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Purification and deodorizing activity against methyl mercaptan of polyphenol oxidase from lettuce (*Lactuca sativa* L.)

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Abstract−Polyphenol oxidase (PPO) from lettuce was isolated to determine the effect of its involvement in deodorizing activity by measuring the reduction in the amount of methyl mercaptan. The enzyme and deodorizing activities of PPO from lettuce were evaluated using different substrates, temperatures, and pH levels. The molecular weight of purified PPO from lettuce was estimated to be 56 kDa. The PPO and deodorizing activities were highest at the pH range of 6.0 at 20 ℃ and 5.0 at 10 ℃, respectively. The deodorizing and PPO activities were greater for *ortho*-diphenols than monophenols and polyphenol. Especially, (+)-catechin, one of *ortho*-diphenols was shown in 100% PPO activity, and chlorogenic acid and caffeic acid eliminated a methylmercaptan odor up to 95%. The deodorizing activity was highest in Cu^{2+} solution, but ascorbic acid and sodium hydrosulfite inhibited deodorizing activity. These basic data will provide an optimum manufacturing condition for commercial products using lettuce extracts.

Keywords: Deodorizing Activity, Methyl Mercaptan, Iceberg Lettuce, Polyphenol Oxidase, Polyphenols

INTRODUCTION

Methyl mercaptan, a volatile compound containing sulfur that is produced by the degradation of sulfur compounds, has been known as a major compound causing off-flavor from foods and the industrial sites [1-4]. Moreover, it is a causative factor of bad breath in the mouth, with the amount of 90% of sulfur compounds in the mouth. Although the threshold is low (0.002 ppm), it is considered a toxic compound [5-8]. It is produced by the proteolitic enzymatic modification of sulfur-containing amino acids such as cysteine and methionine [9]. In daily life, there are desquamated epithelial cells, food debris, bacteria and salivary protein, which provide all the elements for production of volatile sulfide compounds causing halitosis in the human mouth [10]. Kim et al. [11] also noted that there is a highly positive correlation between the concentration of methyl mercaptan and the generation of halitosis.

There has been a growing interest in investigating natural plant sources as efficacious and safe substances for human oral care. Several studies for reducing the amount of sulfur volatile compounds in the mouth are ongoing. According to the results, some natural sources such as mushrooms [12,13], rosemary [14], green tea [15], and algae [16,17] have a deodorizing activity by reducing sulfur compounds. Kita et al. [16] reported that deodorizing activity is generated by the chemical interaction between derivatives of phenol compounds and sulfur compounds. Phenolic compounds are changed into *ortho*-quinone, an intermediate of browning compounds, when these are oxidized, that quinone binds methyl mercaptan and the methyl mercaptan is converted into non-volatile compound because of increasing weight by the quinone. Moreover, the reaction is accelerated by polyphenol oxidase (PPO; EC 1.14.18.1) and oxygen which are oxidizing cofactors [12].

PPO, widely distributed in several fruits and vegetables, is an oxidizing enzyme and that is mainly considered as decreasing the food quality [18,19], and catalyzed the oxidation reaction for producing quinone from phenolic compounds. Among several reports for PPO, there is a study that PPO was purified from mushrooms and applied for reducing activity of methyl mercaptan [12]. Negishi et al. [20] and Zeng et al. [21] also reported PPOs from Ku-ding-cha and green tea have deodorizing activity.

In literature, there are reports of the purification and characterization of PPO from lettuce [18,22]. However, it has not been investigated on deodorizing activity of lettuces PPO against methyl mercaptan. Therefore, our objective was to investigate the effect of the PPO on the deodorizing activity of lettuce extracts against methyl mercaptan.

MATERIALS AND METHODS

1. Materials

Lettuce (iceberg lettuce) was purchased from a local market of Busan in Korea, and stored at 4 ℃ until used. Methyl mercaptan used for measuring the deodorizing activity was purchased from Wako (1 μg/μL in benzene: Wako Pure Chem., Osaka, Japan). *para*-Courmaric acid, feruric acid, catechin, caffeic acid, catechol, chlorogenic acid, L-3,4-dihydroxyphenylalanine (L-DOPA) and gallic acid as substrates of PPO were purchased from Sigma Co. (St. Louis, USA). Other chemical reagents were analytical grade.

2. Purification of PPO from Lettuce

Polyphenol oxidase in lettuce was purified by a modification of Gawlik-Dziki's method [23]. Lettuce was homogenated with four-times volume of 0.1 M phosphate buffer (pH 7.4) and was filtered with cheese cloth. The filtrate was used as a lettuce extract. For PPO purification, lettuce puree with 0.1 M phosphate buffer (pH 7.4)

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Table 1. Stepwise purification results of lettuce PPO

Purification step	Total activity (unit/mL)	Total protein (mg)	Specific activity (unit/mg protein)	Purification fold	Yield (%)
Crude extract	66,700	2,200	30.3	1.0	100.0
25-75% $(NH_4)_2SO_4$	40,000	1,080	37.0	1.2	60.0
Sephacryl S-200 HR	14,500	191	69.8	2.3	21.7
DEAE sepharose	12,000	68	176.5	5.8	18.0

was centrifuged at 8,000 g at 4 ºC for 30 min (SUPRA 30-K, Han-il, Kangleung, Korea). The sediment was discarded. At that time, the upper solution was filtered through glass wool. The residue was used as the crude PPO in this study. Ammonium sulfate was added to the supernatant from 25% to 75% saturation. The protein precipitate, collected by centrifugation (5,000 g, 30 min), was dissolved in a small volume of 10 mM phosphate buffer (pH 7.4) and dialyzed against the same buffer for 24 hrs, with at least three changes of the dialyzing media (M.W.C.O.: 12,000, Sigma, St. Louis, USA). One mL of the dialyzed solution was added to a Sephacryl S-200 HR gel filtration column (2.6×100 cm; Pharmacia Biotech, Piscataway, USA). The S-200 gel was equilibrated with 0.1 M phosphate buffer, pH 7.4. The crude enzyme extract was eluted with the same buffer at a flow rate 25 mL/hr. The eluate was collected in 5 mL fraction and PPO activity was measured and elution volume was determined for each protein by the absorbance measured at 280 nm. The fractions which had PPO activity were loaded to DEAE (diethylaminoethyl) Sepharose fast flow ion-exchange column (1.6×40 cm; Pharmacia Biotech, Piscataway, USA) equilibrated with 0.1 M phosphate buffer, pH 7.4. And 0, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl solutions were eluted to the column; then the fractions which had PPO activity were collected and stored at 4 ºC. Elution volume was detected for each protein by the absorbance measured at 280 nm. Protein concentration was determined by the Lowry's method [24], using a BSA (bovine serum albumin; SIGMA, St. Louis, USA) as a standard. In chromatography, protein was expressed with the absorbance at 280 nm. Molecular weight of PPO from lettuce was estimated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was followed by Laemmli's method [25], and 5% and 7.5% of gels were used at 100 V. Marker proteins used were galactosidase, *E. coli* (M.W. 116,000), phosphorylase b, rabbit muscle (M.W. 97,400), fructose-6-phosphate kinase, rabbit muscle (M.W. 84,000) albumin, bovine (M.W. 66,000), glutamic dehydrogenase, bovine liver (M.W. 55,000) albumin, egg (M.W. 45,000) and glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (M.W. 36,000), which were purchased at Sigma Co. (St. Louis, U.S.A).

3. Measurement of PPO Activity

Activity of PPO was measured by the method of Zenin and Park [26]. Then 2.7 mL 0.01 M sodium phosphate buffer (pH 6.5) containing 10 mM catechol as the substrate solution was added to a cell and stabilized at 25 ºC in a water bath. Next, 0.3 mL enzyme reagent was added and the absorbance was measured at 420 nm on a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Piscataway, USA). One unit was defined as an increase of 0.01 in the absorbance by 1 mL of crude enzyme for a minute.

4. Measurement of Deodorizing Activity

The measurement of deodorizing activity for halitosis inhibition was by Tokita's method [27]. A standard solution of 2 mL methyl mercaptan was dissolved in 198 mL ethanol (purity: 98%) and stored at 4 ºC. The solution was diluted 10 times (1 μg/mL) with deionized water just before the halitosis inhibition was measured. The sample and 1 mL 0.2 M potassium phosphate buffer (PPB) were put into a 30 mL vial. Each solution was adjusted to pH 7.5. One mL of the methyl mercaptan standard solution (1 μg/mL) was added into this solution and promptly capped with a silicone cap. Each vial was vortexed for 5 seconds and incubated at 37 ºC for 6 min. The amount of methyl mercaptan in headspace of the sample vial was detected by a gas chromatography (GC; Hewlett Packard 5890, series II, Agilent Co., Palo Alto, USA) using a flame photometric detector. The column was an HP-1 (5 m×0.53 mm×2.65 μm, Supelco Co., Bellefonte, USA). The temperature program was set for 35 ºC column temperature, 150 ºC injector temperature and 200 ºC detector temperature. The carrier gas, helium, was set at a flow rate of 40 mL/min. The deodorizing activity was calculated from the peak area of methyl mercaptan before and after addition of lettuce extracts. All experimental values are obtained by triplicate.

RESULTS AND DISCUSSION

1. Purification of PPO from Lettuce

PPO was purified from lettuces as described in the methods, and the stepwise purification results are summarized in Table 1. The ammonium sulfate precipitate obtained from 25 to 75% saturation of the crude extract exhibited PPO activity. The precipitate was eluted by Sephacryl S-200 HR column chromatography. Two protein bands appeared by the column chromatography and the PPO activity was

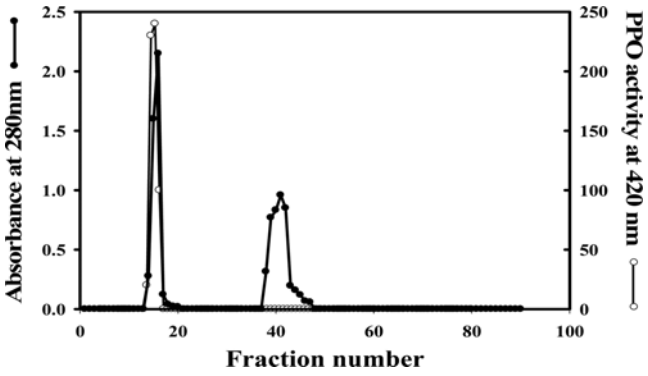

Fig. 1. Elution profile of proteins and PPO activity from lettuces on Sephacryl S-200 HR. The Sephacryl S-200 HR gel was equilibrated with 0.1 M phosphate buffer, pH 7.4. The crude enzyme extract was eluted with the same buffer at a flow rate 25 mL/hr. The eluate was collected in 5 mL fraction until total 90 collections. Fractions for PPO activity and protein were measured at 420 nm and 280 nm, respectively.

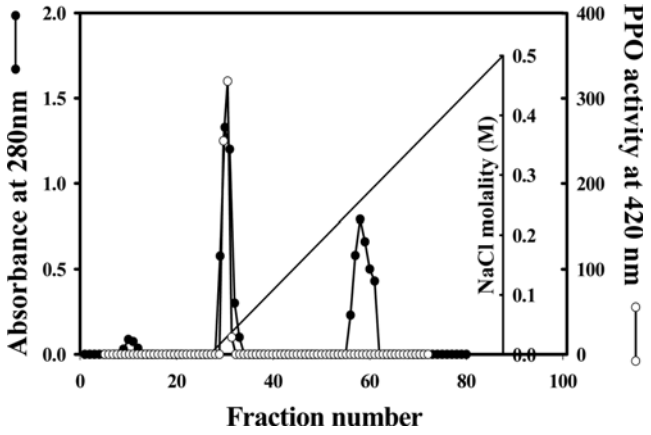

Fig. 2. Elution profile of proteins and PPO activity from lettuces on DEAE sepharose. The eluate showing the PPO activity from Sephacryl S-200 HR gel was loaded to DEAE Sepharose ion exchange chromatography with the gradients of NaCl concentrations. Ionic strength of PPO was 0.1M by the gradient of NaCl concentrations. Fractions for PPO activity and protein were measured at 420 nm and 280 nm, respectively.

shown in the first peak (Fig. 1). The eluate appearing the PPO activity was loaded to DEAE Sepharose ion exchange chromatography with the gradients of NaCl concentrations. Three peaks appeared by ion chromatography and the second peak had PPO activity. Ionic strength of PPO was 0.1 M by the gradient of NaCl concentrations (Fig. 2). The yield was 18% and specific activity was 176.5 unit/mg; Soledad et al. [28] reported that two protein peaks were identified by the gel chromatography and three protein peaks were isolated by the ion chromatography from the PPO of lettuce. The molecular weight of PPO purified by the gel chromatography and the ion exchange chromatography was measured by 7.5% polyacrylamide gel electrophoresis (Fig. 3). The molecular weight of PPO purified from lettuce was estimated to be 56 kDa. Lane 1 contained molecular mass markers of galactosidase (116 kDa), phosphorylase b (97.4 kDa), fructose-6-phosphate kinase (84 kDa), albumin, bovine (66 kDa), glutamic dehydrogenase (55 kDa) albumin, egg (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa). Lane 2 arrows indicate the PPO position (around 56 kDa). Ascorbic acid, sodium hydrosulfate and citric acid were used as inhibitors. The final concentrations of 10 mM were evaluated for deodorizing activity and PPO activity.

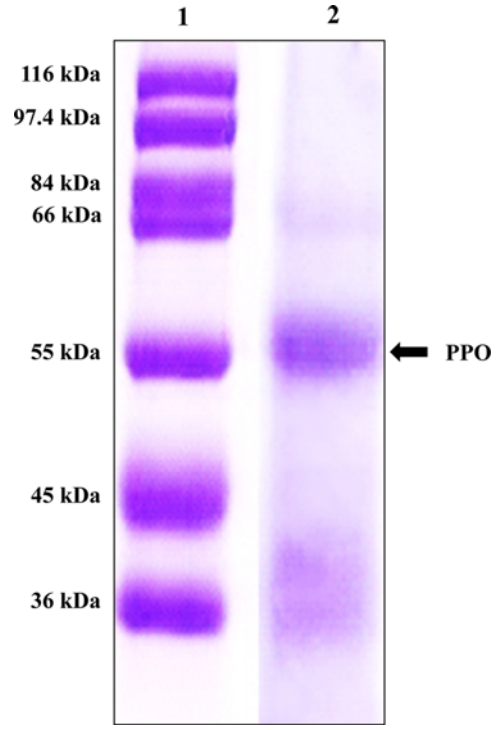

Fig. 3. SDS-PAGE of PPO purified from lettuce. SDS-PAGE was developed by 5% of stacking gel and 7.5% of separating gels at 100 V. Lane 1 was marker proteins which galactosidase (116 kDa), phosphorylase b (97.4 kDa), fructose-6-phosphate kinase (84 kDa) albumin, bovine (66 kDa), glutamic dehydrogenase (55 kDa) albumin, egg (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa), respectively. Lane 2 arrows indicate the PPO position (around 56 kDa).

2. Effects of pH and Temperature on Enzymatic Activity and Deodorizing Activity of PPO

The enzymatic activity and deodorizing activity of lettuce PPO on pH changes were measured and shown in Fig. 4. While the enzymatic activity and deodorizing activity were low at pH 3 and 4, these parameters were high at pH 5, 6 and 7, respectively. Although the enzymatic activity decreased radically at above pH 8, deodorizing activity was maintained compared to enzymatic activity. The effect of temperature on the activity of PPO is shown in Fig. 5. The effect of temperature of deodorization activity changed rapidly. The activity increased from 10 °C to 20 °C, then that was decreased by increasing temperature until 60 °C. Above 60 °C, enzymatic activity did not appear. As temperature increased, the PPO activity also increased rapidly; the highest activity appeared at 20 °C, and then rapidly decreased with only 5% activity at 60 °C.

3. Effects of Polyphenols on Enzyme Activity and Deodorizing Activity of Lettuce PPO

Seven phenolic compounds were evaluated for the effect of substrate specificities of PPO on enzymatic activity and deodorizing

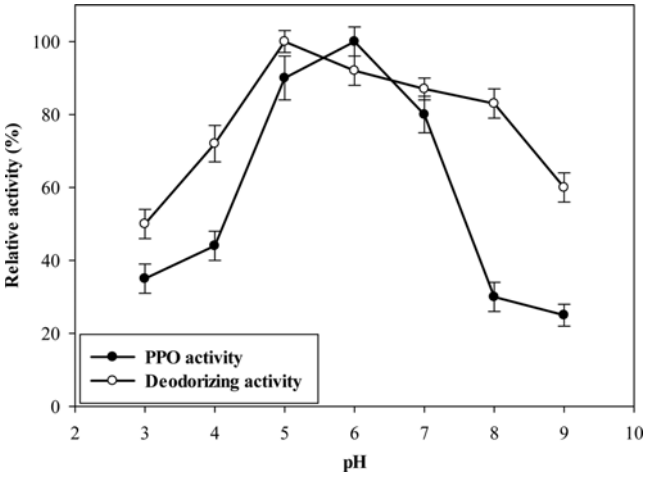

Fig. 4. Effects of pH on PPO and deodorizing activities. Buffers with different pH ranges (3 to 9) were tested and this range was determined by optimal pH range of PPO. All assays were performed at 37 °C.

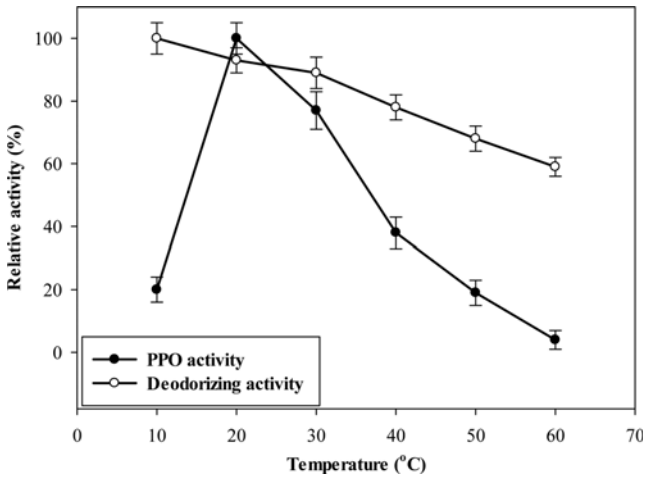

Fig. 5. Effects of temperature on PPO and deodorizing activities. The temperature range (10-60 °C) for the assay was determined by optimal temperature range of PPO.

Table 2. PPO and deodorizing activities on various polyphenol compounds from lettuce

Substrate (10 mM)	Enzymatic activity[a] (%)	Deodorizing activity (%)
Monophenols		
para-Coumaric acid	16±2	15±3
Ferulic acid	21±4	5±1
ortho-Diphenols		
Chlorogenic acid	56±5	95±3
Caffeic acid	67±4	95±4
L-DOPA[b]	42±2	80±3
(+)-Catechin	100±4	56±2
Polyphenol		
Gallic acid	72±3	69±5

[a]The enzymatic activity of polyphenol oxidase on catechol was set to 100

[b]L-DOPA stands for L-3,4-dihydroxyphenylalanine

activity: monophenols of *para*-coumaric acid and ferulic acid, *ortho*-diphenols of chlorogenic acid, caffeic acid, L-DOPA and (+)-catechin and a polyphenol of gallic acid (Table 2). Among the tested substrates, chlorogenic acid and caffeic acid most effectively exhibited deodorizing activities by 95%. *Ortho*-diphenols and polyphenols were determined to be good substrates for PPO as shown by high activities in catechin and gallic acid, which high enzymatic activities for PPO. Identical activities against monophenols lowered not only enzymatic activity but also deodorizing activity.

4. Effect on Metals and Reducing Agents on Enzymatic Activity and Deodorizing Activity of Lettuce PPO

The final concentration of 0.01 M for *para*-courmaric acid, feruric acid, (+)-catechin, L-DOPA, chlorogenic acid, caffeic acid and gallic acid, 0.01 M was used to monitor deodorizing activity and PPO activity in the optimum pH (6.5) and temperature (35 °C). Fe^{2+}, Fe^{3+}, Cu^{2+} and Ca^{2+} were used for catalytic factors. Deodorizing activity and PPO activity as functions of pH and temperature were determined using 0.1 M phosphate buffer (pH 4-9) at 10 °C-60 °C.

Table 3. Effects of metal ions and reducing agents on PPO and deodorizing activities

Compounds	Relative activity (%)	
	Enzymatic activity[a] (%)	Deodorizing activity
Metal ions		
Fe^{2+}	51±3	73±3
Fe^{3+}	84±4	68±4
Cu^{2+}	88±5	100±4
Ca^{2+}	100±3	65±2
Reducing agents		
Ascorbic acid	45±2	25±3
Sodium hyposulfite	41±3	23±2
Citric acid	44±3	58±2

[a]The enzymatic activity of PPO on catechol was set to 100

Various metals and inhibitors were examined to determine their potentials for catalysis and inhibition of PPO activity and their results are shown in Table 3. The concentrations of metal ion mixtures were adjusted to 10 mM and reacted with lettuce PPO for the measurement of the effect on metal ions against to the PPO. The highest enzyme activity appeared in Ca^{2+} solution, and the activity in Fe^{2+} solution was lower compared with the others.

For application of enzymatic deodorization with lettuce PPO for removal of amount of methyl mercaptan that caused bad odors, the effect of methyl mercaptan capturing property by chromatography with adding lettuce PPO was studied. This study demonstrated that, after the oxidation of phenolic compounds by lettuce PPO, elimination of methyl mercaptan appeared and PPO from lettuce strongly repressed the bad smell on methyl mercaptan.

The molecular weight of PPO purified by chromatographic approaches was determined as 56 kDa. Fujita et al. [29], using gel filtration, also reported that lettuce PPO had a molecular weight of 56 kDa. Moreover, Soledad et al. [28] and Gawlik-Dziki et al. [23] also noted that the molecular weights of PPOs purified from iceberg lettuce and butter lettuce were estimated as about 60 kDa. This slight difference between molecular weights of PPOs is likely to be the difference caused by lettuce cultivars. In literature, the molecular weights of PPO purified from other plant sources such as apple [30], banana [31] and Cauliflower [32] were estimated to be about 65, 41, and 60 kDa, respectively. By these results, it was thought that the molecular weights of fruit and plant PPOs were in a range of from 41 to 65 kDa. Gawlik-Dziki et al. [23] also mentioned the different sizes of PPO from different sources that indicate the molecular weight of PPO ranges from 45 to 67 kDa, because PPO has been synthesized as a 60 to 65 kDa protein, which can converted to a 40 to 45 form by proteolysis. Based on the result in this study and literature, the purified homogeneous protein was PPO, suggesting that the common PPO in plant sources have a single polypeptide chain.

In literature, iceberg lettuce PPO had the highest enzyme activity at pH 5 [33]. Heimdal et al. [34] reported that a broad pH optimum of lettuce was found in pH 5-8. The highest pH of deodorizing activity of lettuce PPO was 5. The enzymatic activity decreased rapidly above pH 7, whereas deodorizing activity did slowly to pH 8. Weemaes

et al. [35] reported the results that optimal pH of apples and grapes showed at 6, and that of avocados and plums was 7. Yasuda and Arakawa [36] reported that halitosis inhibition by catechin from green tea was suppressed below pH 5.0. Enzymatic activity of PPO in this study was also highest at pH 5 to 6, and agreed with the literature of PPO in lettuces. The deodorizing activity and PPO activity showed similar with the pH change.

Moreover, the PPO activity increased from 10 °C to 20 °C and deodorizing activity showed at 10 °C decreasing activity as the increasing temperature. This range from 10 °C to 20 °C showed relatively high deodorizing activity even though PPO activity was lowered. It is maybe due to the low volatility of methyl mercaptan at the low temperature. Above 20 °C the deodorizing activity and PPO activity showed a similar pattern. This result demonstrated the deodorizing activity was affecting PPO activity. Wissemann and Lee [37] reported that the optimum temperature of PPO from grape was 25 °C. Therefore, the optimum temperature for PPO from lettuce is lower than that of grapes. In literature, PPOs from plants showed the highest activity in the range of 20-30 °C [31,38-41]. The effect of temperature on the deodorizing activity of PPO was different with the effect on the enzymatic activity of PPO. The highest value for deodorizing was shown at 10 °C in this study. As temperature increased, the deodorizing activity decreased slowly. It was thought that deodorizing activity was not largely affected to the temperature change.

For the substrate specificity test of PPO in this study, catechin and chlorogenic acid and caffeic acid most effectively exhibited the enzymatic and deodorizing activities, respectively. Seol et al. [42] reported that PPO from ginkgo leaves showed the highest enzymatic activity in gallic acid as substrates of polyphenols. Moreover, Seo et al. [43] also reported that polyphenols were good substrates against PPOs from arrow root and pear. On the other hand, Walker and Hulme [44] reported that chlorogenic acid is a good substrate for the browning reaction by PPO in apples. Kim et al. [17] isolated three compounds, eckol, dixinodehydro eckol, and dieckol from Eiseniabicyclis, and reported that dieckol showed the highest activity among them. By the result, it was thought that the high PPO activity against to the chlorogenic acid of lettuce was related to high deodorizing activity in this study. The relationship of enzymatic activity and deodorizing activity did not appear on the substrate specificity.

On the effect of metal ions against PPO and deodorizing activities, Chung et al. [45] reported that apple PPO had high activity in the condition of existence of Cu^{2+}, Mn^{2+} and Fe^{3+}, respectively. Kim et al. [46] also reported that garlic PPO showed activity in the low concentration (0.1-1.0 mM) of Cu^{2+}. Nevertheless, the PPO activity was lower than that of the high concentration (10 mM). In this study, Ca^{2+} and Cu^{2+} increased the activity of lettuce PPO because Ca^{2+} and Cu^{2+} reacted as the cofactor of polyphenol oxidase. The effects of three inhibitors, namely ascorbic acid, sodium hydrosulfite and citric acid, were examined to determine their potential for inhibition of catechol oxidation by the lettuce PPO. Ascorbic acid and sodium hydrosulfite inhibited the deodorizing activity to 25% and 23%, respectively.

This study affirmed that enzymatic activity could be not only applied to the inhibition effected by lettuce, but also other enzymes purified from natural materials such as vegetables, fruits, and fungi could be applied by using this mechanism. Therefore, additional studies are needed to investigate the deodorizing mechanism between PPOs from other natural materials.

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