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Purification and Characterization of Polyphenol Oxidase in the Flesh of the *Fuji* Apple

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Abstract Polyphenol oxidase (PPO) was isolated from the flesh of *Fuji* apples by DEAE-Cellulose, ammonium sulfate precipitation, phenyl-Sepharose CL-4B, and Sephdex G-100 chromatography. The molecular mass of the purified PPO was estimated to be 40 kDa by SDS polyacrylamide gel electrophoresis. With regard to substrate specificity, maximum activity was achieved with chlorogenic acid as substrate, followed by catechin and catechol whereas, there was no detectable activity with hydroquinic acid, resorcinol, or tyrosine as substrate. The optimum pH and temperature with catechol as substrate were 6.5 and 35°C, respectively. The enzyme was most stable at pH 6.0 and unstable at acidic pH. The enzyme was stable when it was heated to 45°C but heating at 50°C for more than 30 min caused 50% loss of activity. Reduced $ZnSO_4$, L-cystein, epigallocatechin-3-o-gallate (EGCG), and gallocatechin gallate (GCG) also inhibited activity.

Keywords: polyphenoloxidase, *Fuji* apple, purification, SDS-PAGE, chromatography

Introduction

Enzymatic browning of fruit is a well-known phenomenon caused by the oxidation of phenolic compounds into quinones (1, 2). This reaction is mainly catalyzed by polyphenol oxidase (PPO, EC 1.14.18.1) in the presence of oxygen (3) and gives rise to a brown pigmentation. This brown discoloration leads to organoleptic and nutritional modifications in the plant tissues, thus causing unfavorable quality changes in the food products (4, 5). The biochemical characteristics of PPO from some apple cultivars have been investigated by several researchers. Harel *et al.* (6) found four isoenzymes of PPO from Grand Alexander apple tissues; three of them in the chloroplasts and one in the mitochondria.

Previous studies have indicated that, although the optimum pH for the activity of PPO extracted from apple mitochondria is around 7, the PPO of the whole apple tissue has a maximum activity at a pH of between 4.5 and 5.5 (7-10). Janovitz-Klapp *et al.* (8) obtained 120-fold purified PPO by ammonium sulfate precipitation and phenyl-Sepharose CL-4B chromatography from *Delicious* apples. Zhou *et al.* (10) obtained 161-fold purified PPO by anion-exchange, dialysis, ammonium sulfate precipitation and phenyl-Sepharose CL-4B chromatography from *Monroe* apple peels.

Although many different types of apples have been investigated in terms of the polyphenol oxidase, the Korean *Fuji* has not been studied yet. The characterization of this enzyme is necessary for both developing a more effective means of controlling enzymatic browning and in better understanding the browning mechanism under specific storage conditions. Such information is important for the post-harvest handling of *Fuji* and, in particular, for minimal processing.

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The aim of this paper was the detection and the kinetic characterization of the PPO in the *Fuji* apple. The enzyme was extracted by using anion-exchange and dialysis, and then purified by the use of ammonium sulfate fractionation, hydrophobic chromatography and Sephadex chromatography. This purified enzyme fraction was then subjected the molecular properties of PPO from fleshed *Fuji* apple, some of the characteristics such as optimum pH and temperature, pH stability, thermal stability, substrate specificity, and Km value, inhibitor, and metal ion effects.

Materials and Methods

Plant materials The *Fuji* apples used in this study were grown at Youngju, Gyeongbuk Province, Korea. They were harvested on November 10 and stored at 1°C in air until used.

Reagents Substrates and Triton X-100, phenylmethane-sulfonyl fluoride (PMSF), DEAE-Cellulose, protinin, amonium sulfate, and phenyl-Sepharose CL-4B were purchased from the Sigma Chemical Co. (St, Louis, MO, USA). All other reagents were analytical grade.

Enzyme extraction The extraction solution was composed of 240 mL of a 0.1 M sodium phosphate buffer (pH 6.5) containing 2% (v/v) Trition X-100 and 1 mM PMSF. Fifty grams (50 g) of hydrated DEAE-Cellulose resin was suspended in the extraction solution as described by Zhou *et al.* (10). The anion-exchange resin was pretreated and equilibrated overnight in a sodium phosphate buffer containing 2% Triton and 1 mM PMSF. The flesh of the apple was lyophilized and ground in a waring blender. Thirty grams (30 g) of the powder was extracted with the extraction solution and suspended in resin for 60 min at 4 °C while stirring gently with a magnetic stirrer. The extract was filtered through glass wool. The filtrate was centrifuged at 15,000×g for 30 min at 4°C, and the super-

natant was collected. After the addition of 0.33 mL of aprotinin/100 mL of supernatant, the crude enzyme was kept at 4°C.

Purification procedures The crude enzyme extract was dialyzed overnight against a 1.0 mM phosphate buffer (pH 6.0). The dialyzed enzyme solution was fractionated with solid ammonium sulfate (30-80% saturation) and the precipitate was collected by centrifugation at 12,500×g for 30 min. The precipitate was redissolved in 6.0 mL of a 0.05 M sodium phosphate buffer (pH 6.5) containing 1.0 M ammonium sulfate and dialyzed overnight against the same buffer. After centrifugation at 15,000×g for 30 min, the total volume of the supernatant was applied to a phenyl-Sepharose CL-4B column (1.0×8.0 cm) previously equilibrated at 5°C with buffer A (0.05 M sodium phosphate buffer, pH 6.5, containing 1.0 M ammonium sulfate and 1.0 M KCl). The PPO was eluted with a stepwise gradient of 1.0, 0.8, 0.5, 0.05 to 0 M buffer A in a 0.05 M sodium phosphate buffer (pH 6.5), followed by 50% ethylene glycol and water. The flow rate was 1.0 mL/min, and of 2.5 mL fractions were collected. Active fractions were pooled, dialyzed against a 0.05 M phosphate buffer (pH 6.5), and concentrated using Microcon YM-10 ultrafiltration cells (Amicon; Millipore, Bedford, MA, USA). The most active fractions were centrifuged at 10,000×g for 10 min at 4°C and loaded on to a Sephardex G-100 gel permeation column (1.6×65 cm) equilibrated with a 0.05 M phosphate buffer (pH 6.5). Active fractions were pooled and concentrated using a Microcon YM-10 ultrafiltration cell. All procedures were done at 4-8°C unless otherwise noted.

Polyacrylamide gel electrophoresis and molecular mass determination SDS-PAGE was performed according to the method of Laemmli (11) using 10% polyacrylamide gels. Protein bands were stained with Coomassie brilliant blue R-250. The molecular mass of the purified enzyme was determined by SDS-PAGE. SDS-PAGE was done by using myosin, β-galactosidase, phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, and aprotinin (Mw equal to 200.00, 116.25, 97.40, 66.20, 45.00, 31.00, 21.50, 14.40, and 6.50 kDa, respectively) as molecular mass marker proteins under denaturing conditions.

Protein determination The amount of protein was determined according to the method of Bradford (12) by using bovine serum albumin as a standard. In order to monitor proteins in the eluted buffer, absorbance at 280 nm was determined.

Enzyme assay PPO activity was assayed with catechol as a substrate according to the spectrophotometric procedure (13). The assay was performed using 0.2 mL of enzyme solution and 2.8 mL of a 0.05 M catechol in a 0.05 M citrate -0.1 M sodium phosphate buffer (pH 5.0). The increase in absorbance at 420 nm at 25°C was measured on a spectrophotometer (V-670; JASCO Co., Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme which caused a change in the absorbance of 0.001/min.

pH optimum and stability The three kinds of buffer solutions used in this study were: 0.1 M citric acid -0.2 M disodium phosphate at pH 3-8; tris (hydroxymethyl) aminomethane at pH 8.5; and 0.1 M bicarbonate buffer at pH 9-10. In order to determine the effect of pH on PPO activity, 0.2 mL of a 0.5 M catechol solution (prepared in 0.1 M citrate - 0.2 M sodium phosphate buffer, pH 5.0) was added to 2.7 mL of various buffer solutions followed by the addition of 0.1 mL of partially purified enzyme solution. Enzyme activity was measured at 420 nm by using the spectrophotometric procedure as previously described.

To determine the effect of pH on PPO stability, 0.2 mL of the enzyme solution was incubated in a 0.4 mL solution of various buffers ranging from pH 2.5 to 10 for 30 min at 25°C. Residual PPO activity was assayed by mixing 2.7 mL of 0.05 M catechol in a 0.05 M citrate - 0.1 M sodium phosphate buffer (pH 5.0) with 0.3 mL of the incubated PPO solution.

Optimum temperature and stability The optimum temperature for PPO activity was determined by adding 0.1 mL of a partially purified enzyme solution to 0.2 mL of 0.5 M catechol solution (prepared in 0.05 M citrate -0.1 M sodium phosphate buffer, pH 5.0) and 2.7 mL of 0.05 M citrate - 0.1 M sodium phosphate buffer (pH 5.0). The substrate and buffer were incubated for 5 min at various temperatures from 10 to 80°C prior to the addition of the enzyme solution. Spectrophotometric measurement for 5 min was carried out at 25°C. For thermal stability studies, 0.1 mL of enzyme solution with 2.7 mL of 0.05 M citrate - 0.1 M sodium phosphate buffer (pH 5.0) was incubated at various temperatures for 30 min, rapidly cooled in an ice bath for 5 min, and then removed to 25°C. When the mixture reached room temperature (25°C), 0.2 mL of 0.5 M catechol was added and the residual enzyme activity assayed and quantified relative to the maximum activity.

Determination of substrate specificity and kinetic parameters The phenols used were catechol, chlorogenic acid, (+)-catechin, dihydroxy phenylalanine (L-dopa), hydroquinone, pyrogallol, resorcinol, and tyrosine. The Michaelis-Menten constant (Km) was determined with various substrate concentrations in a standard reaction mixture, with a maximum absorption wavelength for the correspondent chromophore. The reaction system consisted of a 0.1 mL of enzyme solution and 2.9 mL of various substrate solutions prepared in 0.05 M citrate - 0.1 M sodium phosphate buffer (pH 5.0). The increase in absorbance of the optimum wavelength for each substrate was measured. For each substrate, data were plotted as 1/activity vs 1/substrate concentration. Km was determined as the reciprocal absolute values of the intercepts on the X- and Y-axes of the linear regression curve (14).

Effect of inhibitors To determine the effect of inhibitors on enzyme activity, 2.7 mL of a 10 mM catechol in 0.05 M citrate - 0.1 M sodium phosphate (pH 5.0), and 0.2 mL of inhibitor solution of different concentration amounts were mixed immediately before the addition of a 0.1 mL enzyme solution. Relative enzyme activity was determined from the slope of the reaction curve following any delay in

Table 1. Summary of the extraction and purification of PPO from the flesh of a *Fuji* apple

Purification step	Total Activity (units)	Specific activity (units/mg)	Total protein (mg)	Purification fold	Yield (%)
Crude extract	342400	53.11	6447.4	1.0	100
30-80% $(NH_4)_2SO_4$	75280	465.84	161.6	8.77	21.98
Phenyl-Sepharose CL-4B	11865.6	5742.16	2.0664	108.12	3.46
Sephadex G-100	11140.2	6289.63	1.7712	118.43	3.25

the change of absorbance at 420 nm due to the inhibitors.

Results and Discussion

Extraction and purification of PPO A summary of PPO extraction and purification from the flesh of the *Fuji* apple is shown in Table 1. A 118-fold purification of PPO relative to protein with a yield of 3.25% was achieved. During the extraction of PPO, two main problems were found in obtaining full solubilization of the membrane-bound PPO and in avoiding the oxidation of endogenous phenolic compounds that react with the enzyme extract and cause inactivation and molecular modification. Therefore, Triton X-100 was applied for full extraction of the enzyme (10, 15). The nonionic detergent Triton X-100 has been successfully used for apple PPO extraction due to its disruption of membranes, thus facilitating the release of PPO. An increase of enzyme activity by this detergent has been reported (6, 7). The second problem arises from the simultaneous presence of quinones and their endogenous phenolic precursors in the crude extract of the enzyme. It is essential to minimize the formation of quinines, which may react with the enzyme, resulting in activity loss. Several methods have been described to prevent the reaction of phenols with PPO, including the use of phenol-binding agents such as soluble and insoluble polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP) and anion-exchange resin (10, 15).

The removal of hydrophilic proteins from the crude extract was carried out using DEAE cellulose. Although this procedure results in a partial loss of PPO activity, it ensured a higher homogeneity of the protein solution. Moreover, part of the phenol was adsorbed by the resin resulting in a consistent clearing of the crude extract. Phenyl-Sepharose CL-4B chromatography has been used for the purification of PPO from various fruits and vegetables, such as peaches (16), strawberries (17), bananas (18), and potatoes (19). PPO was eluted from the phenyl-Sepharose CL-4B column within a broad peak at 0.05 M of buffer A (see purification procedures in the Materials and Methods), and within two accompanying peaks at 0.5 and 0.0 M of buffer A in 0.05 M sodium phosphate buffer (pH 6.5). Purification of PPO was achieved by loading the major peak obtained by phenyl-Sepharose CL-4B chromatography onto a Sephadex G-100 column. A sharp peak of activity was observed in gel-filtration, and the pure enzyme was found in the core fractions of the peak (Fig. 1). The recovery of PPO activity from the chromatography was 93%. The major PPO fractions were pooled and examined for kinetic study.

PPO molecular mass determination by SDS-PAGE The molecular mass of the purified PPO was calculated to

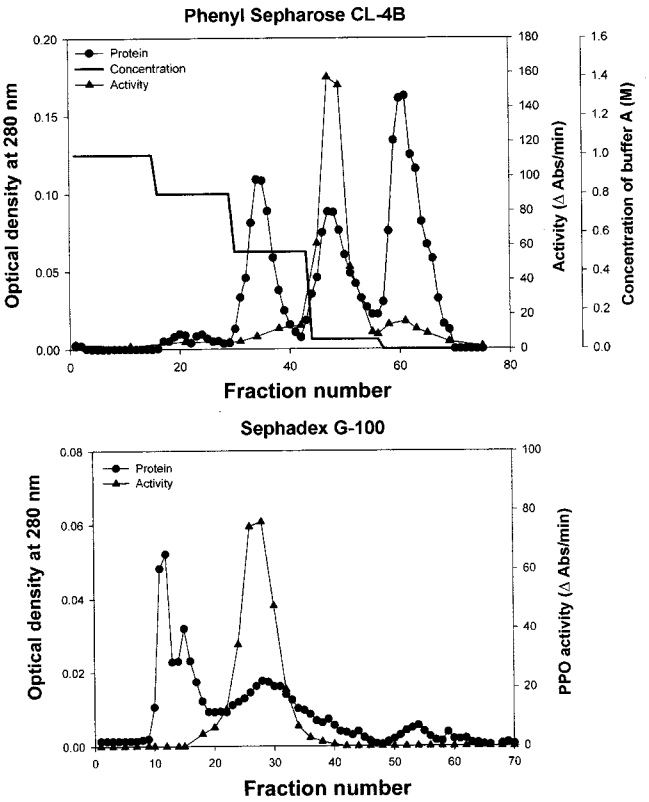

Fig. 1. Elution profiles of PPO and protein after phenyl-Sepharose CL-4B chromatography (upper) and Sephadex G-100 filtration (lower).

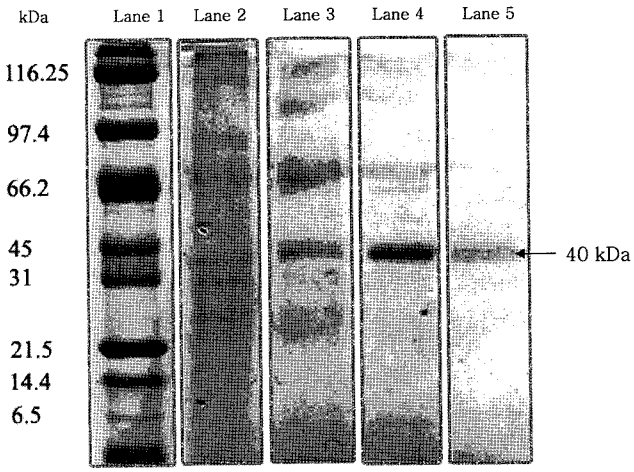

Fig. 2. SDS-PAGE of partially purified and purified PPO; standard solution (lane 1); crude extract (lane 2); ammonium sulfate precipitated solution (lane 3); active fractions eluted from phenyl Sepharose CL-4B (lane 4); purified PPO (lane 5).

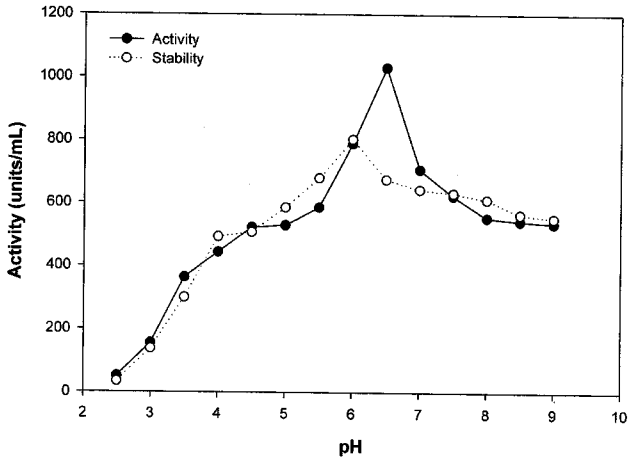

Fig. 3. Determination of the optimum pH and pH stability of PPO from the flesh of a *Fuji* apple.

be approximately 40 kDa by SDS polyacrylamide gel electrophoresis, under completely denaturing conditions (Fig. 2). The molecular weight of PPO from other species via denaturating SDS-PAGE has been reported as follows: Carrots 59 kDa (20); potatoes 69 kDa (19); apple pulp 42 kDa (21); and apples 46 kDa (8). This result indicates that the molecular weights of PPO were similar to those of apple pulp but different from those of carrots and potatoes.

pH optimum and stability The pH activity profile for the oxidation of catechol by purified PPO from the *Fuji* apple is shown in Fig. 3. The optimal pH for PPO activity was observed to be approximately 6.5 at room temperature. The pH activity curve was characterized by a rapid increase at pH 5.5 through 6.5 and a decrease between pH 6.5 and 7.0. Different pH optima of PPO with various substrates were reported to vary between 5.0 and 7.0 depending on the origin of the material. Most of the previously reported assays of apple PPO have established similar limits (2, 10). The optimal pH for PPO activity in *Starking* apples was 6.1 and for *Jonathan* apples it was 6.2 with catechol (22). Differences in pH optima with different substrates have been reported for PPO from strawberries (17) and *Rome beauty, Winesap* and *Cortland* apples (23). Harel *et al.* (6) reported that the apple PPO has two pH optima, 5.1 from chloroplast PPO and 7.3 from mitocondria PPO. Based on these reports, PPO purified from *Fuji* apples in this study appear to be from the mitochondria. The PPO was most stable at a pH of 6.0 and less stable with an acidic pH. The pH stability of PPO increased between a pH of 2.0 and 6.0 and then decreased from 7.0 to pH 8.5 (Fig. 3).

Optimum temperature and stability The PPO from the flesh of the *Fuji* apple had an optimum temperature of 35°C (Fig. 4). The enzyme activity increased with temperature from 10 to 35°C, and then decreased from 35 to 80°C. The temperature stability curve of PPO is shown in Fig. 4. The apple enzyme was stable up to 40°C and less stable at lower temperatures. The temperature stability of PPO decreased above 50°C. The relative activity of PPO decreased from

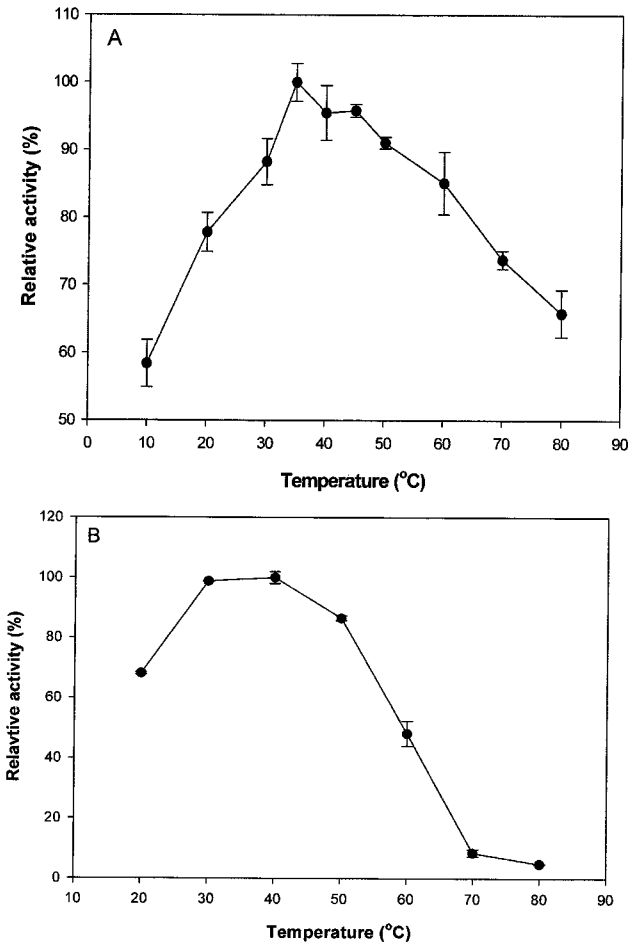

Fig. 4. The optimum temperature (A) and thermal stability (B) of PPO from the flesh of a *Fuji* apple.

100 to 40% when the temperature increased from 40 to 60 °C. In this respect, the *Fuji* apple PPO was similar to the PPO extracted from *Monroe* apple (10) and longan fruits (24).

Substrate specificity and kinetic parameters PPO activity, using different substrates, is shown in Table 2. The optimum wavelength of each substrate was measured and relative activity of PPO was calculated using catechol as the basis of comparison. The results indicated that the *Fuji* PPO did not have any activity towards tyrosine and hydroquinic acid or resorcinol, which was consistent with results obtained by previous studies performed with *Starking* (25) and *Jonagored* apples (26). The highest activity was detected with chlorogenic acid as substrate, followed by (+)-catechin and catechol. The Km value for catechol, chlorogenic acid, L-DOPA, (+)-catechin, and pyrogallol as calculated by the Lineweaver-Burk plot (data not shown) were 9.7, 3.2, 41.4, 13.05, and 18.1 mM, respectively. The Km value of chlorogenic acid is over three times lower than that obtained with different substrates. The lower Km value for PPO from *Fuji* apples obtained with chlorogenic acid indicates a relatively high affinity of the enzyme for this substrate. This Km value differs from the value reported for apples, because assay methods, apple varieties, origins of the same variety and the

Table 2. Substrate specificity of PPO from *Fuji* apple flesh

Substrate	Contc. (mM)	Wavelength (nm)	Activity (units/mL)	Relative activity compared to that of catechol (%)	Km
Monophenol					
Tyrosine	2.5	475	0	0	-
Diphenol					
o-Phenols					
Catechol	10	420	110	100	9.7
Chlorogenic acid	10	420	422.4	384	3.2
L-DOPA	10	475	46.91	42.65	41.4
(+)-Catechin	10	420	260	236.36	13.05
Caffeic acid	10	420	9.44	8.58	-
m-Diphenol					
Resorcinol	10	460	0	0	
p-Diphenol					
Hydroquinone	10	460	0	0	-
Triphenol					
Pyrogallol	10	334	104.2	94.73	18.1

Table 3. Effect of various compounds on PPO activity from the flesh of a *Fuji* apple

Compounds	Relative activity (%)	
	0.1 mM	1.0 mM
$CaCl_2$	96.54	83.64
$CuSO_4$	-	97.17
$MgSO_4$	-	96.74
$FeCl_2$	-	93.30
$MnCl_4$	-	94.65
$ZnSO_4$	56.27	1.04
Ascorbic acid	97.05	23.06
Benzoic acid	99.95	81.56
Kojic acid	68.57	54.89
L-Cysteine	77.64	3.74
4-Hexylresorcinol	93.37	61.95
EGCG	85.73	15.38
ECG	98.49	43.23
GCG	73.88	7.80

extraction pH were different (2, 8, 26).

Inhibitors The effect of different PPO inhibitors on apple PPO is shown in Table 3. The general inhibitors of PPO inhibition have been reviewed previously (21-24), and the inhibitory effects of the green tea catechins (EGCG), epigallocatechin gallate (ECG) and (GCG) were reported by No *et al.* (27). With catechol as the substrate, a lag period was observed when the inhibitors L-cysteine, EGCG, GCG, and ascorbic acid were used at a concentration of 1.0 mM. Residual PPO activity was determined from the slope of the reaction curve after the lag period. Cysteine can easily form complexes with o-quinones, and PPO was inhibited by the formation such as, ascorbic acid, chelating agent, and sulfur-containing amino acids (28-30). Cysteine has been suggested to prevent enzymatic browning in processed fruit products. Since the 1986 FDA ban on the use of sulfites for fresh fruits and vegetables, the food industry has sought a replacement for sulfites (31). In addition, PPO activity was markedly inhibited by $ZnSO_4$, but not by $MgSO_4$ or $CaCl_2$. Jimenez *et al.* (32) reported that divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} increased grape PPO activity. In this study, L-cysteine was most effective at inhibiting *Fuji* apple PPO activity, followed by GCG and EGCG.

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