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Research Article

Prevention of Enzymatic Browning by Inhibiting Polyphenol Oxidase with Some Natural Compounds and Benzenethiol

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Abstract

Background: In recent years consumers' awareness of the health benefits of fresh vegetables and fruits have increased and demand for ready-made food has increased. That's why, fresh cut vegetables and fruits have become a popular. An important concern in this process is enzymatic browning, which cause changes in the texture, taste, and color of freshly cut product. Physical and chemical-based anti-browning methods to prolong the shelf life of freshly cut products are remarkable. In this study, it was aimed to prevent enzymatic browning via inhibition of polyphenol oxidase (PPO) enzyme.
Method: Firstly, potato was used as the source of polyphenol oxidase and enzyme purified by affinity chromatography. Sepharose 4B L-tyrosine p-amino benzoic acid (Sepharose 4B L-tyr-p-ABA) was used as affinity column. SDS-PAGE was used to check enzyme purity. Then, the inhibition effect of baicalin, bailcalein, phloridzin, phloretin natural compounds and benzenthiol sulfur compound were investigated.
Results: PPO was purified with 13.94% fold from potato. In inhibition studies K_i values of phloridzin, phloretin and benzenethiol were determined as 0.120±0.0157, 0.027±0.0054, 0.008±0.0014 mM respectively. According to our results, the order of inhibition is as follows: pholoridzin > phloretin > benzenethiol. While baicalin and bailcalein did not show any inhibition effect, phloridzin and phloretin showed non-competitive, benzentiol showed competitive inhibition.

Keywords: Enzymatic Browning, Inhibition, Polyphenol Oxidase, Natural Compound, Benzenethiol.

Bazı Doğal Bileşikler ve Benzentiyol ile Polifenol Oksidaz İnhibisyonu Yoluyla Enzimatik Kararmanın Önlenmesi

Öz

Arkaplan: Taze kesilmiş sebzeler ve meyveler, son yıllarda tüketicilerin taze sebzelerin ve meyvelerin sağlık yararları konusundaki farkındalığının artması ve hazır gıda talebinin artması nedeniyle popüler hale gelmiştir. Önemli bir endişe, taze kesilmiş ürünlerin renk, tat ve dokusunda değişikliklere yol açabilen ve bu nedenle kalite bozulması ile ilişkili enzimatik esmerleşmedir. Taze kesilmiş ürünlerin raf ömrünü uzatmak için fiziksel ve kimyasal temelli anti-esmerleşme yöntemleri dikkat çekicidir. Bu çalışmada, enzimatik esmerleşmeden sorumlu polifenol oksidaz (PPO) enzimi için inhibitörler önerilmiştir.
Yöntem: İlk olarak patates, Sepharose 4B L-tirozin p-amino benzoik asit (Sepharose 4B L-tyr-p-ABA) afinite kromatografisi ile polifenol oksidaz saflaştırılmasında enzim kaynağı olarak kullanıldı. Enzim saflığı SDS-PAGE ile kontrol edildi. Daha sonra, baicalin, bailcalein, phloridzin, phloretin doğal bileşikleri ve benzentiol sülfür bileşiğinin inhibisyon etkisi araştırıldı.
Sonuçlar: PPO, patatesden % 13.94 kat saflaştırıldı. İnhibisyon çalışmalarında phloridzin, phloretin ve benzenethiol'ün Ki değerleri sırasıyla 0.120 ± 0.0157, 0.027 ± 0.0054, 0.008 ± 0.0014 mM olarak belirlenmiştir. Sonuçlarımıza göre, inhibisyon sırası aşağıdaki gibidir: pholoridzin > phloretin > benzenethiol. Baicalin ve bailcalein bir inhibisyon etkisi göstermezken, phloridzin, phloretin yarşmasız, benzentiol yarışmalı inhibisyon gösterdi.

Anahtar Kelimeler: Enzimatik Kararma, İnhibisyon, Polifenol Oksidaz, Doğal Bileşikler, Benzentiyol.

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1. Introduction

As a result of physical and mechanical processes during storage and post-harvest processing, the appearance of color changes in vegetables and fruits that can turn from yellow to brown is known as enzymatic browning. Enzymatic browning reactions are mainly performed by polyphenol oxidase (PPO), which is commonly found in fungi and high plants (Mayer, 2006). This enzyme is responsible enzymatic browning in seafood, fruits, vegetables and melanin formation in human skin. Therefore, it attracts attention in the fields of food science, plant physiology and cosmetic development (Bayrak, Öztürk, Demir, Alım, & Küfrevioglu, 2020; Mishra & Gautam, 2016). PPO is an intracellular diphenol oxidase containing copper and catalyzes the oxidation of polyphenolic substrates to quinone groups. Then, brown melanin pigments are occured from the quinones with a non-enzymatic reaction. Consequently, enzymatic browning causes not only to color change and antioxidant degradation, but also to loss of color, odor and nutritional value due to condensation of quinones with compounds such as phenols, sugar, amino acids, and proteins (Jiang, 1999). Because browning reduces the sensory and nutritional qualities in foods, various physical and chemical methods have been developed to control enzyme activity of PPO. These methods include one or more essential components such as oxygen, copper ion, enzyme substrates, products, and even the enzyme itself, which are required for the reaction to occur (Queiroz, Lopes, Fialho, & Valente-Mesquita, 2008). Depending on the inhibition mechanism, different effectors classified as acidulants, reducing agents, chelating agents, complexing agents, enzyme inhibitors, and enzymatic browning can control with these effectors (Tinello & Lante, 2018). Sulphiting agents are the most commonly used agents for browning control (Kahn, Ben-Shalom, & Zakin, 1999). Sulfur dioxide (SO_2) are the most common inhibitors for the PPO enzyme, but especially asthmatics people may be sensitive to sulfide (M. V. Martinez & Whitaker, 1995; Sapers, 1993). Ascorbic and citric acid formulations are sulphite alternatives used in the food industry (Hsu, Shieh, Bills, & White, 1988). However, these formulations are less effective than sulphiting agents. Therefore, the inhibition effect of SH or thiol (sulfhydryl) compounds on the PPO enzyme is often studied at the various source such as atemoya (Chaves, Ferreira, Da Silva, & Neves, 2011), tea leaf (Öztürk, Aksoy, & Küfrevioglu, 2020), peach (Garro & Gasull, 2010), eggplant (Mishra, Gautam, & Sharma, 2012), potato (Duangmal & Apenten, 1999).

Food biotechnology and bioprocess is an extremely dynamic research area and is constantly developing. Researches are developing new and safe application areas at all stages before food processing, storage and consumption (Karasu, 2015). Inhibitors used to prevent browning is limited due to the effectiveness of the inhibition, as well as the reasons such as economic feasibility, off-flavors/odors, and food safety (ESKIN, 1971.; McEvily, Iyengar, & Otwell, 1992; Sapers, 1993). So, it is interesting to find additional natural, safe, and effective anti-browning agent(s) to prevent browning reactions in vegetables and fruits. Due to natural and safe, the use of flavonoids in PPO inhibition has attracted attention in recent years (Xiong, Liu, Zhou, Zou, & Chen, 2016). Many natural compounds such as curcumin analogs (S. N. Bukhari et al., 2014), morin (Wang, Zhang, Yan, & Gong, 2014), apigenin (Xiong et al., 2016), quercetin (Chen & Kubo, 2002), resveratrol (Shin et al., 1998) have been used for inhibition of PPO enzyme in preventing enzymatic browning.

In this study, it was aimed to investigate the inhibition effect of phloridzin, phloretin, baicalin, and baicalein phenolic compounds and a thiol compound benzenethiol on potato PPO. Baicalein and its analogue baicalin are a flavone, a type of flavonoid (Wei et al., 2015) and baicalein is aglycon of baicalin. Phloridzin and its analogue phloretin are a dihydrochalcone. Dihydrochalcones are the bicyclic flavonoid family. Phloretin is an aglycon of phloridzin (Ehrenkranz, Lewis, Kahn, & Roth, 2005). Benzenethiol is an organosulfur compound containing a sulfhydryl group (-SH) covalently bonded to an aromatic ring (Saboury, Zolghadri, Haghbeen, & Moosavi-Movahedi, 2006).

In this study, PPO was purified from potato by Sepharose 4B L-tyr-p-ABA column. Enzyme purity was checked with SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis). The inhibitory potency and IC_{50} values of phloridzin, phloretin, baicalin, baicalein, and benzenethiol on potato PPO activity were measured. In addition, the binding constant (K_i) and inhibition type of these inhibitors were determined.

2. Material and Method

2.1. Materials

Potatoes (*Solanum tuberosum L.*) were purchased from local market. Phloridzin, phloretin, baicalin, and baicalein were obtained from Sigma-Aldrich.

2.2. Preparation of Homogenates

Potatoes were grated and mashed under liquid nitrogen for 10 minutes. The mashed potato was homogenized with 0.1 M (pH 6.0) acetate buffer solutions that contain 0.5% PEG (polyethylene glycol), 10 mM ascorbic acid, 1 mL of Triton X-100. Four layers of cheesecloth were used to filter the homogenate. After filtration, it was centrifuged at 15000xg for 35 min and the supernatant was used in subsequent studies.

2.3. Affinity Chromatography

Sepharose 4B L-tyr-p-ABA affinity gel was used to purify the PPO enzyme from potato. This gel has been synthesized many times according to the method synthesized by Arslan et al (Arslan, Erzengin, Sinan, & Ozensoy, 2004) and used in our previous studies (Aksoy, 2020). The affinity gel was equilibrated with 0.05 M pH 6.0 PBS buffer and the supernatant was applied to affinity gel. The 0.05 M pH 6.0 PBS buffer was used for washing the affinity gel. 0.1 M pH 8.5 Tris/HCl buffer-1 M KCI solution was used to elute the PPO enzyme from the column.

2.4. Determination of Protein Amount

Bradford method (Bradford, 1976) was used for determination of protein content. The standard graph was drawn using bovine serum albumin.

2.5. Determination of PPO Activity

The absorbance increase at 420 nm was recorded during conversion of the phenolic substrate (catechol) to quinones for

measurement of potato PPO activity (Flurkey, 1986). A 0.001 increase in absorbance was defined as one enzyme unit.

2.6. Determination of Enzyme Purity

SDS–PAGE was used for determination of enzyme purity using Laemmli method (Laemmli, 1970) according to previous studies (Türkeş, Demir, & Beydemir, 2020). Silver staining was used to display the protein bands. The resulting pattern was photographed.

2.7. Determination of Inhibition Parameters

The effects of baicalin, baicalein, phloridzin, phloretin and benzenethiol inhibitors on PPO activity were investigated. Enzyme activity was measured at a fixed substrate (catechol) and at least five different inhibitor concentrations to determine the inhibitor concentration (IC_{50}) that halves the enzyme activity.

Lineweaver-Burk plots were drawn at three different concentrations of each inhibitor using five different substrat concentrations from 100 mM stock catechol solution to determine K_i (dissociation constant) values and type of inhibition.

Table 1. The purification profile summary of the potato PPO

Purification steps	Total volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Spesific activity (EU/mg)	Yield (%)	Purification fold
Homogenate	6	7200	14.14	84.84	43200	509.19	100	1
Sepharose 4B L-tyr-p-ABA affinity chromotography	1.5	2200	0.31	0.465	3300	7096.77	7.6	13.94

3. Results and Discussion

Potato (*Solanum tuberosum*) is one of the most consumed vegetables in the world, so it is of great commercial importance. Due to the presence of PPO enzyme in potato, enzymatic browning is very common. In this study, purification of the PPO enzyme from potato was performed in one step via affinity chromatography (Sepharose 4B L-tyr-p-ABA). The purification profile summary of the PPO was given in Table 1.

Purity of the potato PPO enzyme checked with SDS-PAGE. It was observed single band at electrophoresis photograph (Figure 1).

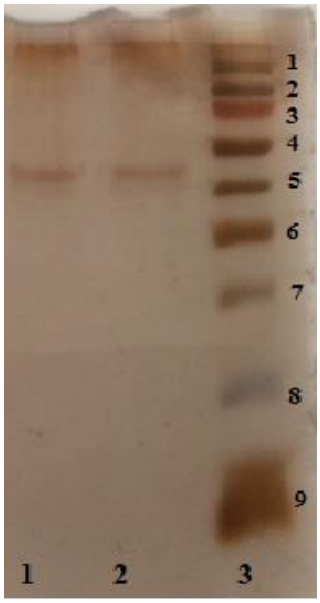

Figure 1. Sodium dodecyl sulfate (SDS)-PAGE: Lane 1 and 2, purified enzyme from Sepharose 4B L-tyr-p-ABA column, Lane 3 indicates marker (**1**: 180000 Da, **2:** 130000 kDa, **3:** 95000 Da, **4:** 72000 Da, **5:** 55000 Da, **6:** 43000 Da, **7:** 38000 Da, **8:** 26000 Da **9:** 10000 Da)

Physical and mechanical processes during storage and post-harvest processing cause enzymatic browning in fruits and vegetables. This event is a result of the reaction catalyzed by PPO (Mayer, 2006). This is undesirable since it causes nutritional losses and commercial damage to fruits and vegetables. One way to prevent enzymatic browning is to inhibit the PPO enzyme. These inhibitors should be selected from natural products that will not harm human health (Loizzo, Tundis, & Menichini, 2012). Phenolic compounds more attention as natural compounds that can be used in enzyme inhibition. Some polyphenols can affect their activity by binding to protein through the hydrogen bond, thanks to their hydroxyl groups. These groups in polyphenols have been suggested to perform nucleophilic attack on copper ions of the active site of PPO, which may then lead to inhibition of PPO (Xiong, Liu, Zhou, Zou, & Chen, 2016). In this study, the inhibition effect of phloridzin, phloretin, baicalin, baicalein natural compound was also investigated on potato PPO. The IC_{50} values of phloridzin and phloretin were determined to be 0.22 and 0.02 mM, respectively. Baicalin and baicalein did not show any inhibition effect for PPO. The inhibition types and K_i constants of phloridzin and phloretin were reached from the Lineweaver-Burk graphs (Fig. 2). Non-competitive inhibition was found for phloridzin and phloretin, and the K_i constants were 0.120±0.0157 and 0.027±0.0054 mM, respectively. In my previous study, the inhibition of potato PPO of curcumin and quercetin natural compounds was investigated and the IC_{50} value is 0.018mM for curcumin and 0.029mM for quercetin (Aksoy, 2020). In one study, the inhibition of synthetic curcumin compounds into the mushroom tyrosinase enzyme was investigated and it was found that most of the compounds inhibit the enzyme below 100 µM (Bukhari et al., 2014). In the study in which the effect of quercetin on mushroom tyrosinase activity was examined, IC_{50} value was found to be $(3.08 \pm 0.74)x10^{-5}$ M for diphenolase activity (Fan,

Zhang, Hu, Xu, & Gong, 2017). Ascorbic acid is known to be an effective inhibitor of PPO. It has been demonstrated in several studies that ascorbic acid inhibits potato PPO by 30% at a concentration of 0.7 mM and 80% at a concentration of 2.5 mM (Lourenco, Neves, & Dasilva, 1992). In addition, 14% inhibition was observed at a concentration of ascorbic acid of 0.7 mM for potato PPO using catechol as substrate. (Duangmal & Apenten, 1999).

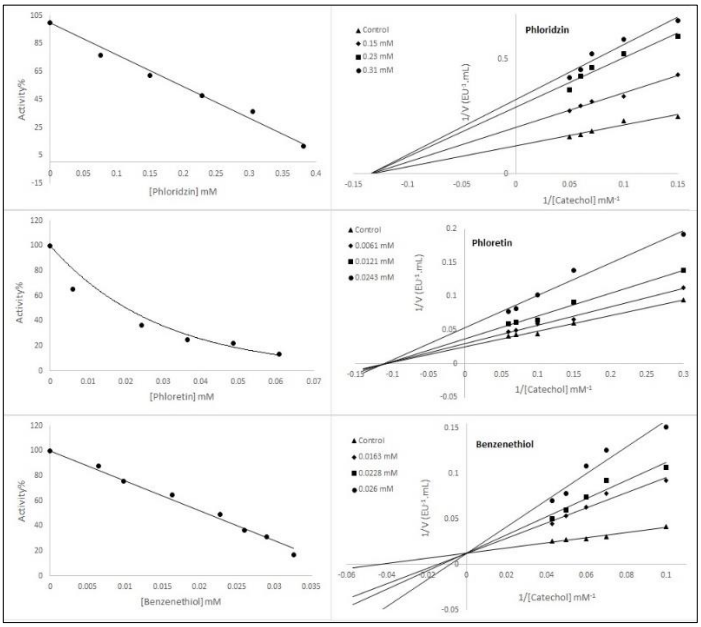

Figure 2. Activity% - [Inhibitor] and Lineweaver-Burk graphs that drawn to determine inhibition parameters

Until now, the inhibition effect of thiol groups on PPO enzymes from different source has been extensively studied (Bravo & Osorio, 2016; Duangmal & Apenten, 1999; Gonzalez, de Ancos, & Cano, 1999; Nagai & Suzuki, 2001; Negishi & Ozawa, 2000). One of the inhibition mechanisms of thiol groups is the copper-nitrogen bond cleavage in the active site. There are sulfhydryl groups a strong affinity for copper ions, they assume that they displace the histidine amino acid residues that are ligand to the copper ion of the active PPO region and/or remove the copper completely from the enzyme (Lerch, 1987; Martinez et al., 1986). This type of interaction causes competitive type inhibition. In this study, benzenethiol was shown competitive-type inhibition. IC_{50} value and K_i constant of benzenethiol were found 0.02 mM and 0.008±0.0014 mM, respectively. In one study, the inhibitory effect of benzenethiol on cresolase and catecholase activities of mushroom tyrosinase was investigated at two different temperatures (20 and 30ºC) and at two different pH (pH 5.3 and 6.8). Competitive-type inhibition has been found with benzenethiol for both activities of mushroom tyrosinase (Saboury, Zolghadri, Haghbeen, & Moosavi-Movahedi, 2006). In a study with atemoya fruit, the inhibition effect of sulfur compounds such as diethyl dithiocarbamate, sodium metabisulfite, sodium sulfite, β-mercaptoethanol, dithioerythritol were investigated. Diethyl dithiocarbamate, sodium metabisulfite, sodium sulfite at a concentration of 0.033 µM showed inhibition of 46.95%, 31.09% and 7.6%, respectively. β-mercaptoethanol showed 43.5% inhibition at 0.1 µM concentration and dithioerythritol showed 34.5% inhibition at 0.07 µM concentration (Chaves, Ferreira, Da Silva, & Neves, 2011). The inhibition profile of phloridzin, phloretin, baicalin, baicalein, and benzenethiol are summarized in Table 2.

Table 2. Inhibition parameters for potato PPO

Inhibitors	IC_{50} (mM)	K_i (mM)	Inhibition type
Phloridzin	0.22	0.120±0.0157	Noncompetitive
Phloretin	0.02	0.027±0.0054	Noncompetitive
Baicalin	NI*		
Baicalein	NI*		
Benzenethiol	0.02	0.008±0.0014	Competitive

*NI: No Inhibiton

4. Conclusions and Recommendations

The use of reliable anti-browning agents is essential in the food industry. According to the results of the *in vitro* inhibition studies, it can be said that phloridzin, phloretin, and benzenethiol are the inhibitors of potato PPO. Therefore, the use of these inhibitors as an antibrowning agent in the food industry is of great importance.

5. Acknowledge

The author report no declarations of interest.

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