (7). The present study examined tyrosinase stability inhibition in the presence of the extracts with both chemical and thermal denaturation. The thermal stability reduction of the enzyme in the presence of these extracts was confirmed by chemical and thermal denaturation. The fluorescence techniques also showed the unfolding and fluctuation of the tertiary structure of the enzyme in a concentration-dependent manner in the presence of different propolis extracts. According to the results, ethanolic, methanolic and hexanolic extracts of propolis are proper inhibitors of tyrosinase. Quantitatively, the ethanolic extract had a better effect and the methanolic extract had a weaker effect. All three extracts reduced enzyme thermal and chemical stability and the thermodynamic evidence was consistent with the structural changes. Regarding the thermal and chemical denaturation of tyrosinase in the presence and absence of the inhibitors, it should be noted that the thermodynamic denaturation of the protein is the basis of understanding protein stability. The denaturation of the protein is a process in which the normal spatial arrangement of macromolecules is changed without destroying the primary covalent bonds. That is, in this process, macromolecules' basic structure (sequence of amino acids) does not change, but the spatial arrangement (three-dimensional structure) changes. The spatial arrangement is the special arrangement of atoms in a three-dimensional space and the length of binding angles (22). In the present study, the intrinsic fluorescence intensity of the enzyme reduced in the presence of ethanolic, methanolic and hexanolic extracts of propolis. This shows fluorescence quenching by different concentrations of the extracts. The intrinsic fluorescence of the enzyme is induced by the bases of tryptophan and tyrosine amino acids, and the fluorescence emission intensity of both depends on the surrounding environment. When the protein is excited at a wavelength of 280, where these amino acids are affected, the tertiary structure of the proteins is altered (23). The fluorescence measurements can provide information about the mechanism of small molecules' binding to a protein, binding constants, binding sites, etc. for macromolecules (24). Fluorescence quenching depends on several factors such as molecular interactions including excited state reactions, molecular rearrangement, energy transfer, and complex formation (25, 26). Propolis extracts contain numerous compounds, especially flavonoids and probably the binding site of these flavonoids to the enzyme is the tryptophan amino acid and hence lead to conformational changes in the enzyme (27). Partial unfolding of of proteins by increasing the concentration of these flavonoids is an important factor in the reduction of enzyme activity, which is consistent with fluorescence results.

Conclusion

The results showed that all three ethanolic, methanolic and hexanolic extracts of propolis had proper inhibitory effects on tyrosinase. Therefore, they can be used in the treatment of hyperpigmentation and similar problems, although more studies are needed for their clinical use.

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