

# Inhibitory effect of garlic extracts on polyphenoloxidase

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## Abstract

*In this study, the inhibition effect of heat-treated garlic extract on polyphenol oxidase (PPO) obtained from Mentha piperita L. (mint) was investigated. In addition that the investigation was extended using inhibitors such as ascorbic acid, L-cysteine, glutathione, ethylenediaminetetraacetic acid (EDTA), and sodium azide using catechol, 4-methylcatechol and pyrogallol as substrates. PPO activity was significantly decreased by different reducing agents such as ascorbic acid, sodium azide, glutathione, L-cysteine and EDTA. The enzyme was also inhibited by the addition of garlic extract heat treated at different temperature (room temperature, 50 and 100 °C). Garlic extract heat-treated at 100 °C showed higher inhibition effect than the other garlic extracts. Ascorbic acid showed the strongest inhibitory activity among these inhibitors. This paper represents a new inhibitory source for preventing of enzymatic browning.*

**Keywords:** Polyphenol oxidase, mint, garlic, inhibition.

## Sarımsak ekstraktlarının polifenol oksidaz üzerine inhibitör etkisi

### Öz

*Bu çalışmada, Isıl işlem görmüş sarımsak özütlerinin, Mentha piperita L.' (nane) den elde edilen polifenoloksidaz (PPO) üzerindeki inhibisyon etkisi araştırılmıştır. Ek olarak, araştırma askorbik asit, L-sistein, glutatyon, etilendiaminetetraasetik asit (EDTA) ve sodium azid inhibitörleri ile katekol, 4-metikatekol ve pirogallol substratları kullanılarak genişletilmiştir. PPO aktivitesi askorbik asit, sodyum azid, glutatyon, L-sistein ve EDTA gibi farklı indirgeyici ajanlar tarafından kuvvetli bir şekilde durdurulmuştur. Enzim ayrıca farklı sıcaklıklarda (oda sıcaklığı, 50 ve 100 °C) ısı*

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*işlem görmüş sarımsak ekstraktı ile inhibe edildiği tespit edilmiştir. 100 °C'de ısı işlem görmüş sarımsak özütü, diğer sarımsak özütlerinden daha yüksek inhibisyon etkisi göstermiştir. Askorbik asit, bu inhibitörler arasında en güçlü inhibitör aktivite göstermiştir. Bu makale, enzimatik esmerleşmenin önlenmesi için yeni bir engelleyici kaynağı sunmaktadır.*

**Anahtar kelimeler:** Polifenol oksidaz, nane, sarımsak, inhibisyon.

## 1. Introduction

Polyphenol oxidase (PPO; EC 1.14.18.1) is a metallo-enzyme including copper ions responsible for browning in plants and melanization in animals. PPO has different names depending on the substrate specificity, product and its form such as phenol oxidase, phenolase, tyrosinase, *o*-diphenol oxidase and catechol oxidase. Amino acids and proteins that are endogenous, catalyzes hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones resulting in formation of complex brown pigments, [1]. Browning can be a critical problem in fruits and vegetables, occurring color alterations after bruising, cutting or during storage that reduce the commercial product value and even make them undesirable to the consumer [2]. The browning mechanism is well characterized in some studies related to PPO enzyme that are aimed to stop the enzymatic browning [1-9]. Generally, synthetic inhibitors such as EDTA, sodium azide, glutathione, sulfite used to stop enzymatic browning. Whereas, most of these inhibitors are highly harmful for human health. For example, it was determined that sulfite-containing additives caused the oxidation of unsaturated fatty acids [3]. Recently, there is an increasing interest in natural compounds displaying inhibiting food deterioration, antimicrobial activity or avoiding oxidative processes for preventing the quality loss of processed products [2]. Unlike additives, the use of extracts does not pose a problem for consumers. As food components, there is a growing demand for customers related to using of natural ingredients instead of synthetic components. The polyphenol oxidase inhibitors obtained from natural resources were studied in several plants, but the development of natural and efficient polyphenol oxidase inhibitors is still needed. Lee et al (2002) studied inhibitory effect of onion extracts on PPO activity changing with heating temperature and time. They reported that heat-treatment of onion extract at 100 °C for 10 min exhibited stronger inhibitory effect on potato PPO activity than the fresh one. They suggested that onion extracts could be used as a natural inhibitor for PPO enzymes to be isolated from potatoes and different sources [1].

*Allium sativum* (Garlic) which is a species in the Alliaceae family is native to central Asia. Garlic extracts have been used for medicinal objectives for thousands of years. Garlic has a rich potential control agent for infectious organisms due to their antimicrobial properties [4]. Their use had determined as a treatment by Hippocrates for treating pneumonia and inflamed wounds. The using of garlic extracts for treatment such ailments as gastric catarrh, typhoid, cholera and dysentery has continued in several parts of the world. Besides, they have been asserted to be more effective than penicillin for the remedy of certain throat infections [4]. Additionally, Kumar and Berwal studied the garlic inhibitory activity against to *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. They investigated minimal inhibitory concentration of garlic at 80% inhibition level for these bacteria and found that garlic

was a potent inhibitor of food pathogens [5]. Davis et al demonstrated that *A. sativum* compounds possessed influential anti-cryptococcal activity and acts synergistically with amphotericin B under in vitro conditions to inhibit growth and wiped out *Cryptococcus neoformans* [6]. Wilson et al developed a rapid test to identify antifungal activity in plant extracts and essential oils. Extract solutions obtained from 345 plants and 49 essential oils were formed an estimate of their antifungal activity against *B. cinerea*. Among 345 plant extracts analyzed, 13 extracts showed high levels of antifungal activity among 345 plant extracts and, red thyme, palmarosa, clove buds, and cinnamon leaf among the 49 essential oils demonstrated the most antifungal activity against *B. cinerea* [7]. As can be seen from the explanations above, no studies have been found in the literature about polyphenol oxidase inhibition by garlic extracts. The aim of this paper is to develop alternative, reliable and natural inhibitors for preventing the enzymatic browning. Besides, the inhibition activity of the heat-treated garlic extracts at different temperatures was measured and compared with the inhibitory effects of ascorbic acid, L-cysteine, glutathione, sodium azide and EDTA compounds.

## 2. Materials and method

### 2.1 Materials

Mint (*Mentha piperita* L.) and garlic (*Allium sativum* L.) used in this study were obtained from a local market in Balıkesir (Turkey). The chemicals were analytical grade and were purchased from Sigma Chem. Co. and Merck.

### 2.2 Extract of PPO

Firstly, the mint was treated with liquid nitrogen and then disintegrated by blender. 10 g ground mint was homogenized with 100 mL of 0.1 M sodium phosphate buffer containing 10 mM ascorbic acid and 4% polyethylene glycol at pH 7.0 for 2 min. The homogenate was filtered, and the filtrate was centrifuged at 15 000 x g for 10 minutes at 4 °C. Then, the supernatant was precipitated by ammonium sulfate (70%) and the pellet was dissolved in 0.1 M, phosphate buffer (pH 7.0) and dialyzed at 4 °C in 0.01 M sodium phosphate buffer for overnight to remove excess ammonium sulfate ions. Finally, the obtained sample was used as the PPO enzyme source in the further experiments [11].

### 2.3 Preparation of garlic extract

100 g cloves of garlic was homogenized with 0.01 M sodium phosphate buffer (100 mL) at pH 7.0, and the homogenate was centrifuged at 15 000 x g for 10 min. The supernatant was collected to use as a fresh garlic extract. Supernatant was separated into three parts. The first part of the supernatant was separated for room temperature analysis. The other parts were heat-treated by heating at 50 and 100 °C for 10 min [1].

### 2.4 Enzyme assay

The PPO activity was measured by monitoring the formation of quinones spectrophotometrically (A perkin Elmer Lambda-35 UV-Visible Spectrophotometer) at room temperature. To determine the enzymatic activity, catechol ( $10^{-1}$ M), 4-methylcatechol ( $10^{-1}$ M) and pyrogallol ( $10^{-1}$ M) were used as substrates, and analysis were carried out spectrophotometrically at 420, 420 and 320 nm, respectively. The total assay mixture containing PPO solution, substrate and buffer was 3 mL. The reaction was started by adding aliquots of extract in the test medium. The blank solution was

also prepared in same way using only substrate and buffer solutions. Enzyme activity data were calculated as averages of triplicate measurements [12].

### 2.5 Enzyme kinetics and substrate specificity

The procedure of the catalytic activity of PPO was assayed and evaluated as function of substrate including 4-methylcatechol, catechol, L-tyrosine, and pyrogallol. Additionally, PPO reaction rate was monitored using increasing substrate concentrations. The assessment of the experimental was achieved by using the Michaelis-Menten equation. The Michaelis-Menten equation was transformed into the double-reciprocal form (the Lineweaver-Burk plot) and provided a more reliable determination of  $V_{\max}$  and  $K_m$  [8].

### 2.6 Inhibition assay

Garlic extract was evaluated for its effectiveness as a inhibitor of mint PPOs using the same substrates. Furthermore, L-cysteine ( $10^{-3}$ M), EDTA ( $10^{-1}$ M), ascorbic acid ( $10^{-3}$ M for catechol and pyrogallol,  $10^{-4}$ M for 4-methylcatechol), glutathione ( $10^{-3}$ M for 4-methylcatechol and pyrogallol,  $10^{-4}$  M for catechol) and sodium azide ( $10^{-2}$ M), which are known as a inhibitors of PPO, was also used to compare the inhibition potential of garlic extract [13].

## 3. Results and discussion

Monophenolic and diphenolic substrates were assayed for substrate specificity of mint PPO. 4-methylcatechol, catechol, and pyrogallol as diphenolic substrates were oxidized considerably by mint PPO, but L-tyrosine which is a monophenolic substrate was unable to oxidize. Several plant PPO, such as broad bean, potato, and mushroom react with both the oxidation of *o*-diphenols and the hydroxylation of monophenols. Whereas a lot of PPOs absence monophenol activity[9]. Substrate specificities clearly demonstrate that the measured activity is diphenolase activity as noted for other plant PPOs sources like artichoke[10–12]. For every diphenolic substrate, substrate saturation curves signified that mint PPO follows Michaelis-Menten kinetics. Regression coefficients are approximately 0.99 and close to 1. Individual  $V_{\max}$  and  $K_m$  values were resulted with Lineweaver-Burk plots for each substrate for the kinetic analysis of the reaction rates at a series of concentrations.  $V_{\max}/K_m$  ratio as catalytic efficiency were evaluated for substrate specificities[13]. From Table 1,  $V_{\max}/K_m$  which is the catalytic efficiency values, showed that pyrogallol had higher catalytic activity as substrate for mint PPO. These findings are similar to previous studies which were reported peppermint, lettuce, lemon balm [11,13-14].

Table 1.  $K_m$  and  $V_{\max}$  values of mint PPO for different substrates.

Substrates	$K_m$ (mM)	$V_{\max}$ (EU/mL.min)	$V_{\max}/K_m$
Catechol	15	5000	333
4-methyl catechol	12.5	2500	200
Pyrogallol	5	5000	1000

In this work, the inhibition effect of heat-treated garlic extract solutions on PPO activity using pyrogallol, catechol, and 4-methyl catechol as substrates which were obtained from mint was investigated. Heat treatment of garlic extracts was carried out at room

temperature, 50 and 100 °C. Additionally, the inhibition effects of L-cysteine, EDTA, ascorbic acid, glutathione and sodium azide on PPO activity were also studied. Many compounds change an enzyme activity by combining with it in a way that affects the binding of substrate and/or its turnover number. Such compounds that reduce activity of an enzyme by this way are identified as inhibitors. Compared with substrate, inhibitors are general compounds that structurally have resemblance to enzyme's substrate which react very slowly or do not react. These inhibitors are usually used to examine thoroughly the chemical and structural nature of a substrate-binding site as part of an struggle to explain the enzyme's catalytic mechanism [14]. In some cases such as inactivation of PPO, elimination of one of the substrates for the reaction and the role of inhibitors on enzyme-substrat reaction products to inhibit the formation of colored products in secondary reactions may cause the inhibition of browning [15]. The prevention of enzymatic browning may be explained via various mechanisms such as single or multiple reaction mechanisms performed by a specific inhibitor.

Tablo 2. IC<sub>50</sub> values of mint PPO by garlic extracts.

Substrates	Parameters (°C)	IC <sub>50</sub> (mg/mL)
Catechol	Room temperature	0.093
	50	0.104
	100	0.051
4-methyl catechol	Room temperature	---
	50	---
	100	---
Pyrogallol	Room temperature	---
	50	0.182
	100	0.137

Tables 2 and 3 show the IC<sub>50</sub> values of garlic extract and inhibitors such as ascorbic acid, glutathione, sodium azide, L-cysteine and EDTA on mint PPO enzyme activity using catechol, 4-methyl catechol and pyrogallol as substrate, respectively. Table 2 shows the effect of inhibition of garlic extracts, which is an anti-browning agent, subjected to heat treatment at different temperatures, on mint PPO enzyme activity. Garlic extract showed different behaviors according to both substrates and heat treatment temperatures. The highest inhibition activity of the garlic extract was observed for the heat-treated sample at 100 °C. When catechol was used as a substrate for this example, IC<sub>50</sub> value was calculated to be 0.051 mg/mL. Using the catechol as a substrate, the IC<sub>50</sub> values of the heat-treated garlic extracts at 25 and 50 °C were calculated as 0.093 and 0.104 mg/mL, respectively. As similar to results of this study, Myin-Kyung et al. reported that onion extract heat treated at 100 °C exhibited a higher inhibitory effect on potato polyphenol oxidase than those of fresh and other heat treated onion extracts [1]. At another study, it was reported that clove essential oil treatment with fresh-cut lettuce could prevent the browning induced by PPO, which catalyzes phenols into brown quinones [16]. When pyrogallol was used as a substrate, heat treated garlic extracts at 50 and 100 °C showed more inhibition effect on the mint PPO. IC<sub>50</sub> values of both of them were measured as 0.137 mg/mL for heat treated sample at 100 °C and 0.182 mg/mL, for heat treated sample at 50 °C, respectively. But, the fresh garlic extract did not exhibit any inhibitory effect on mint PPO. On the other hand, all

heat treated garlic extracts did not show any inhibition effect on mint PPO when 4-methyl catechol was used as substrate.

Tablo 3. IC<sub>50</sub> values of some inhibitors on mint PPO.

Substrates	Inhibitors	IC <sub>50</sub> (mM)
Catechol	Ascorbic acid	0.087
	Glutathione	0.103
	Sodium azide	1.410
	L-cysteine	0.109
	EDTA	15.6
4-methyl catechol	Ascorbic acid	0.011
	Glutathione	0.114
	Sodium azide	2.051
	L-cysteine	0.105
	EDTA	----
Pyrogallol	Ascorbic acid	0.013
	Glutathione	0.234
	Sodium azide	-----
	L-cysteine	0.162
	EDTA	----

Table 3 summarizes the IC<sub>50</sub> values for inhibition of mint PPO activity with ascorbic acid, L-cysteine, glutathione, sodium azide, and EDTA inhibitors using 4-methylcatechol, pyrogallol, and catechol substrates. The results showed that ascorbic acid had the most inhibitory power on PPO with 4-methyl catechol, followed by pyrogallol and catechol. IC<sub>50</sub> values were 0.011, 0.013, 0.087 mM for 4-methyl catechol, pyrogallol, catechol, respectively. In literature, it was found that ascorbic acid was the most effective inhibitory for *Prunus spinosa* PPO when using 4-methyl catechol as a substrate, and its IC<sub>50</sub> value was 0.04 mM [17]. It was determined that glutathione and L-cysteine show less inhibitory effect for PPO, than the ascorbic acid. Both of them significantly performed the prevention of enzymatic browning. IC<sub>50</sub> values of glutathione were found as 0.103 mM, 0.114 mM and 0.234 mM with catechol, 4-methyl catechol and pyrogallol as substrates, respectively. For L-cysteine, IC<sub>50</sub> values were 0.105, 0.109 and 0.162 mM using 4-methyl catechol, catechol and pyrogallol, respectively. Sodium azide and EDTA were found to be the weakest inhibitors among them. Moreover, it was determined that EDTA had not any inhibitory effect on mint PPO with pyrogallol and 4-methyl catechol. On the other hand, sodium azide showed the inhibitory activity with catechol and 4-methyl catechol and these IC<sub>50</sub> values were 1.410 and 2.051 mM, respectively. But, it did not show any inhibitory activity with pyrogallol.

#### 4. Conclusion

From the experimental results, garlic extracts can be used as an inhibitor source for preventing the enzymatic browning of different PPOs. The fact that garlic extracts subjected to heat treatment at high temperatures show the higher inhibition effect may

be due to the increasing kinetic energies of the substances reacting with increasing temperature and the denature of the structures of some substances that prevent inhibition in garlic extracts with increasing temperature. In comparison with the values in the literature, garlic extracts had a very high inhibitory potency against PPO activity. Among the inhibitors of PPO known in the literature and used in this study, ascorbic acid had the greatest inhibitory potency for mint PPO.

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