STUDIES ON POLYPHENOL OXIDASE (PPO) FROM SOME CRUCIFERAE VEGETABLES

(アブラナ科野菜のポリフェノール酸化酵素（PPO）に関する研究)

ANDI NUR FAIDAH RAHMAN

2012

Studies on Polyphenol Oxidase (PPO) from Some Cruciferae Vegetables

(アブラナ科野菜のポリフェノール酸化酵素（PPO）に関する研究)

By

Andi Nur Faidah Rahman

(アンディ　ヌル　ファイダ　ラーマン)

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor

THE UNITED GRADUATE SCHOOL OF
AGRICULTURAL SCIENCES
KAGOSHIMA UNIVERSITY
JAPAN

September 2012

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Undesirable browning of damaged tissue occurs by enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenol oxidase (EC 1.10.3.1, *o*-diphenol: oxygen oxidoreductase, PPO). Enzymatic browning can cause considerable losses in the food and agricultural sector, which results in decreased the marketability. To prevent the browning, many investigations have been conducted to purify and characterize the PPOs of many fruits and vegetables. Most of PPO strongly oxidized *o*-diphenol, however, a new type PPO, which only oxdized 1,3,5-trihydroxybenzenes such as phloroglucinol (Phl) have been found in turnip and cabbage which belong to cruciferae vegetables. The Phl oxidizing enzymes (PhO) also have peroxidase (EC 1.11.1.7; hydrogen peroxidase oxidoreductase, POD) activity. PPO and POD play important roles in deterioration of color and flavor. However, little is known about the PhO from cruciferae vegetables except for turnip and cabbage PPOs. The objectives of these studies are: to investigate the distribution of PhO in other cruciferae vegetables, and to purify and characterize the Phl oxidizing enzyme from Japanese radish, cauliflower, and broccoli.

In **chapter 2**, the PPOs of various cruciferae vegetables were prepared, and the distribution of PPO was determined. PPOs in fruits and vegetables vary depend on the plant source. Cabbage, turnip, broccoli, and cauliflower PPOs strongly oxidized 1,3,5-trihydroxybenzenes such as Phl and phloroglucinol carboxylic acid, but not oxidized *o*-diphenol and 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. On the other hand, mizuna, takana, leaf mustard,

katsuona, and komatsuona also strongly oxidized Phl, but had small activity toward pyrogallol. In addition, Japanese radish root strongly oxidized Phl, but a little oxidized pyrogallol. It seems that the PPOs distributed in all cruciferae vegetables are a group of a new type of PPO that is “phloroglucinol oxidase (PhO)”. In addition, these PPOs have dual activities of PhO and POD. PhO distributed in all cruciferae vegetables with different activity level, from 15 varieties of vegetables tested showed wide variation in PhO activity ranging between 1.0 to 127.0 unit/mg protein. Japanese radish had the highest activity toward PhO compared to other cruciferae vegetables PPO tested, that is 127 unit/mg protein. The activity level of PhO from various cruciferae vegetables in order as follows: Japanese radish root > turnip > cabbage > broccoli > cauliflower > broccoli sprout > nabana > Chinese cabbage > shingensai > daikon sprout > mizuna > takana > komatsuna > Chinese mustard > katsuona. On the other side, turnip PPO showed the highest activity toward POD compared to others PPO, that is 432 unit/mg protein. The activity level of POD from cruciferae vegetables in order as follows: turnip > cabbage > Japanese radish root > broccoli > cauliflower > nabana > broccoli sprout > mizuna > daikon sprout > Chinese cabbage > shingensai > takana > Chinese mustard > komatsuna > katsuona.

In **chapter 3**, PPO was purified from the Japanese radish root by ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. The enzyme was purified about 192-fold with a recovery rate of 15%. The purified enzyme appeared as a single band on SDS-PAGE. The molecular weight of the purified PPO was estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE. The

purified enzyme quickly oxidized phloroglucinol (1,3,5-trihydroxybenzene) with a K_m of 2 mM. The enzyme also oxidized 1,2,3-trihydroxybenzenes, such as pyrogallol and gallic acid; however, it not oxidized *o*-diphenols, such as chlorogenic acid and dopamine. POD activity was also present in the purified enzyme preparation with the final preparation having a purification and recovery rate of 259-fold and 20%, respectively. The optimum pH for the PhO and POD activities was 8.0 and 5.0, respectively, and the measured activities were stable at 5°C for 20 h in the pH ranges of 3.0–10.0 and 3.0–11.0, respectively. Both enzyme activities were stable up to 50°C after heat treatment for 10 min and were strongly inhibited by treatment with sodium diethyldithiocarbamate, potassium cyanide, L-ascorbic acid, chlorogenic acid, and hydroquinone at a final concentration of 10 mM.

In **chapter 4**, PPO of cauliflower was purified to 282-fold with a recovery rate of 8.1%, using phloroglucinol as a substrate. The enzyme appeared as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The estimated molecular weight of the enzyme was 60 kDa and 54 kDa by SDS–PAGE and gel filtration, respectively. The purified enzyme (PhO) oxidized phloroglucinol (K_m = 3.3 mM) and phloroglucinol carboxylic acid. The enzyme also had peroxidase (POD) activity. At the final step, the activity of purified cauliflower POD was 110-fold with a recovery rate of 3.2%. The PhO and POD showed the highest activity at pH 8.0 and pH 4.0, and were stable in the pH range of 3.0–11.0 and 5.0 –8.0 at 5°C for 20 h, respectively. Optimum temperature was 55°C for PhO and 20°C for POD, both activities were stable at a temperature range of 20–50°C. The most effective inhibitor for PhO was sodium diethyldithiocarbamate at 10 mM

(IC_{50} 0.64 and K_i 0.15 mM) and for POD was potassium cyanide at 1.0 mM (IC_{50} 0.03 and K_i 29 μM).

In **chapter 5**, PPO from broccoli was purified using phloroglucinol as a substrate. The purified enzyme appeared as a single band on SDS-PAGE. The enzyme was purified about 80-fold with a recovery rate of 26%. The estimated molecular weight of the enzyme was 63 kDa and 65 kDa by SDS−PAGE and gel filtration, respectively. The purified enzyme oxidized phloroglucinol with a K_m value of 11 mM. POD activity was also present in the enzyme preparation which was purified about 33-fold with a recovery rate of 11%. The PhO and POD showed the highest activity at pH 8.0 and pH 7.0, respectively. The activities of PhO and POD were stable in the pH ranges of 5.0−10.0 at 5°C for 20 h. Optimum temperature was 60°C for PhO and 20°C for POD, both activities were stable at temperature range of 30−60°C. The activities of PhO and POD were strongly inhibited by sodium diethyldithiocarbamate, KCN, L-ascorbic acid, chlorogenic acid, and hydroquinone at 1 mM.

In **chapter 6**, we compared the characterics of PPO (PhO) from cruciferae vegetables with those of *o*-dipehnolase from other fruits and vegetables, and then carried out general discussion.

CHAPTER 1

INTRODUCTION

Undesirable browning in damaged tissues of many fruits and vegetables is induced by enzymatic oxidation of polyphenols, catalyzed by polyphenol oxidase (EC 1.10.3.1; *o*-diphenol: oxygen oxidoreductase, PPO). PPO is a group of copper containing proteins and is widely distributed in plant and animal tissues.[1] When the tissue is damaged, the rupture of plastids where PPO is located, occur, and the rupture leads to the enzyme contact with the phenolic compounds (PP) released from the vacuole, where these compounds are stored.[2]

The oxidation of PP by PPO is thought to be the major cause of the brown discoloration of many fruits and vegetables during ripening, handling, storage, and processing. **Figure 1** shows the mechanism of the brown discoloration. The enzyme catalyzes two distinct reactions: the *o*-hydroxylation of monophenols to *o*-diphenols (so called as cresolase) and the oxidation of *o*-diphenols to *o*-quinones (so called as catecholase).[1] Oxidation of PP by these enzymes caused enzymatic browning during storage and processing of the fruits and vegetables in resulting the quality and marketability of the foods will decrease because of damaging PP which have been associated with off-flavor and discoloration of the fruits and vegetables. **Figure 2** shows the structure of some PP. To prevent such browning, PPOs have been purified and characterized in many fruits and vegetables, because these enzymes are very important in food quality and economics of fruits and vegetables.[3]

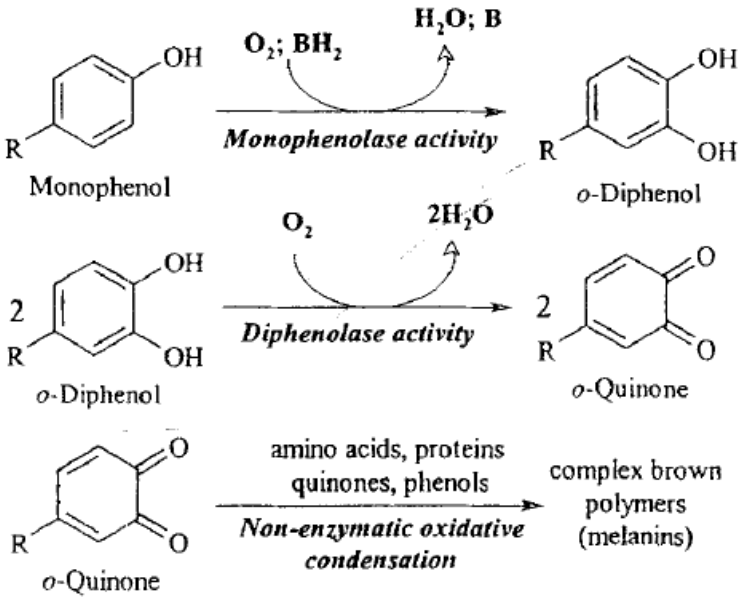
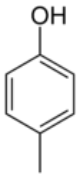
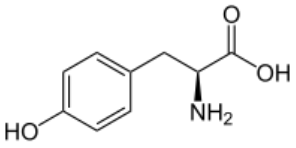


Figure 1. Reaction scheme of tyrosine; both the cresolase and catecholase are shown.[1]

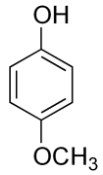
Mono phenols



p-cresol

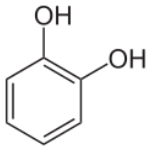


tyrosine

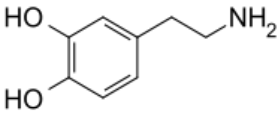


4-methoxyphenol

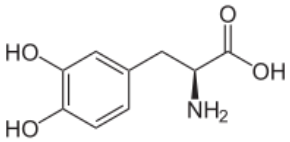
Ortho diphenols



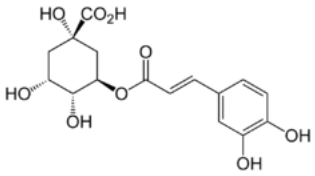
catechol



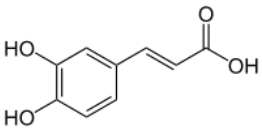
dopamine



L-dopa

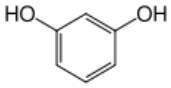


chlorogenic acid

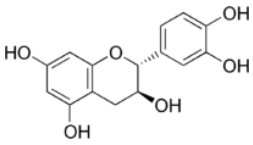


caffeic acid

Meta diphenols



resorcinol

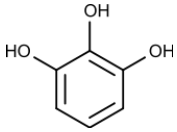


catechin

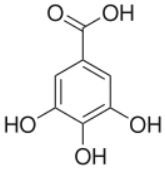
Para diphenols

hydroquinone

1,2,3-trihydroxybenzene

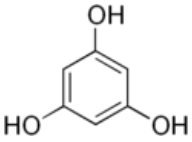


Pyrogallol

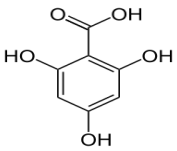


Gallic acid

1,3,5-trihydroxybenzene



phloroglucinol



phloroglucinol carboxylic acid

Figure 2. Structures of some phenolic compounds

PPO that originates from distinct plant sources displays different substrate specificites. Most of PPOs oxidize *o*-diphenols such as catechol, chlorogenic acid, DL-dopa, and dopamine. For example, apple,[4] garland chrysanthemum,[5] and edible burdock[6] strongly oxidize chlorogenic acid; banana pulp and peel[7,8] strongly oxidize dopamine; mango pulp[9] and mamey[10] strongly oxidize catechol; broccoli florets[11] and butter lettuce[12] strongly oxidize catechol and 4-methylcatechol; nettle oxidizes catechol, 4-methylcatechol, L-dopa, L-tyrosine, pyrogallol, catechin, and trans-cinnamic acid;[13] and artichoke heads[14] oxidize catechol, 4-methylcatechol, DL-dopa, L-dopa, and gallic acid. In contrast the plants PPO from soybean[15] and one of the purified enzyme of edible burdock[16] did not show the substrate specificities toward *o*-diphenol, but they strongly oxidized pyrogallol (1,2,3-trihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene). However, a novel type of PPO, which only oxidizes 1,3,5-trihydroxybenzene, such as phloroglucinol and phloroglucinol carboxylic acid but not oxidize *o*-diphenol and 1,2,3-trihydroxybenzene was found in fruit Satsuma mandarin[17] and cruciferae vegetables, such as turnip[18] and cabbage.[19,20] In these experiments, many of the enzymes were partially purified and characterized.

The enzymatic browning occurred due to the presence of the essential components such as enzyme, oxygen, copper or substrate.[2] Because the browning decreases the quality of plant product, the method of control essential components caused the browning in plant products have been done widely. **Figure 3** showed the general classification of methods for inhibition of enzymatic browning.[21] Heat

treatment is one of the methods for inactivating the enzyme activity. This method have been applied widely in fruits and vegetables such as pineapple puree[22] and showed the activity of its enzyme reduced 60% after treated at 40–60°C for 30 min, and increased rapidly at temperature above 75°C. In mango pulp[9], the 98% of the PPO activity was lost after the extract was heated for 5 min at 80°C. In butter lettuce[12], the 50% inactivation of activity at 50°C, 60°C, and 70°C were found to be about 30, 20, and 5 min, respectively. The effect of heat treatment in some plant products caused the destruction of thermosensitive nutrients.[2] Lado and Yousef[23] try the alternative for food-preservation technologies by using high pressure processing, ionizing radiation, pulsed electric field, and ultraviolet radiation. The results showed that the nutrition and sensory qualities of plant product can be maintained. **Figure 4** shows miscellaneous and non conventional methods for the treatment of enzymatic browning.[21] Plaza et al.[24] combined the high-pressure with additives such as citric acid and sodium chloride on tomato puree. The results showed the enzyme activity significantly inhibited by these combined treatments at high values of pressure and concentration of additives. Fang et al.[25] tried to the inhibition of browning of kiwi fruits by the combination of the high-pressure and heat treatment. The results showed the enzyme inactivation at 400 MPa and mild heat (≤ 50°C) for ≤ 15 min, no great effect for prolongation of exposure time after the first 15 min. **Figure 5** showed the another inhibition of enzymatic browning by using chemical methods.[21] Ascorbic acid is widely used for browning control. This compound inhibits the browning by decreasing pH of PPO reaction. Ascorbic acid also acts as an antioxidant because

it reduces the quinone produced before it undergoes secondary reactions that lead to browning.[2] **Figure 6** showed the inhibition of browning by using reducing agent.[21] Chen et al.[26] combined meta-bisulfite and ascorbic acid with honey to inhibit the browning in which the results showed the honey increases the effectiveness of compounds. Liu et al.[27] inhibited the peroxidase and polyphenol oxidase in red beet extract with continuous high pressure carbon dioxide (HPCD) treatment at 7.5 MPa (55°C, 30 min) in which the results showed the POD and PPO activities reduce approximately 73% and 93%, respectively.

As described previously, most of PPOs oxidize *o*-diphenols such as catechol, chlorogenic acid, DL-dopa, and dopamine. However, a novel type of PPO, which only oxidized 1,3,5-trihydroxybenzene, such as phloroglucinol and phloroglucinol carboxylic acid but not oxidized *o*-diphenol and 1,2,3-trihydroxybenzenes was found in fruit Satsuma mandarin[17] and cruciferae vegetables, such as turnip[18] and cabbage.[19,20] Cruciferae vegetables, such as cabbage, broccoli, cauliflower, Japanese radish root, and turnip, belong to a group of brassicaceae. The family of brassicaceae consists of 350 genera and about 3500 species.[28] These vegetables are consumed all over the world and economically important, because they have many positive impact and widely usage of many side such as vegetable, fodder crop, condiment, edible and industrial oil seed, green manure covers, and bio-fumigants by using their buds, inflorescences, leaves, roots, seeds, and stems.[28,29] In addition, cruciferae vegetables have many benefits for human health. As reported by Soengas et al.,[30] cruciferae vegetables are a good resource of dietary antioxidants, including water-soluble antioxidants (phenolic compounds,

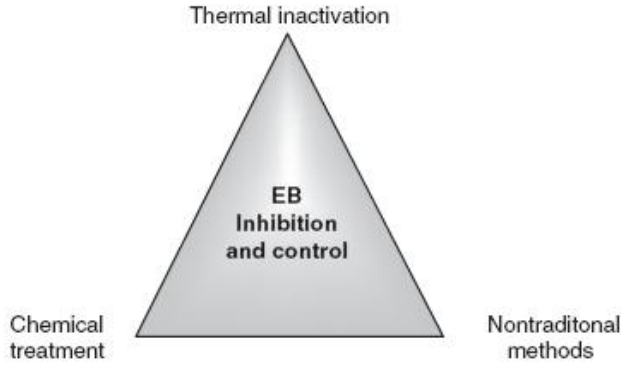


Figure 3. Description of chemical methods for the inhibition of enzymatic browning (EB).[21]

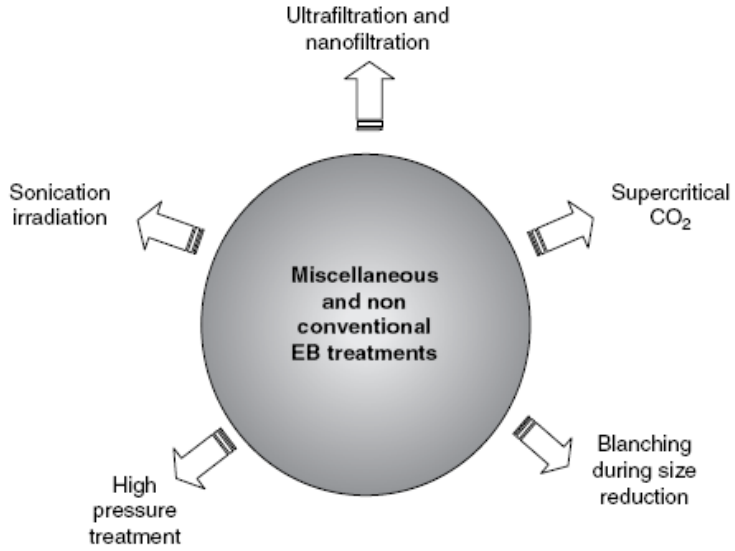


Figure 4. Miscellaneous and non conventional methods for the treatment of enzymatic browning.[21]

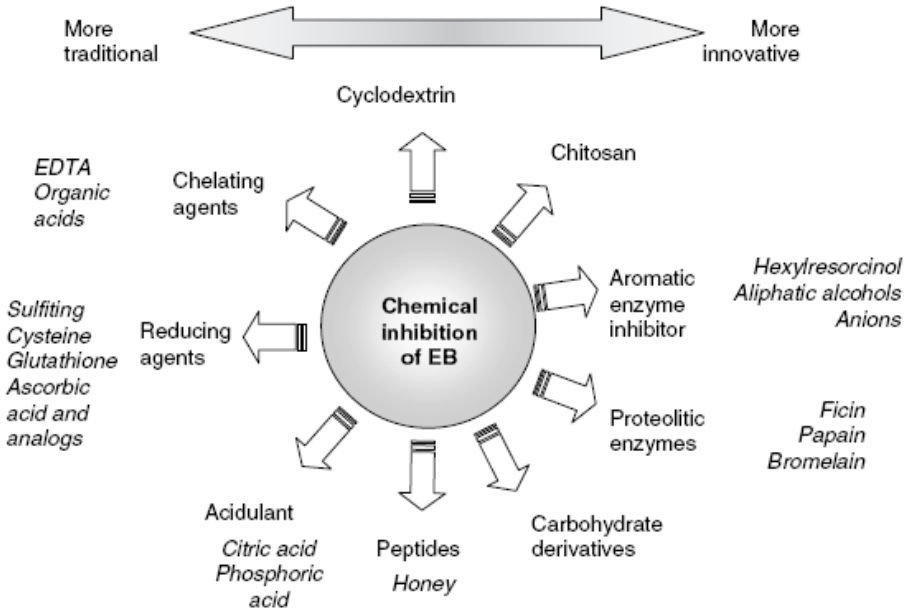


Figure 5. Description of chemical methods for the inhibition of enzymatic browning.[21]

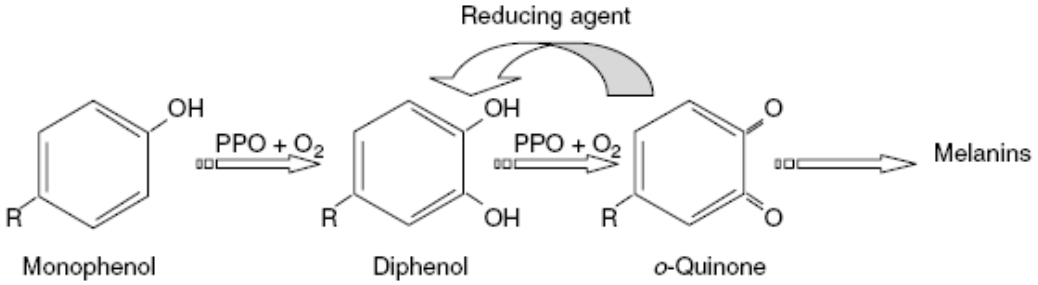


Figure 6. Effect of reducing agents on the first stages of enzymatic browning.[21]

vitamin C, and folic acid) and lipid-soluble antioxidants (carotenoids and vitamin E). Strong epidemiological evidence shows that the intake of a good deal of these vegetables can reduce the risk of several types of cancer and prevent the other human diseases. In addition, Cartea et al.[28] reported that the high antioxidant activity of cruciferae vegetables was due to the presence of PP which have been associated with flavor and color of fruit and vegetables. PPs involved in plant foods were potentially protective factors against cancer and heart diseases, because of their potential anti-oxidative properties. Naturally, when the tissue of fruits and vegetables damaged, the oxidation of PP by PPO occurred and lead to undesirable browning. The impact shows that the nutrition and quality of fruit and vegetables will decrease due to the type of browning. To prevent such browning that results in decreased marketability, this enzyme has been purified and characterized in many fruits and vegetables.[4¬20] Purification and characterization of this enzyme is important to identify its biochemical properties and function and in turn to understand how to prevent its deteriorative action during storage and processing.

As mentioned above, PPO from soybean[15] and edible burdock[16] did not show the substrate specificities toward *o*-diphenol, but they strongly oxidize pyrogallol (1,2,3-trihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene). In contrast that, a new type PPO, which only oxdize 1,3,5-trihydroxybenzene such as phloroglucinol and phloroglucinol carboxylic acid, has been found in Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The phloroglucinol oxidizing enzymes (PhO) also have peroxidase (EC 1.11.1.7; hydrogen peroxidase oxidoreductase,

POD) activity.[17¬20] PPO and POD play important roles in deterioration of color and flavor.[4¬20] However, little is known about the PPO from other cruciferae vegetables except for turnip and cabbage PPOs. The objectives of these studies are: to purified the PPO from other cruciferae vegetables such as Japanese radish, cauliflower, and broccoli and to investigate the properties and distribution. The properties and distribution of PPO will be also compared those of *o*-diphenol oxidase.

The whole manuscript consists of seven chapters. The introduction is mentioned in chapter 1. In chapter 2, the distribution of PPO and PhO activities in fruits and vegetables was studied. The purification and characterization of polyphenol oxidase from Japanese radish (*Raphanus sativus* L.) root was presented in chapter 3. In chapters 4 and 5, the purification and characterization of polyphenol oxidase from cauliflower (*Brassica oleracea* L.), and from broccoli (*Brassica oleracea L*. var. *botrytis*), respectively were presented. The general discussion and conclusions were also presented in chapters 6 and 7, respectively.

CHAPTER 2
DISTRIBUTION OF POLYPHENOL OXIDASE IN FRUITS AND VEGETABLES

2.1 Introduction

Polyphenol oxidase oxidizes phenolic compounds to produce undesirable browning of damaged tissues in many fruits and vegetables. Because the browning decreases the marketability of fruits and vegetables, many studies for PPO have investigated to prevent this discoloration.[4–20] Most of PPO strongly oxidized *o*-diphenols such as chlorogenic acid, dopamine, and catechol.[4–10] The PPOs of apple,[4] garland chrysanthemum,[5] and edible burdock[6] strongly oxidized chlorogenic acid. In addition, banana PPO[7,8] strongly oxidized dopamine; and the PPOs of mango[9] and mamey[10] strongly oxidized catechol. In contrast, purified PPOs of soybean[15] and one of the purified enzyme of edible burdock[16] oxidized pyrogallol (1,2,3-trihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) but not oxidized *o*-diphenols. On the other hand, a novel type of PPO which only oxidizes 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, was found in citrus fruits such as Satsuma mandarin[17] and cruciferae vegetables such as turnip[18] and cabbage.[19,20] These purified phloroglucinol oxidizing PPO (PhO) also has strong POD activities. In contrast that, in broccoli (*Brassica oleracea* L.) which is one of the cruciferae vegetables, the PPO and POD have been purified and characterized separately.[11,31] The purified broccoli florest PPO strongly oxidized catechol and 4-methylcatechol, and had not POD activity.[11] The crude enzyme extracts of turnip[18] and

cabbage[19,20] had strong PhO and POD activities. These enzymes were also concerned with the browning of these cruciferae vegetables. However, no other detailed study has been reported on the distribution of PPO in other cruciferae vegetables. Hence in the present chapter, the distributions of PPOs of cruciferae vegetables were firstly investigated.

2.2 Materials and Methods

2.2.1 Chemicals and Materials

Fresh variation of cruciferae vegetables (**Table 1)** were purchased from a local market in Saga City, Japan. Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

2.2.2 Enzyme extraction

The edible parts of the cruciferae vegetables were homogenized with 0.1 M phosphate buffer (pH 7.0) at 5°C. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10,300 × g for 20 min at 5°C, and the supernatant was brought to 80% ammonium sulfate saturation. After 24 h, the precipitated protein was collected by centrifugation (10,300 × g), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed at 5°C in the same buffer for 36 h during which the buffer was changed four times. After dialysis the dialyzed solution was centrifuged again at the same condition, and the supernatant was used as the enzyme solution.

2.2.3 Measurement of enzyme activity

PhO Activity. The activity of PhO was measured by the spectrophotometric method based on a difference in spectra.[32] The reaction mixture was consisted of 0.5 mL of 20 mM aqueous solution of phloroglucinol, 1.4 mL of 0.1 M potassium phosphate/0.1 M sodium hydrogen phosphate buffer (pH 7.0), and 0.1 mL of the enzyme solution. After 10 min of incubation at 30°C, 0.5 mL of the reaction mixture was taken out and added to 4.5 mL of distilled water. Immediately after being added to water, the enzyme activity was measured at 272 nm against an enzyme blank. One unit of enzyme activity was defined as a change in absorbance of the mixture at 272 nm (△A272) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

PPO Activity. The activity of PPO was measured by the colorimetric method.[7] The reaction mixture was consisted of 0.5 mL of 10 mM aqueous solution of various polyphenols, 4.0 mL of 0.1 M phosphate buffer (pH 7.0), and 0.5 mL of the enzyme solution. After 5 min of incubation of the mixture at 30°C, the increase in absorbance was measured at 420 nm. One unit of enzyme activity was defined as a change in absorbance of the mixture at 420 nm (△A420) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

POD Activity. The activity of POD was determined by the colorimetric method.[19] The reaction mixture was consisted of 0.5 mL of 0.1 M aqueous solution of guaiacol, 4.1 mL of 0.1 M phosphate buffer (pH 6.0), 0.2 mL of 0.1% hydrogen peroxide, and 0.2 mL of the enzyme solution. After 2 min of incubation of the mixture at 30°C, the increase in absorbance at 470 nm was measured. One

unit of enzyme activity was defined as a change in absorbance of the mixture at 470 nm (△A470) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

2.2.4 Determination of protein

Determination of Protein. The protein content was determined according to the method of Lowry which was modified by Hartree[33] using bovine serum albumin (BSA, fraction V; Katayama Chemical Company, Osaka, Japan) as a standard. In chromatography, protein was measured at absorbance 280 nm.

2.3. Results and Discussion

Table 1 showed the substrate specificity of PPO from crude enzyme of cruciferae vegetables. Cabbage, turnip, broccoli, and cauliflower PPOs strongly oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid, but not oxidized 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. On the other hand, PPOs of mizuna, takana, leaf mustard, katsuona, and komatsuona also strongly oxidized 1,3,5-trihydroxybenzenes, but have small activity toward pyrogallol. In addition, Japanese radish root PPO has small activity toward pyrogallol and gallic acid. **Table 1** also showed that all the cruciferae vegetables PPOs tested did not show activity toward *o*-diphenol. On the other hand, as listed in **Table 2**, most of PPO studied strongly oxidized *o*-diphenol, however, the amount of activity for each compound was different from those of plant sources. For example, garland chrysanthemum[5] and Japanese butterbur[34] strongly oxidized chlorogenic acid; banana pulp,[7] banana peel,[8] and edible yam[35]

strongly oxidize dopamine; red Swiss chard leaves strongly oxidized L-DOPA.[36] Catechol was found the main substrate in broccoli florest,[11] butter lettuce,[12] and cherry pulp.[37] The main substrates of fruits and vegetables are different from plant source (**Tables 1 and 2**). According to Yoruk and Marshall,[1] the PPO is active to those substrates with the high preference to the enzyme. Nature of the side chain, numbers of hydroxyl groups and their position in the benzene ring of the substrate have a major effect on the catalytic activity of the enzyme.

As shown in **Table 3**, the enzyme from these vegetables showed dual activities of phloroglucinol oxidase (PhO) and peroxidase (POD). From 15 varieties of vegetables tested showed wide variation in PhO activity, ranging between 1.0 to 127.0 unit/mg protein. Japanese radish had the highest activity toward PhO compared to other cruciferae vegetables PPO tested, that is 127 unit/mg protein. The level activity of PhO from various cruciferae vegetables in order as follows: Japanese radish root > turnip > cabbage > broccoli > cauliflower > broccoli sprout > nabana > Chinese cabbage > shingensai > daikon sprout > mizuna > takana > komatsuna > Chinese mustard > katsuona. On the other hand, turnip PPO showed the highest activity toward POD compared to others PPO, that is 432 unit/mg protein. The activity level of POD from cruciferae vegetables in order as follows: turnip > cabbage > Japanese radish root > broccoli > cauliflower > nabana > broccoli sprout > mizuna > daikon sprout > Chinese cabbage > shingensai > takana > Chinese mustard > komatsuna > katsuona.

2.4 Conclusion

The PPO of various cruciferae vegetables were prepared by using 80 % ammonium sulphate saturation and the distribution of PhO was determined. Cabbage, turnip, broccoli, and cauliflower PPO strongly oxidized 1,3,5-trihydroxybenzenes, but not oxidized *o*-diphenol and 1,2,3-trihydroxybenzenes. On the other hand, mizuna, takana, leaf mustard, katsuona, and komatsuona also strongly oxidized 1,3,5-trihydroxybenzenes, but have small activity toward pyrogallol. In addition, Japanese radish root strongly oxidized 1,3,5-trihydroxybenzenes, but a little oxidized 1,2,3-trihydroxybenzenes.

From these results, it seems that the PPOs distributed in all cruciferae vegetables are a group of a new type of PPO that is “phloroglucinol oxidase (PhO)”. In addition, these PPO have dual activities of PhO and POD.

Table 1. Substrate specificity of crude PPO from various cruciferae vegetables

substrates	relative activity (%)									
	mizuna	takana	cabbage	turnip	broccoli	leaf mustard	katsuona	komatsuna	cauliflower	Japanese radish root
1,3,5-trihydroxybenzenes:										
phloroglucinol	100	100	100	100	100	100	100	100	100	100
phloroglucinol carboxylic acid	45	99	71	47	86	34	25	40	42	46
1,2,3-trihydroxybenzenes:										
pyrogallol	15	18	0	0	0	4	2	26	0	2.5
gallic acid	0	0	0	0	0	0	0	0	0	0.4
***o*-diphenols**										
catechol	0	0	0	0	0	0	0	0	0	0
chlorogenic acid	0	0	0	0	0	0	0	0	0	0
DL-DOPA	0	0	0	0	0	0	0	0	0	0
dopamine	0	0	0	0	0	0	0	0	0	0
***m*-diphenols**										
resorcinol	0	0	0	0	0	0	0	0	0	0

Above data were averages of three trials

Table 2. Substrate specificity of PPO from fruits and vegetables

substrates	relative activity (%)								
	garland chrysanthemum[5]	banana pulp[7]	banana peel[8]	broccoli florets[11]	butter lettuce[12]	Japanese butterbur[34]	edible yam[35]	red Swiss chard leaves[36]	cherry pulp[37]
***o*-diphenols**									
catechol	76	54	34.0	100	100	39	35	86	100
4-methylcatechol				62.76	88.50				
chlorogenic acid	100	24.5	5.3	1.89	53.40	100	6.5	80	
DL-DOPA	72	12.3	8.0			2	6.5		
L-DOPA								100	
dopamine	74	100	100			9	100		
resorcinol	0	0	0			0	0		0
caffeic acid		2.0	0.7	5.92	2.30	42			
D-catechin	70	35.6	11.5			37			
epicatechin	100	22.7	9.3			94	20		
p-dimethylphenol								10	
m-dimethylphenol								11	
ferulic acid				1.10	0.20				
1,2,3-trihydroxybenzenes:									
pyrogallol	70	5.5	1.4			0	15		
gallic acid	72	0	0			0	0	73	70.1
1,3,5-trihydroxybenzenes:									
Phloroglucinol	0	0	0	0.16		0	0		0

Table 3. Distribution of PhO and POD in the cruciferae vegetables

various of cruciferae vegetables	specific activity (unit/mg protein)	
	PhO	POD
komatsuna (*Brassica rapa* var. perviridis)	1.0	5.2
mizuna (*Brassica rapa* var. nipposinica)	1.9	10.5
takana (*Brassica juncea* Czern. et Coss. (Integlifolia Group))	1.1	7.8
turnip (*Brassica compestris* L.)	96.8	432.0
Chinese cabbage (*Brassica campestris* L.)	3.1	9.4
nabana (*Brassica napus* L.)	4.6	26.5
Chinese mustard (*Brassica campestris* L.)	0.9	5.8
shingensai (*Brassica rapa* L. (Chinensis Group))	2.7	9.0
katsuona (*Brassica juncea* Czern.)	0.3	1.5
daikon sprout (*Raphanus sativus* L.)	2.0	9.5
broccoli sprout (*Brassica oleracea* L.)	5.4	19.3
cauliflower (*Brassica oleracea* L.)	16.8	41.3
broccoli (*Brassica oleracea* L.)	36.0	45.0
Japanese radish root (*Raphanus sativus* L.)	127.0	216.0
cabbage (*Brassica oleracea* L.)[19]	37.0	292.0

Above data were averages of three trials.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM JAPANESE RADISH (*Raphanus sativus* L.*)* ROOT

3.1 Introduction

The undesirable browning of damaged tissues in fruits and vegetables occurs by the enzymatic oxidation of polyphenols. Such oxidation is mainly caused by polyphenol oxidase. Because browning can decrease the marketability of variety of fruits and vegetables, many studies have investigated PPO with the goal of preventing these discoloration.[4¬20] PPOs that originate from distinct plant sources display different substrate specificities. Most PPOs oxidize *o*-diphenols, such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methylcatechol. For example, purified PPOs from apple,[4] garland chrysanthemum,[5] and edible burdock,[6] are known to strongly oxidize chlorogenic acid, while banana PPO[7,8] strongly oxidizes dopamine. In contrast, purified PPOs of soybean[15] and edible burdock[16] oxidize pyrogallol (1,2,3-trihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) but not oxidized *o*-diphenols. However, a novel type of PPO, which only oxidizes 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, was found by our laboratory in Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] These purified phloroglucinol -oxidizing PPOs (PhOs) also have strong peroxidase activities. While purified soybean PPO[15] has been shown to have dual PPO and POD activities, edible burdock PPO[16] was not found to have POD activity. As shown in chapter 1, PhO activity

was found in crude extracts of the Japanese radish (*Raphanus sativus* L.) root, which is considered a cruciferous vegetable similar to cabbage and turnip. In addition, crude PPO that was prepared from the Japanese radish root was found to have dual PPO and POD activities, which is similar to that of soybean,[15] turnip,[18] and cabbage[19,20] PPOs; however, a detailed characterization of Japanese radish root PPO has not previously been conducted. In this chapter, Japanese radish root PPO was purified using phloroglucinol as a substrate, and the properties of the purified enzyme were investigated.

3.2 Materials and Methods

3.2.1 Chemicals and Materials

Fresh Japanese radish (*Raphanus sativus* L. cv. Aokubi soufuto-L.) root was purchased from a local market in Saga City. DEAE-Toyopearl 650-M, Butyl Toyopearl 650-M, and Toyopearl HW55-s were obtained from Tosoh Co., Tokyo, Japan. Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

3.2.2 Measurement of enzyme activity

PhO activity. The activity of PhO was measured by the spectrophtometric method based on a difference in spectra[32] described in section 2.2.3. One unit of enzyme activity was expressed as an increase in absorbance at 272 nm (ΔA272) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

PPO activity. The activity of PPO was measured by the colorimetric method[7] described in section 2.2.3. One unit of enzyme activity was defined as 0.1 (ΔA420) per min and per mL enzyme solution (1.0 cm light path).

POD activity. The activity of POD was determined by the colorimetric method[19] described in section 2.2.3. One unit of enzyme activity was defined as 0.1 (ΔA470) per min and per mL enzyme solution (1.0 cm light path).

3.2.3 Assay of enzyme properties

Optimum pH. The activity of PhO and POD was measured at 30°C in 0.2 M sodium phosphate/0.1 M citric acid buffer (McIlvaine buffer) in the pH range of 3.0–8.0 and also in 0.1 M boric acid-potassium chloride/0.1 M sodium carbonate (Atkins-Pantin buffer) in the pH range of 9.0–11.0. The enzyme activity was expressed as the percentage of maximum activity.

pH stability. The enzyme of cauliflower was pre-incubated at 5°C for 20 h in McIlvaine buffer in the pH range of 3.0–8.0 and in Atkins-Pantin buffer in the pH range of 9.0–11.0. The remaining PhO and POD activities were measured under the standard conditions (PhO: pH 7.0, 30°C; POD: pH 6.0, 30°C). The enzyme activity was defined as the percentage of the maximum activity level.

Optimum temperature. The activities of PhO and POD were measured at pH 7.0 in the temperature range of 20–80°C and at pH 6.0 in the temperature range of 10 –60°C, respectively. The enzyme activity was defined as the percentage of the maximum activity level.

Thermal stability. Enzyme solution was pre-heated for 10 min at 20–80°C.

The remaining activity of PhO and POD was measured under the standard conditions (PhO: pH 7.0, 30°C; POD: pH 6.0, 30°C). The enzyme activity was defined as the percentage of the maximum activity level.

Effect of various compounds. The activities of PhO and POD were measured in the absence of 13 kinds of compounds showed in **Table 6** under the standard conditions (PhO: pH 7.0, 30°C; POD: pH 6.0, 30°C).

3.2.4 Determination of protein

Determination of protein was carried out with the same methods as described in section 2.2.4.

3.2.5 Molecular weight determination

Molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration was performed following the method of Andrews[38] with the use of α-globulin (160 kDa), serum albumin (bovine; 65 kDa), ovalbumin (chicken egg; 44 kDa), and cytochrome c (12.4 kDa) as marker proteins. SDS-PAGE was carried out as described by Weber and Osborn[39] with the use of myosin (209 kDa), α- galactosidase (124 kDa), serum albumin (bovine; 80 kDa), ovalbumin (chicken egg; 49.1 kDa), carbonic anhydrase (34.8 kDa), soybean trypsin inhibitor (28.9 kDa), and lysozyme (20.6 kDa) as marker proteins. The proteins were stained by 0.25% coomassie brilliant blue (CBB).

3.2.6 Purification of the Japanese radish root enzyme

Extraction and ammonium sulfate fractionation. All steps were carried out at 5°C. Whole roots from the Japanese radish (10 kg; 9¬10 radishes) were homogenized with a Japanese-style grater without using buffer. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10,300 × g at 4°C for 20 min. The supernatant was then brought to 80% saturation with ammonium sulfate. Precipitated protein was collected by centrifugation (10,300 × g), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed against the same buffer for 36 h with the four changes of the dialysis buffer.

DEAE-Toyopearl chromatography. After another round of centrifugation (10,300×g), the dialyzed solution was applied to a DEAE-Toyopearl 650-M column (4.5×15 cm) that was equilibrated with 0.01 M phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions containing active PPO, which were not adsorbed onto the column, were pooled, and brought to 1 M saturation with ammonium sulfate.

Butyl-Toyopearl chromatography. The active fraction obtained from DEAE-Toyopearl chromatography applied to a butyl-Toyopearl 650-M column (1.6 × 15 cm) that was equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. The column was eluted using a linear gradient of ammonium sulfate (1 to 0 M ammonium sulfate in 0.01 M phosphate buffer, pH 7.0). Fractions containing PPO activity were pooled and dialyzed with 0.01 M phosphate buffer (pH 7.0).

Toyopearl HW 55s chromatography. Above dialyzed solution was concentrated using a membrane filter (Amicon YM-10, Millipore Japan Co., Tokyo, Japan), added to a Toyopearl HW 55-s column (1.6 × 80 cm) that was equilibrated with 0.1 M phosphate buffer, and then eluted using the same buffer. Fractions containing enzyme activity were collected and used for enzyme characterization.

3.3 Results and Discussion

3.3.1 Purification of the Japanese radish root enzyme

Figure 7 shows a typical elution pattern of PPO on a gel filtration column. Enzyme activity eluted in one peak, and the peak fractions from several columns were pooled as purified enzyme. A typical stepwise purification of PPO is shown in **Table 4**. After the final purification steps, the enzyme preparation contained a specific activity that was increased 192-fold compared to that of the initial homogenate with a recovery rate of 15%.

3.3.2 Characterization of the purified enzyme

The purified enzyme appeared as a single band on SDS-PAGE (**Figure 8**). As shown in **Figure 9**, the molecular weight of PPO was estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE. These results indicate that the purified enzyme is monomeric. While the molecular weight of the purified Japanese radish root PPO was similar to soybean PPO (47 kDa)[15] and leaf lettuce PPO (46 kDa),[40] it differed from those of edible burdock (40 kDa),[6] broccoli

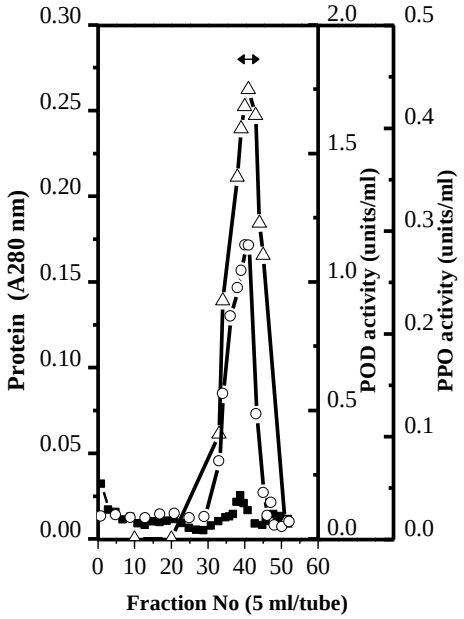


Figure 7. Elution pattern of the Japanese radish root enzyme on HW Toyopearl 55-s. (↔) fraction pooled; (■) protein; (○) PPO activity; (Δ) POD activity.

Table 4. Purification of the Japanese radish root enzyme

enzyme	PhO						POD			
	volume (mL)	total activity (units)	total protein (mg)	specific activity (unit/mg protein)	purification (fold)	recovery (%)	total activity (units)	specific activity (unit/mg protein)	purification (fold)	recovery (%)
crude Extract	6890	689345	35812	19	1.0	100	1302210	36	1.0	100
crude Enzyme	490	302967	2379	127	6.6	44	514338	216	6.0	40
DEAE-Toyopearl 650-M	1225	379922	266	1428	74.2	55	456404	1716	47.2	35
butyl-Toyopearl 650-M	931	143421	50	2875	149.4	21	328696	6589	181.2	25
toyopearl HW 55-s	1250	103167	28	3694	192.0	15	262938	9415	259.0	20

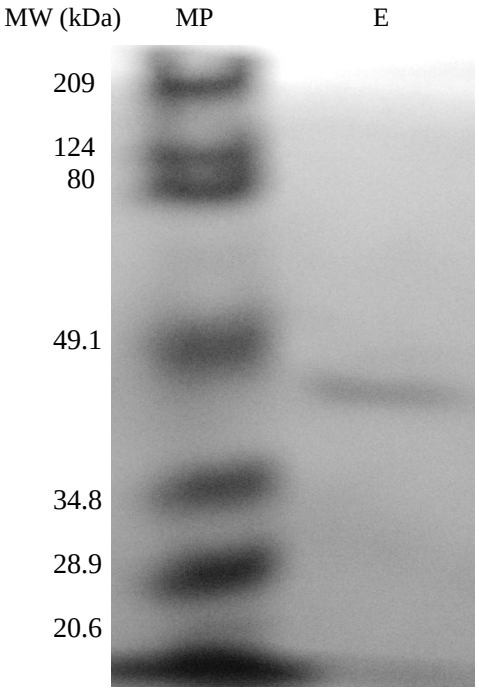


Figure 8. SDS-PAGE of the purified Japanese radish root enzyme. MW, Molecular Weight; MP, Marker Protein; E, Enzyme.

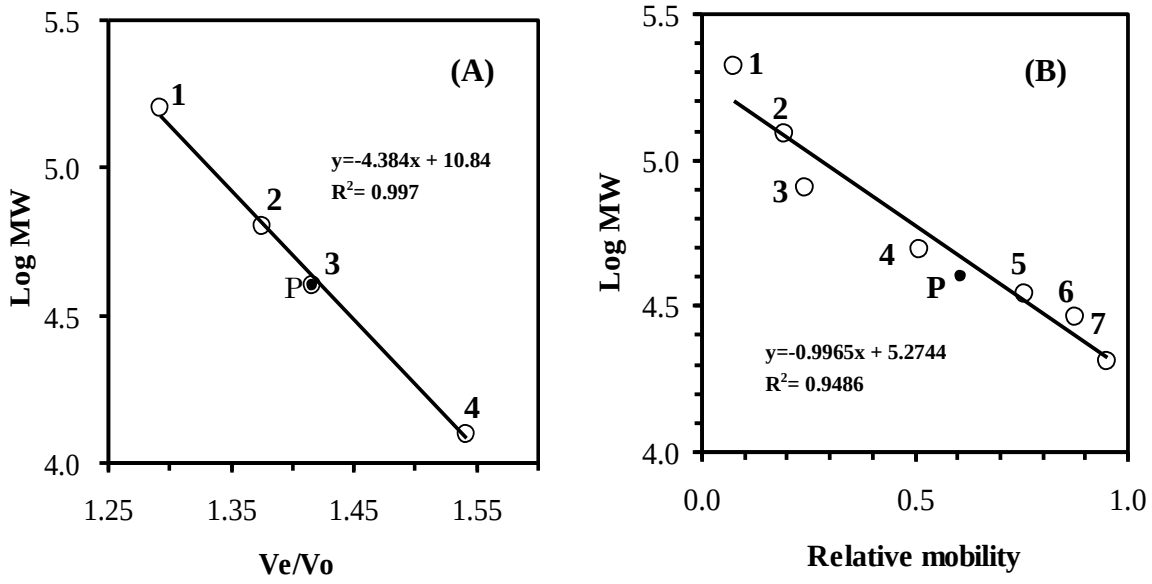


Figure 9. Molecular weight estimation of the Japanese radish root enzyme by gel filtration on a Toyopearl HW 55-s column (A) and by SDS-PAGE (B).

(A) Vo, void volume of the column; Ve, elution volume of the substance; MW, molecular weight: 1. α-globulin (160 kDa), 2. serum albumin (bovine; 65 kDa), 3. ovalbumin (chicken egg; 44 kDa), and 4. cytochrome c (12.4 kDa); P, purified enzyme.

(B) MW, molecular weight in kDa: 1. myosin (209 kDa), 2. β-galactosidase (124 kDa), 3. serum albumin (bovine; 80 kDa), 4. ovalbumin (chicken egg; 49.1 kDa), 5. carbonic anhydrase (34.8 kDa), 6. soybean trypsin inhibitor (28.9 kDa), and 7. lysozyme (20.6 kDa); P, purified enzyme.

florest (57 kDa),[11] turnip (27 kDa),[18] cabbage F-IA (40 kDa),[19] cabbage F-IB (43 kDa),[20] Japanese pear (56 kDa),[41] and black radish (66 kDa).[42]

Most PPOs oxidize *o*-diphenols, such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methylcatechol. PPOs purified from apple,[4] garland chrysanthemum,[5] and edible burdock[6] strongly oxidize chlorogenic acid, while banana PPO[7,8] strongly oxidizes dopamine. As shown in **Table 5**, purified Japanese radish root PPO strongly oxidizes 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid.

The Michaelis constant (K_m) of the enzyme for the oxidation of phloroglucinol was 2 mM (**Figure 10**). This value differed from that of other plant PPOs, specifically, Satsuma mandarin PPO (0.67 mM),[17] cabbage PPO F-IA (6.4 mM),[19] and cabbage PPO F-IB (8.5 mM).[20] PPO purified from the Japanese radish root also oxidized 1,2,3-trihydroxybenzenes, such as pyrogallol and gallic acid; however, it not oxidized *o*-diphenols, such as catechol, chlorogenic acid, and dopamine, or *p*-diphenols, such as resorcinol. This enzyme was found to share a similar substrate specificity as that of soybean PPO[15] and edible burdock PPO.[16] However, the substrate specificities of these three enzymes were different from the PPOs of Satsuma mandarin,[17] turnip,[18] and cabbage[19,20] which only oxidized 1,3,5-trihydroxybenzenes.

The purified Japanese radish root enzyme preparation was red in color with an absorption maxima at 405, 490, and 630 nm, which was similar to that of soybean PPO.[15] Similar results were obtained using purified PPOs of Satsuma mandarin,[17] turnip,[18] and cabbage[19,20] which contain iron. Soybean, Satsuma

Table 5. Substrate specificities of Japanese radish root PPO

substrates	specific activity (unit/mg protein)
phloroglucinol[1]	3694
phloroglucinol carboxylic acid[1]	1692
pyrogallol[2]	91
gallic acid[2]	16
catechol[2]	0
chlorogenic acid[2]	0
DL-Dopa[2]	0
dopamine[2]	0
resorcinol[2]	0

[1]Measured by a spectrophotometric method based on differences in spectra.[32]
[2]Measured by a colorimetric method.[19]

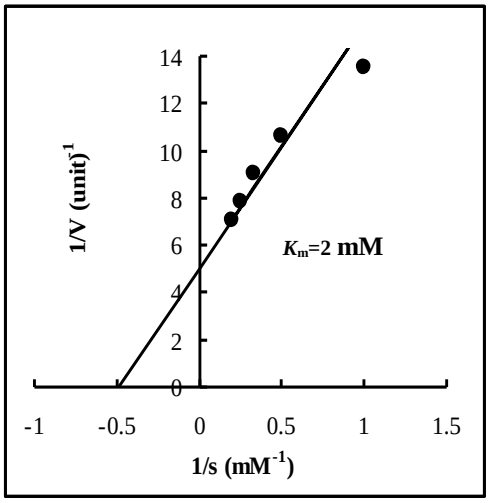


Figure 10. Lineweaver-Burk plots of phloroglucinol oxidation by the Japanese radish root enzyme.

mandarin, cabbage, and turnip enzymes had dual PPO and POD activities. As shown in **Figure 7**, activities of both PPO and POD were detected in the same peak fraction with the final purification of the Japanese radish root enzyme. Furthermore, the purified enzyme was visualized as a single protein band on SDS-PAGE (**Figure 8**). Therefore, we reasoned that the Japanese radish root enzyme has dual PPO and POD activities, similar to the soybean,[15] turnip,[18] and cabbage[19,20] enzymes. The final preparation of the purified enzyme showed a 259-fold increase in POD activity and had a recovery rate of 20% for POD (**Table 4**). Further characterization of the PPO and POD activities of the enzymatic preparation was conducted. The effects of pH on the activity and stability of both PPO and POD activities of the purified enzyme are shown in **Figure 11**. The optimal pH for PPO and POD activities was 8.0 and 5.0, respectively (**Figure 11 A**). The effects of changes in the pH on PPO and POD activities of the PPO enzyme have also been demonstrated for PPOs in soybean,[15] Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The optimal pH for the activity of the Japanese radish root PPO enzyme was similar to PPOs purified from soybean,[15] turnip,[18] cabbage F-IA,[19] and cabbage F-IB and F-II,[20] which were shown to function best in the optimal pH range of 7.4¬7.6. However, the optimal pH for our purified Japanese radish root PPO differed from that of butter lettuce PPO (pH 5.5)[12] and vanilla bean PPO (pH 3.0).[43] The optimal pH for POD activity in our purified Japanese radish root enzymatic preparation was also similar to that of Satsuma mandarin[17] and turnip,[18] but it differed from cabbage enzyme F-IA (pH 6.4)[19] and F-II (pH 6.7).[20]

As shown in **Figure 11 B**, both PPO and POD activities of the Japanese radish root enzyme were stable in wide pH ranges. Eighty percent of the PPO and POD activities were retained in the pH ranges of 3.0¬10.0 and pH 3.0¬11.0, respectively. The ranges of pH stability for both PPO and POD activities differed slightly from those of cabbage F-IA PPO,[19] in which both activities were stable in the range of pH 5.0¬11.0. Similar results were found for Satsuma mandarin PPO[17] and turnip PPO.[18]

The PPO enzyme from Satsuma mandarin,[17] turnip,[18] and cabbage[19,20] had a very high thermal stability. **Figure 12** shows the thermal stability of the purified Japanese radish root PPO enzyme. After heating at 80°C for 10 min, only 20% of the PPO activity remained; however, POD activity was almost completely lost. The thermal stability of this preparation of PPO enzyme was lower than that of the above enzymes specifically, that of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] In contrast, the soybean enzyme[15] had a similar thermal stability of PPO and POD activities as the Japanese radish root enzyme.

Table 6 shows the effects of different compounds on the PPO and POD activities of the purified enzyme. Both the PPO and POD activities were markedly inhibited by sodium diethyldithiocarbamate and potassium cyanide when used at a 10 mM final concentration. Additionally, L-ascorbic acid inhibited both PPO and POD activities at 10 mM but not at 1 mM. $CuSO_4$ inhibited PPO activity, but not inhibited POD activity. Remarkably, $MnCl_2$ was found to activate PPO in edible burdock,[16] turnip,[18] and cabbage;[19,20] however, little activation was found for Japanese radish root PPO. Both PPO and POD activities were markedly inhibited

by chlorogenic acid (an *o*-diphenol) and hydroquinone (a *p*-diphenol) which are similar to studies using purified from satsuma mandarin[17] and cabbage.[19,20]

3.4 Conclusion

Japanese radish root PPO was purified using phloroglucinol as a substrate. The purified enzyme quickly oxidized 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, and 1,2,3-trihydroxybenzenes, such as pyrogallol; however, it not oxidized *o*-diphenols, such as chlorogenic acid and dopamine, which was similar to soybean PPO[15] and edible burdock PPO.[16] Furthermore, the substrate specificities of these enzymes differed from those of Satsuma mandarin PPO,[17] turnip PPO,[18] cabbage PPO,[19,20] which only oxidized 1,3,5-trihydroxybenzene. POD activity was also found in the purified Japanese radish root PPO. Due to the different effects of pH, temperature, and various compounds on PPO and POD activities of the purified Japanese radish root enzyme, it probable that this enzyme has separate active sites for PPO and POD activities, and this may also be the case for soybean,[15] turnip,[18] and cabbage[19,20] PPOs.

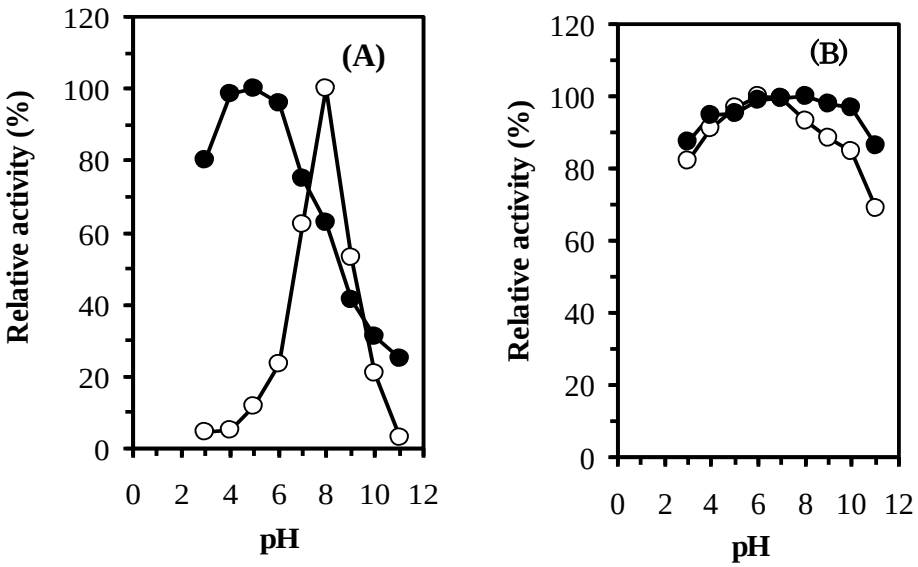


Figure 11. Effect of pH on the activity (A) and stability (B) of the Japanese radish root enzyme. (○) PPO activity; (●) POD activity.

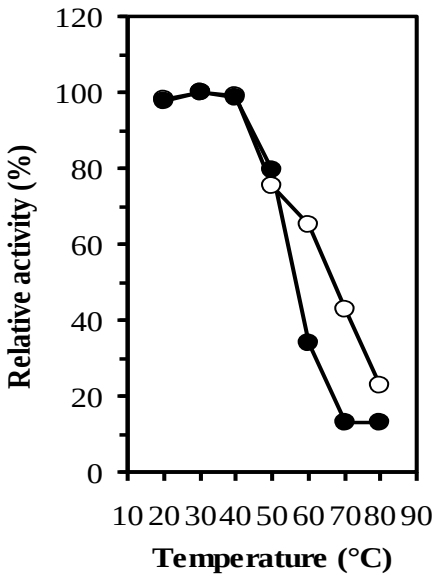


Figure 12. Effect of temperature on the stability of the Japanese radish root enzyme. (○) PPO activity; (●) POD activity.

Table 6. Effects of various compounds on the activities of PPO and POD of Japanese radish root

compounds	relative activity (%)			
	PPO activity		POD activity	
	1 mM[a]	10 mM[a]	1 mM[a]	10 mM[a]
none	100	100	100	100
sodium diethyldithiocarbamate	11	0	89	33
KCN	87	32	0	0
EDTA	98	94	94	92
NaF	105	110	103	83
NaCl	103	100	97	92
$MnCl_2$	128	132	93	90
$CuSO_4$	37	13	111	96
$BaCl_2$	115	97	108	119
$ZnSO_4$	109	107	105	103
L-ascorbic acid	109	0	97	1
chlorogenic acid	21	16	48	1
resorcinol	116	104	104	96
hydroquinone	28	26	33	1

[a]Final concentration of compound.

CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM CAULIFLOWER (*Brassica oleraceae* L.)

4.1 Introduction

Polyphenol oxidase oxidizes phenolic compounds to produce undesirable browning of damaged tissues in many fruits and vegetables. Their quality and marketability decrease due to this type browning during storage and processing. To prevent such browning, PPO has been widely investigated in many fruits and vegetables.[4¬10] Most of the PPOs studied strongly oxidized *o*-diphenols such as catechol, dopamine, and chlorogenic acid. A new type of PPO, which oxidizes only 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid, has been found in Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The phloroglucinol oxidizing enzyme (PhO) also has strong POD activity.[17–20] The PPO and POD play important roles in deterioration of color and flavor. As discussed by Rayan et al.,[44] the PPO is responsible for browning in many fruits and vegetables. On the other hand, soybean PPO[15] and Japanese radish PPO (Chapter 3) oxidized not only 1,3,5-trihydroxybenzenes but also 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. These enzymes also have POD activity. As shown in chapter 2, the crude enzyme extract of cauliflower (*Brassica oleracea* L.), which belongs to cruciferae vegetables including cabbage and Japanese radish had strong PhO and POD activity. The cauliflower POD has been purified and characterized, in which the result showed

that the cauliflower is a good resource of POD.[45] The cauliflower enzymes decrease the quality of this vegetable during storage and processing in an appropriate condition. Determination of PPO and POD is important to identify its biochemical properties and function and in turn to understand how to prevent its deteriorative action during storage and processing. However, little is known about the characteristics of cauliflower enzymes PPO. In this chapter, the PPO of cauliflower was purified using phloroglucinol as substrate, and characterized the PPO and POD activities of the purified enzyme.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

Fresh cauliflower (*Brassica oleracea* L.), which was purchased from a local market in Saga City, Japan, was used. DEAE-Toyopearl 650-M, CM-Sephadex C-50, butyl Toyopearl 650-M, and Toyopearl HW55-s were obtained from Tosoh Co., Tokyo, Japan. Other reagents were purchased from Wako Pure Chemical Company, Osaka, Japan.

4.2.2 Measurement of enzyme activity

PhO activity. The activity of PhO was measured by the spectrophtometric method based on a difference in spectra[32] described in section 2.2.3. One unit of enzyme activity was defined as a change in absorbance of the mixture at 272 nm (△A272) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

PPO activity. The activity of PPO was measured by the colorimetric method[7] described in section 2.2.3. One unit of enzyme activity was defined as a change in absorbance of the mixture at 420 nm (△A420) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

POD activity. The activity of POD was determined by the colorimetric method[19] described in section 2.2.3. One unit ofenzyme activity was defined as a change in absorbance of the mixture at 470 nm (△A470) of 0.1 per minute and per mL enzyme solution (1.0 cm light path).

4.2.3 Assay of enzyme properties

Assay of enzyme properties were performed using several methods described in section 3.2.3.

4.2.4 Determination of protein

Determination of protein was carried out with the same methods as described in section 2.2.4.

4.2.5 Molecular weight determination

The molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), which were carried out with the same methods as described in section 3.2.5.

4.2.6 Purification of the cauliflower enzyme

Extraction and ammonium sulfate fractionation. The edible parts of cauliflower (8.0–9.0 kg) were homogenized with 0.1 M phosphate buffer (pH 7.0) at 5°C. After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10,300 × g for 20 min at 5°C, and the supernatant was brought to 80% ammonium sulfate saturation. After 24 h, the precipitated protein was collected by centrifugation (10,300 × g), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed at 5°C in the same buffer for 36 h during which the buffer was changed four times.

DEAE-Toyopearl 650-M column chromatography. The dialyzed enzyme solution was added to a DEAE-Toyopearl 650-M column (4.5 × 10 cm), equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with the same buffer.

CM-Sephadex C-50 column chromatography. The active fractions of PPO from DEAE-Toyopearl were pooled and applied to a CM-Sephadex C-50 column (4.5 × 10 cm), equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with a linear gradient of sodium chloride from 0 to 1.0 M in 0.01 M phosphate buffer (pH 7.0). The PPO active fraction was collected and brought to a 1.0 M ammonium sulfate concentration.

Butyl Toyopearl 650 M chromatography. The above collected fraction was applied to a butyl-Toyopearl 650 M column (1.6 × 15 cm), equilibrated with 0.01M phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in 0.01 M phosphate buffer (pH 7.0). The enzyme active fraction was pooled, dialyzed with 0.01 M

phosphate buffer (pH 7.0), and concentrated with a membrane filter (Amicon YM-10, Millipore Japan Co., Tokyo, Japan).

Toyopeal HW 55-s chromatography. The final purification was done by Toyopearl HW 55-s column (1.6 × 80 cm). After concentration, the solution was added to the column and equilibrated with 0.1 M phosphate buffer (pH 7.0), and eluted using the same buffer. The PPO active fraction was pooled as the purified enzyme of cauliflower and used for enzyme characterization.

4.2.7 Statistical analysis

All experiments were conducted in triplicate and all the measurements were performed in triplicate. Standard deviation of the data was indicated.

4.3 Results and Discussion

4.3.1 Purification of the cauliflower enzyme

The enzyme of cauliflower was purified by ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. **Figure 13** shows a typical elution pattern of the enzyme on a final gel filtration. The enzyme activity showed a sharp single peak on the column. The active fraction of enzyme from several columns was pooled as the purified enzyme. The activity and yield of PPO in the course of purification are presented in **Table 7**. Finally, the PPO was purified up to 282 ± 16.5 fold purification with a recovery rate 8.1 ± 0.5 % as compared with the crude extract. The obtained

enzyme was used for further studies.

4.3.2 Characterization of the Purified Enzyme

As shown in **Figure 14**, the purified enzyme of cauliflower produced a single band on SDS−PAGE. The molecular weight of the enzyme was estimated to be 60 ± 0.036 kDa by SDS−PAGE and 54 ± 0.025 kDa by gel filtration (**Figure 15**). This result indicates that the purified enzyme is a monomer protein. The molecular weight of the purified cauliflower PPO was different from the molecular weight of the PPOs of another plants such as edible burdock (40 kDa),[6] turnip (27 kDa),[18] cabbage F-IA (40 kDa),[19] and cabbage F-IB (43 kDa).[20] The molecular weight of the purified cauliflower PPO was also different from the molecular weight of Japanese radish root (46 kDa), which was shown in chapter 3. While, it was similar to the molecular weights of PPO of broccoli florest (57 kDa),[11] butter lettuce (60 kDa),[12] and Japanese pear (56 kDa).[41]

As shown in **Table 8**, the purified cauliflower PPO oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid but not oxidized *o*-diphenols. Similar substrate specificity of cauliflower PPO had been found in the purified enzymes of Satsuma mandarin,[17] turnip,[18] cabbage F-IA,[19] and cabbage F-IB.[20] In addition, the substrate specificity of cauliflower enzyme differed from the specificity of the PPOs of soybean,[15] edible burdock,[16] and Japanese radish root (Chapter 3) which oxidized both 1,3,5-trihydroxybenzenes and 1,2,3-trihydroxybenzenes but not oxidized *o*-diphenols. These results indicate that the cauliflower PPO is a group of a new type of PPO that is “phloroglucinol oxidase (PhO)”.

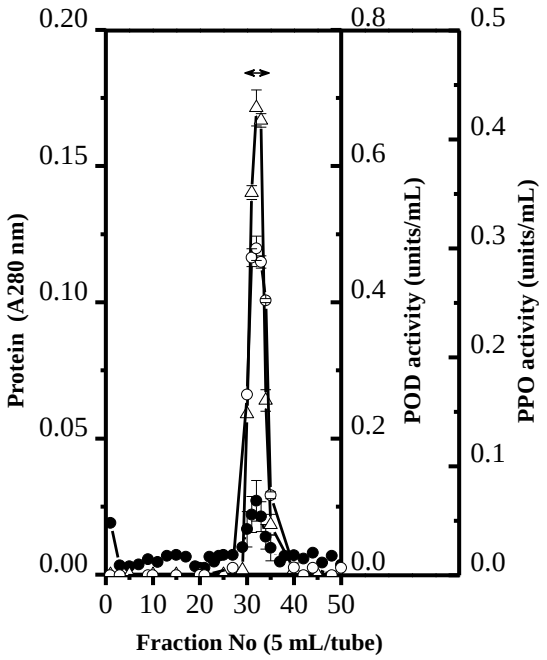


Figure 13. Elution pattern of the cauliflower enzyme on Toyopearl HW 55-s column. (↔) Fraction pooled; (●) Protein; (○) PhO Activity; (Δ) POD Activity.

Table 7. Purification of the cauliflower enzyme

enzyme	PhO					POD			
	volume (mL)	total activity (unit)	total protein (mg)	specifffic activity (unit/mg protein)	purification (fold)	recovery (%)	total activity (unit)	specifffic activity (unit/mg protein)	purification (fold)	recovery (%)
crude Extract	1890	228135 ± 4441	17614 ± 527.8	13 ± 1	1 ± 0.0	100.0 ± 0.0	369968 ± 2741	21 ± 1	1.0 ± 0.0	100.0 ± 0.0
crude Enzyme	99	39594 ± 371	865 ± 13.1	46 ± 1	4 ± 0.1	17.4 ± 0.4	100980 ± 1648	117 ± 3	5.6 ± 0.1	27.3 ± 0.5
DEAE-Toyopearl 650 M	312	33073 ± 953	50 ± 2.7	662 ± 50	51 ± 4.1	14.5 ± 0.5	40850 ± 1894	817 ± 55	38.9 ± 1.8	11.0 ± 0.5
CM-Sephadex C-50M	347	28258 ± 716	33 ± 2.0	853 ± 30	66 ± 3.5	12.4 ± 0.5	33934 ± 2408	1024 ± 37	48.8 ± 0.4	9.2 ± 0.7
butyl Toyopearl 650-M	495	22579 ± 240	15 ± 0.0	1443 ± 16	111 ± 4.2	9.9 ± 0.3	28256 ± 913	1806 ± 59	86.0 ± 5.3	7.6 ± 0.2
HW Toyopearl 55-s	146	18448 ± 1063	5 ± 0.2	3656 ± 206	282 ± 16.5	8.1 ± 0.5	11698 ± 433	2318 ± 64	110.4 ± 6.2	3.2 ± 0.1

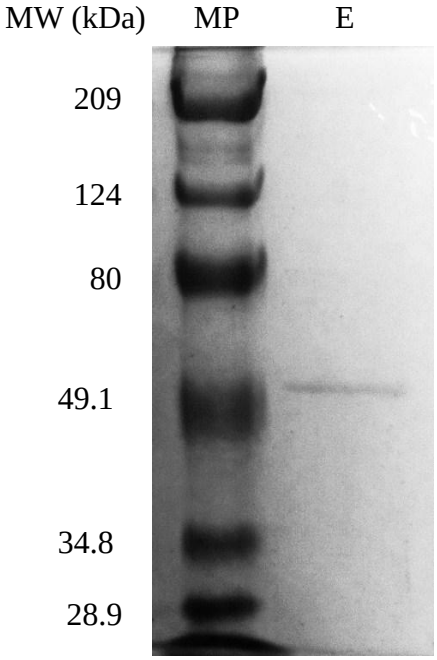


Figure 14. SDS–PAGE of the purified cauliflower enzyme. MW, Molecular Weight; MP, Marker Protein; E, Enzyme.

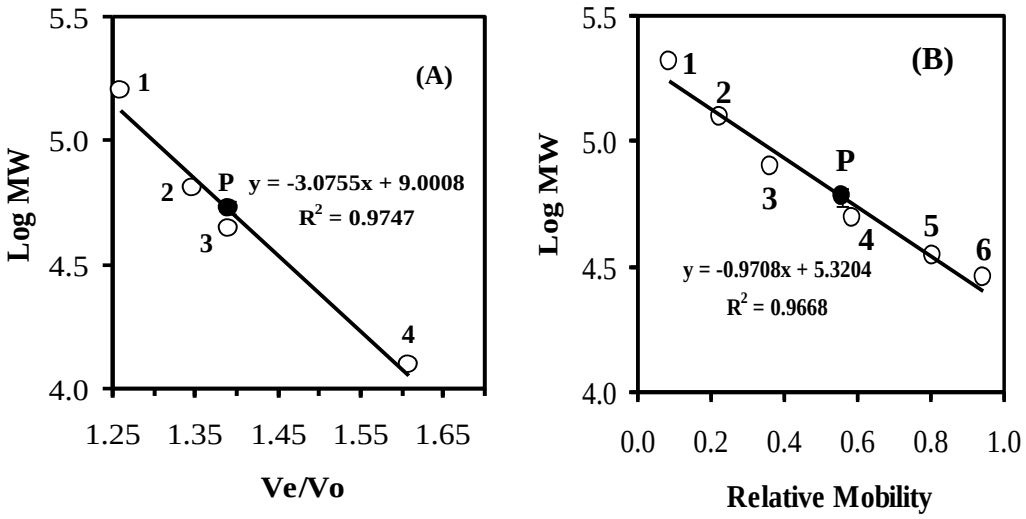


Figure 15. Molecular weight estimation of the cauliflower enzyme by gel filtration on Toyopearl HW 55-s (A) and SDS–PAGE (B).

(A) Vo, void volume of the column; Ve, elution volume of the substance; MW, molecular weight in kilo daltons: 1. α-globulin (160 kDa), 2. serum albumin (bovine; 65 kDa), 3. ovalbumin (chicken egg; 44 kDa), and 4. cytchrome c (12.4 kDa); P, purified enzyme.

(B) MW, molecular weight in kDa: 1. myosin (209 kDa), 2. β-galactosidase (124 kDa), 3. serum albumin (bovine; 80 kDa), 4. ovalbumin (chicken egg; 49.1 kDa), 5. carbonic anhydrase (34.8 kDa), and 6. soybean trypsin inhibitor (28.9 kDa); P, purified enzyme.

Table 8. Substrate specificities of cauliflower PPO

substrates	specific activity (unit/mg protein)
phloroglucinol[1]	3656 ± 206
phloroglucinol carboxylic acid[1]	1926 ± 45
pyrogallol[2]	0 ± 0
gallic acid[2]	0 ± 0
catechol[2]	0 ± 0
chlorogenic acid[2]	0 ± 0
D,L-dopa[2]	0 ± 0
dopamine[2]	0 ± 0
resorcinol[2]	0 ± 0

[1]Measured by the spectrophotometric method based on the difference in spectra.[32]
[2]Measured by the colorimetric method.[7]

As shown in **Figure 16**, the Michaelis constant (K_m) value of phloroglucinol oxidation of the purified cauliflower enzyme was 3.3 mM. This K_m value was larger than the purified turnip PhO (0.67 mM),[18] but smaller than the cabbage PhO F-IA (6.4 mM)[19] and the cabbage PhO F-IB (8.5 mM).[20] The value of K_m indicated the affinity of the enzyme for enzyme-substrate complex formation. The K_m value of cauliflower enzyme was larger than turnip PhO, but smaller than cabbage PhO. It was indicated that cauliflower PhO has the greater affinity compared to cabbage PhO, but it reverses to turnip PhO.

The purified enzyme solution of the cauliflower showed a red color with absorption maxima at 405, 490, and 630 nm. Similar results were found in the purified enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage,[19,20] contained iron and had POD activity. As shown in **Figure 13**, the highest activity of PhO and POD of cauliflower was eluted in a similar fashion at the final step of purification.

The purified enzyme gave a single band of protein on SDS-PAGE (**Figure 14**). This result shows that the purified cauliflower enzyme has a dual activity of PhO and POD, just like the enzymes from Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] At the final step of purification, the POD activity of cauliflower enzyme increased to 110 ± 6.2 fold with a recovery rate of 3.2 ± 0.1% (**Table 7**).

As shown in **Figure 17 A**, the effect of pH on the activity of PhO and POD of purified cauliflower was greatly affected by pH. The optimum pH of PhO and POD of cauliflower was 8.0 and 4.0, respectively. The pH-dependent activity of PhO and POD had been also found in the enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The optimum pH of cauliflower PhO was almost the

same with that of turnip,[18] cabbage F-IA,[19] cabbage F-IB,[20] and cabbage F-II;[20] the optima of these enzymes were in the range of pH 7.4–7.6. The optimum pH of cauliflower POD was close to that of turnip,[18] broccoli,[31] and cauliflower buds,[44] with guaiacol as substrate, and but different from that of cabbage F-IA (pH 6.4)[19] and cabbage F-II (pH 6.7).[20]

As shown in **Figure 17 B**, the activity of PhO of cauliflower enzyme was more stable than POD in the wide range of pH. Over 80% of PhO activity remained in the pH range of 3.0–11.0. However, the POD activity was stable at pH 5.0–8.0 and declined rapidly at the pH below 8.0 after 20 h incubation at 5°C. The stability of PhO and POD of cauliflower was slightly different from that of cabbage F-IA enzyme[19] in the range of pH 5.0–11.0. Gülçin and Köksal found the pH stability of cauliflower buds[45] by using several substrates in the range of pH 8.0–9.0. In the present result, it was found close pH stability from that of cauliflower buds by using guaiacol as substrate that is pH 8.5.

Figure 18 shows the effect of temperature on the activity and stability of PhO and POD of the purified cauliflower enzyme. The optimum temperature of PhO and POD was 55°C and 20°C, respectively (**Figure 18 A**). The optimum temperature of cauliflower PhO was almost same with that of soybean (50°C).[15] However, it was different from that of banana peel (30°C)[8] and cabbage F-IA (40°C).[19] The optimum temperature of cauliflower POD was different from that of cabbage F-IA (45°C)[19] and slightly different from that of Turkish black radish (30°C).[42]

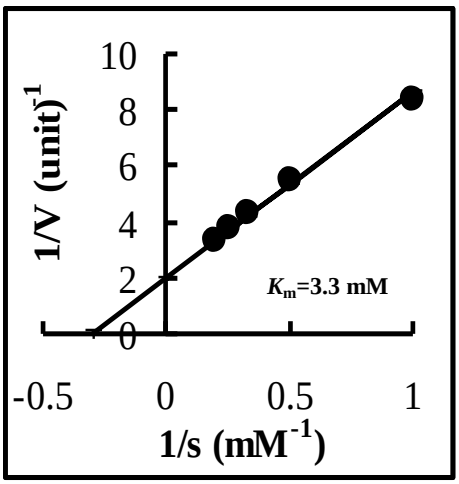


Figure 16. Lineweaver-Burk plots of phloroglucinol oxidation by the cauliflower enzyme.

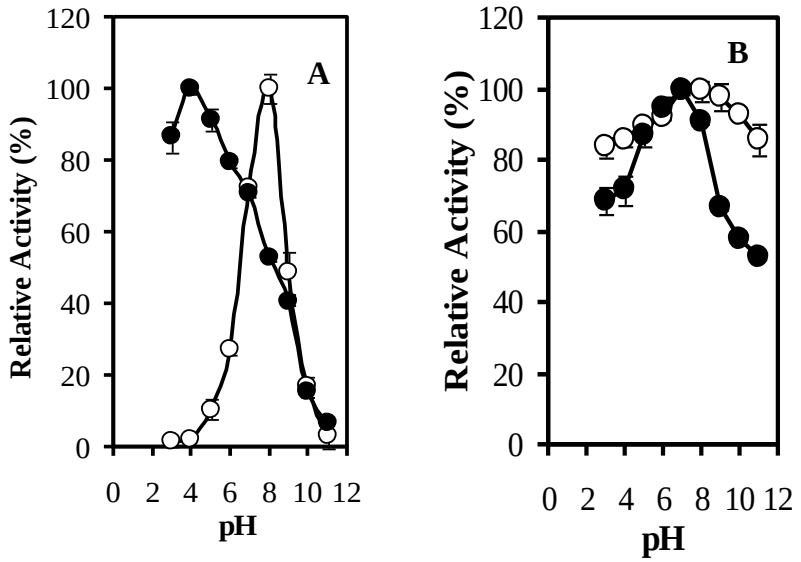


Figure 17. Effect of pH on the activity (A) and stability (B) of the cauliflower enzyme. (○) PhO activity; (●) POD activity.

Very high thermal stability of PhO had been found in the PhOs of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] Contrarily, the purified enzyme of cauliflower showed a relatively low thermal stability. The activity of PhO and POD was stable at the temperature range of 20–50°C. At the higher temperature than 50°C, the activity of POD was almost lost. Contrarily, the PhO was more heat-stable and 60% of its activity remained after heating at 80°C for 10 min (**Figure 18 B**). The POD activity was close with that of found by Rayan et al.[44] in which showed that the heat inactivation kinetic for POD cauliflower was at temperature range from 65–85°C. It indicated that the POD activity will be inactivated at temperature above 65°C.

Table 9 shows the effect of 13 kinds of compounds on the activity of PhO and POD of purified cauliflower enzyme. Sodium diethyldithiocarbamate inhibited PhO (IC_{50} 0.64; *Ki* 0.15 mM) and POD (IC_{50} 6.61; *Ki* 1.64 mM) activities of the enzyme competitively. The inhibition rate for PhO was higher than that for POD at 1 mM. Competitive inhibitor of sodium diethyldithiocarbamate was also found in the PPO of nettle (*Ki* 1.79×10^{-9} mM).[13] KCN completely inhibited POD activity (IC_{50} 0.03; *Ki* 29 µM), and also strongly inhibited PhO activity at 10 mM (IC_{50} 7.88; *Ki* 1.95 mM). KCN inhibited both PhO and POD activities at 1 mM. These results indicate that the purified enzyme is a metallic enzyme, and the metal is seemed to be iron as mentioned previously. L-Ascorbic acid competitively inhibited the activity of PhO and POD at 10 mM but not at 1 mM. The IC_{50} for PhO and POD activities were 3.55 ± 0.07 mM and 3.67 ± 0.66 mM, respectively. $CuSO_4$ noncompetitively inhibited PhO activity

(IC_{50} 0.56 ± 0.05) and slightly inhibited POD activity at 1 mM and 10 mM. Similar results were obtained for the PhOs from cabbage F-IA,[19] cabbage F-IB, and cabbage F-II.[20] EDTA inhibited PhO and slightly activated POD of cauliflower. $BaCl_2$ and $ZnSO_4$ showed a weak activation of PhO and a weak inhibition of POD. However, the strong activation of PhO was induced by $MnCl_2$. These results were similar from the PhOs of turnip[18] and cabbage,[19,20] which showed a remarkable activation by $MnCl_2$. The activity of PhO and POD of cauliflower was markedly inhibited by chlorogenic acid (*o*-diphenols) and hydroquinone (*p*-diphenol) in 1 mM and 10 mM just like the enzymes of Satsuma mandarin[17] and cabbage.[19,20] Chlorogenic acid showed competitive inhibition for PhO activity and noncompetitive inhibition for POD activity. Hydroquinone competitively inhibited both PhO and POD activities of the purified enzyme. It is interesting that the type of inhibition of chlorogenic acid to the cauliflower enzyme for both PhO and POD activities were different. Even though both activities appeared in the same peak, chlorogenic acid inhibited the PhO activity with noncompetitive type and inhibited the POD activity with competitive type. It is indicated that both PhO and POD have separate active sites.

4.4 Conclusion

PhO of cauliflower was purified to a homogeneous state on SDS–PAGE. The purified cauliflower enzyme oxidized 1,3,5-trihdroxybenzenes but not oxidized 1,2,3-trihydroxybenzens and *o*-diphenols. These results may indicate that this enzyme is a group of a new type of PPO, “phloroglucinol oxidase”, which is

similar to the enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The purified cauliflower enzyme showed a dual activity of PhO and POD. The activity of PhO and POD was affected in a different manner by pH, temperature, various compounds, and type of inhibition tested. Based on these results, we speculate that the purified cauliflower enzyme has separate active sites for PhO and POD. The results of biochemical properties of cauliflower enzyme were also beneficial in an attempt to inhibit or control PhO and POD activities in cauliflower during storage and processing.

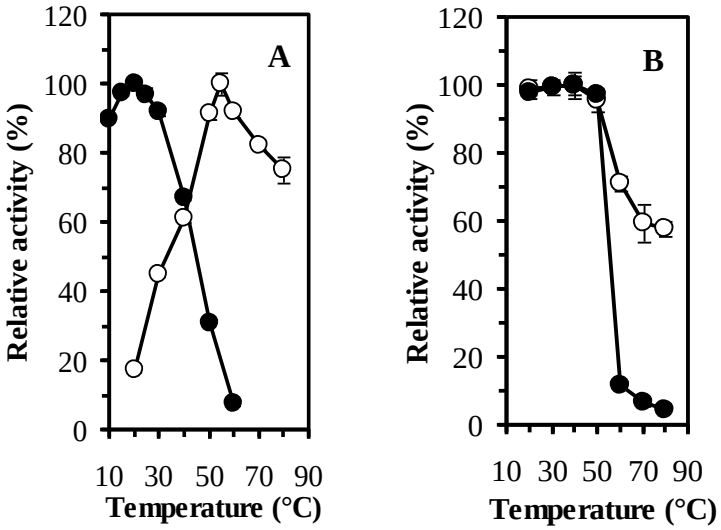


Figure 18. Effect of temperature on the activity (A) and stability (B) of the cauliflower enzyme. (○) PhO activity; (●) POD activity

Table 9. Effect of various compounds on the activities of PhO and POD of cauliflower

compounds	relative activity (%)											
	PhO						POD					
	1 mM[a]			10 mM[a]			1 mM[a]			10 mM[a]		
none	100	±	0.39	100	±	0.39	100	±	1.38	100	±	1.38
$NaS_2CN(C_2H_5)_2$[b]	12	±	1.07	11	±	0.79	91	±	4.66	14	±	0.19
KCN	94	±	0.73	40	±	2.94	1	±	0.09	0	±	0.01
EDTA	69	±	6.41	68	±	7.58	117	±	0.47	119	±	12.72
NaF	101	±	1.01	102	±	4.01	113	±	11.08	122	±	1.93
NaCl	106	±	0.80	101	±	0.39	107	±	9.51	129	±	3.94
$MnCl_2$	238	±	5.13	278	±	1.62	122	±	9.42	127	±	1.99
$CuSO_4$	35	±	4.71	21	±	1.50	99	±	1.02	92	±	0.97
$BaCl_2$	104	±	0.33	128	±	0.32	98	±	1.08	94	±	0.56
$ZnSO_4$	114	±	1.17	123	±	1.11	98	±	0.47	91	±	1.24
L-ascorbic acid	95	±	1.60	0	±	0.31	103	±	0.97	0	±	0.36
chlorogenic acid	18	±	0.40	6	±	0.66	16	±	0.62	0	±	0.14
resorcinol	109	±	2.08	97	±	1.33	108	±	0.92	94	±	0.76
hydroquinone	73	±	3.24	46	±	0.69	23	±	0.59	1	±	0.05

[a]Final concentration of compound, [b]sodium diethyldithiocarbamate.

Table 10. Inhibition of some compounds on the activities of PhO and POD of cauliflower

compounds	PhO			POD		
	Ki (mM)[a]	IC_{50} (mM)	type of inhibition	*Ki* (mM)[a]	IC_{50} (mM)	type of inhibition
sodium diethldithiocarbamate	0.15 ± 0.01	0.64 ± 0.04	competitive	1.64 ± 0.03	6.61 ± 0.14	competitive
KCN	1.95 ± 0.13	7.88 ± 0.54	competitive	2.9×10^{-2} ± 0.2×10^{-2} [b]	0.03 ± 0.002	competitive
$CuSO_4$	0.16 ± 0.01	0.56 ± 0.05	non competitive	not measured[c]		
L-ascorbic acid	0.88 ± 0.02	3.55 ± 0.07	competitive	0.91 ± 0.020	3.67 ± 0.660	competitive
chlorogenic acid	0.19 ± 0.08	0.25 ± 0.10	non competitive	0.06 ± 0.002	0.24 ± 0.008	competitive
hydroquinone	1.93 ± 0.10	7.76 ± 0.41	competitive	0.16 ± 0.001	0.64 ± 0.005	competitive

[a]Final concentration of compound (10 mM), [b] final concentration of compound (1.0 mM), [c]slightly inhibited.

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM BROCCOLI (*Brassica oleraceae* L. var *botrytis)*

5.1 Introduction

Polyphenol oxidase oxidizes phenolic compounds to produce undesirable browning in damaged tissues of many fruits and vegetables. Because browning decrease the marketability of fruits and vegetables, many studies have been conducted on PPO aiming at preventing tissue discoloration of plant tissue. In fruits and vegetables most of PPO strongly oxidized *o*-diphenols such as chlorogenic acid, dopamine, and catechol.[4–10] The PPOs of apple,[4] garland chrysanthemum,[5] and edible burdock[6] strongly oxidized chlorogenic acid. In contrast, the purified PPOs of soybean,[15] edible burdock,[16] and Japanese radish root (Chapter 3) oxidized pyrogallol (1,2,3-trihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) but not oxidized *o*-diphenols. It found that a novel type of PPO with oxidizing activity of such 1,3,5-trihydroxybenzenes as phloroglucinol and phloroglucinol carboxylic acid in citrus fruits such as Satsuma mandarin[17] and in cruciferae vegetables such as turnip[18] and cabbage.[19,20] In chapter 3 and 4, same type of PPO were found in Japanese radish and cauliflower. These purified phloroglucinol oxidizing PPO (PhO) had strong POD activities as well. In contrast, PPO and POD in broccoli (*Brassica oleracea* L.) had been purified and characterized separately.[11,31] The purified broccoli florest PPO strongly oxidized catechol and 4-methylcatechol but not showed POD activity.[11]

As shown in chapter 2, we found that the crude enzyme extract from broccoli had strong PhO and POD activities which related with the browning of broccoli just like the cauliflower PPO (Chapter 4). However, no other detailed information is available on the broccoli PPO. Hence in the present chapter, we purified the PPO of broccoli using phloroglucinol as substrate, and characterized the activity of the purified enzyme.

5.2 Materials and Methods

5.2.1 Chemicals and Materials

Fresh broccoli (*B. oleracea* L.var. *botrytis*) was purchased from a local market in Saga City, Japan. DEAE-Toyopearl 650-M, CM-Sephadex C-50, butyl Toyopearl 650-M, and Toyopearl HW55-s were obtained from Tosoh Co., Tokyo, Japan. Other reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

5.2.2 Measurement of enzyme activity

PhO Activity. The activity of PhO was measured by the spectrophtometric method based on a difference in spectra[32] described in section 2.2.3. One unit of enzyme activity was defined as a change in absorbance of the mixture at 272 nm (△A272) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

PPO Activity. The activity of PPO was measured by the colorimetric method[7] described in section 2.2.3. One unit of enzyme activity was defined as a change in

absorbance of the mixture at 420 nm (△A420) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

POD Activity. The activity of POD was determined by the colorimetric method[19] described in section 2.2.3. One unit of enzyme activity was defined as a change in absorbance of the mixture at 470 nm (△A470) of 0.1 per min and per mL enzyme solution (1.0 cm light path).

5.2.3 Assay of enzyme properties

Assay of enzyme properties were performed using the same methods as described in section 3.2.3.

5.2.4 Determination of protein

Determination of protein was carried out with the same methods as described in section 2.2.4.

5.2.5 Molecular weight determination

The molecular weight of the purified enzyme was determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), which were carried out with the same methods as described in section 3.2.5.

5.2.6 Purification of the broccoli enzyme

Extraction and ammonium sulfate fractionation. All steps of purification were carried out at 4–5°C. The edible parts of broccoli (7 kg) were homogenized with

0.1 M phosphate buffer (pH 7.0). After filtration of the homogenate through a cotton cloth, the filtrate was centrifuged at 10,300 × g at 4°C for 20 min. The supernatant was then brought to 80% saturation of ammonium sulfate. Precipitated protein was collected by centrifugation (10,300 × g), dissolved in a small volume of 0.01 M phosphate buffer (pH 7.0), and then dialyzed against the same buffer for 36 h with the four changes of the dialysis buffer.

DEAE-Toyopearl 650-M chromatography. After another round of centrifugation (10,300 × g), the dialyzed solution was applied to a DEAE-Toyopearl 650-M column (4.5 × 15 cm) that was pre-equilibrated with 0.01 M phosphate buffer (pH 7.0) and eluted with the same buffer. The active fractions of PPO were pooled.

CM-Sephadex C-50 chromatography. Above active fractions were applied to a CM-Sephadex C-50 column (4.5 × 10 cm) pre-equilibrated with 0.01 M phosphate buffer (pH 7.0), and eluted with a linear gradient of sodium chloride from 0 to 1.0 M in 0.01 M phosphate buffer (pH 7.0). All of the PPO active fractions were collected and brought to a 1.0 M ammonium sulfate concentration.

Butyl-Toyopearl chromatography. Above collected fraction was applied to a butyl-Toyopearl 650-M column (1.6 × 15 cm) pre-equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 1.0 M ammonium sulfate, and eluted with a linear gradient of ammonium sulfate from 1 to 0 M in 0.01 M phosphate buffer (pH 7.0). The enzyme active fraction was pooled, dialyzed with 0.01 M phosphate buffer (pH 7.0), and concentrated using a membrane filter (Amicon YM-10, Millipore Japan Co., Tokyo, Japan).

Toyopearl HW 55-s chromatography. The final purification was done using a Toyopearl HW 55-s column (1.6 × 80 cm). After concentration, the solution was added to the column and equilibrated with 0.1 M phosphate buffer (pH 7.0), and eluted using the same buffer. The PPO active fraction was pooled as the purified enzyme of broccoli and used for enzyme characterization.

5.2.7 Statistical analysis

All experiments were conducted in triplicate and all the measurements were performed in triplicate. Standard deviation of the data was indicated.

5.3 Results and Discussion

5.3.1 Purification of broccoli enzyme

The broccoli PPO was purified by precipitating with 80% ammonium sulfate, ion exchange chromatography, hydrophobic chromatography, and gel filtration. **Figure 19** shows a typical elution pattern of PPO on a final gel filtration column. The enzyme activity showed a sharp single peak on the column. The active fractions of enzyme from several columns were pooled as the purified enzyme. The activity and yield of PPO in the course of purification are presented in **Table 11**. Finally, the PPO was purified up to 80 ± 15 fold with a recovery rate 26 ± 4.5% as compared with the crude enzyme. The purified enzyme was used for further studies.

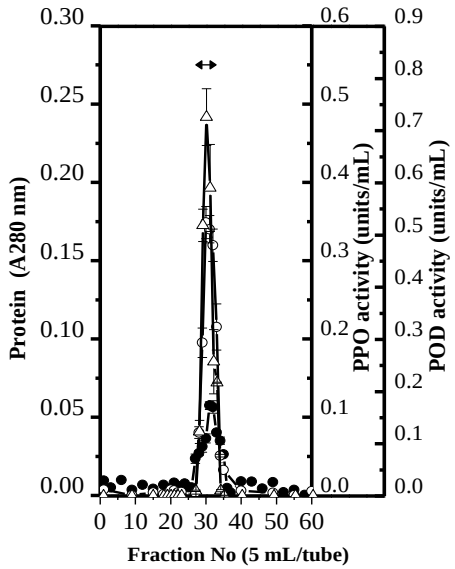


Figure 19. Elution pattern of the broccoli enzyme from a Toyopearl HW 55-s column. (↔) fraction pooled; (●) protein; (○) PPO activity; (△) POD activity.

Table 11. Purification of the broccoli enzyme

enzyme	PhO														POD								
	volume (mL)	total activity (unit)			total protein (mg)			specific activity (unit/mg protein)			purification (fold)			recovery (%)			total activity (unit)			specific activity (unit/mg protein)			purification (fold)			recovery (%)		
crude Enzyme	100	30600	±	2456	843	±	24.9	36	±	3	1	±	0	100	±	0.0	37917	±	2458	45	±	2	1	±	0.0	100	±	0.0
DEAE-Toyopearl 650 M	253	22770	±	2172	35	±	0.9	654	±	78	18	±	1	75	±	6.2	12239	±	171	351	±	8	8	±	0.6	32	±	2.0
CM-Sephadex C-50M	313	19250	±	282	23	±	1.2	833	±	51	23	±	2	63	±	5.4	12729	±	690	552	±	53	12	±	1.5	34	±	3.4
butyl Toyopearl 650-M	317	9191	±	353	8	±	0.4	1154	±	75	32	±	5	30	±	3.4	5081	±	269	639	±	67	14	±	1.0	13	±	0.2
HW Toyopearl 55-s	103	7902	±	783	3	±	0.1	2875	±	359	80	±	15	26	±	4.5	4147	±	412	1508	±	178	33	±	2.9	11	±	0.9

5.3.2 Characterization of the purified enzyme

As shown in **Figure 20**, the purified enzyme produced a single band on SDS-PAGE. The molecular weight of purified enzyme was estimated to be about 63 kDa by SDS-PAGE and 65 kDa by gel filtration (**Figure 21**). These results suggest that the purified PPO is a monomer protein. Gawlik-Dziki et al.[11] reported the monomer PPO in broccoli florest and estimated its molecular weight to be about 51.3 kDa by SDS-PAGE and 57 kDa by gel filtration. The difference in the estimated molecular weights may due to the different PPO isoenzyme: the broccoli florest PPO was purified using catechol as a substrate. Almost same molecular weight of purified PPO were found in apple (65 kDa),[4] butter lettuce (60 kDa),[12] and Turkish black radish (66 kDa).[42] And lower molecular weight of the purified PPOs were found in soybean (47 kDa),[15] turnip (27 kDa),[18] cabbage F-IA (40 kDa),[19] cabbage PhO F-IB (45 kDa) and PhO F-II (33 kDa),[20] and leaf lettuce (46 kDa).[40]

As shown in **Table 12**, purified broccoli PPO strongly oxidized 1,3,5-tryhdroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid but not oxidized 1,2,3-trihydroxybenzenes and *o*-diphenols. Similar substrate specificities were found for the PPOs of Satsuma mandarin,[17] turnip,[18] cabbage,[19,20] and cauliflower in chapter 4 which only oxidized 1,3,5-trihydroxybenzenes. The purified PPOs of soybean[15] and edible burdock[16] oxidized both 1,3,5-trihydroxybenzenes and 1,2,3-trihydroxybenzenes, but not oxidized *o*-diphenols. As shown in chapter 3, the same substrate specificity was found in the purified PPO of Japanese radish root. In case of the PPOs of fruits

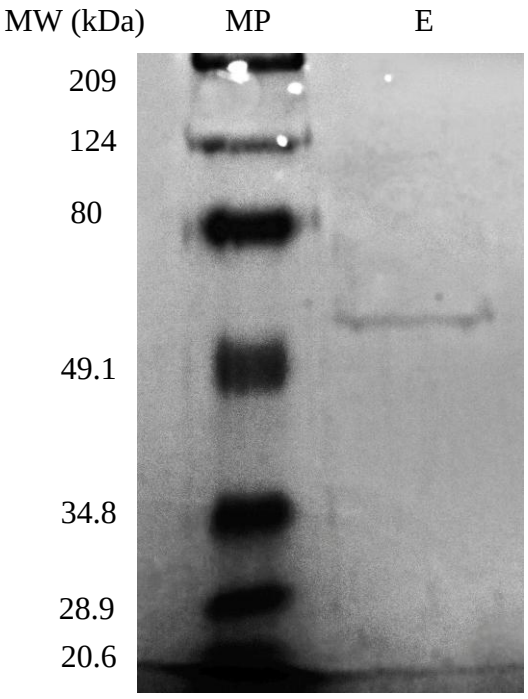


Figure 20. SDS-PAGE of the purified broccoli enzyme. MW, Molecular Weight; MP, Marker Protein; E, Enzyme.

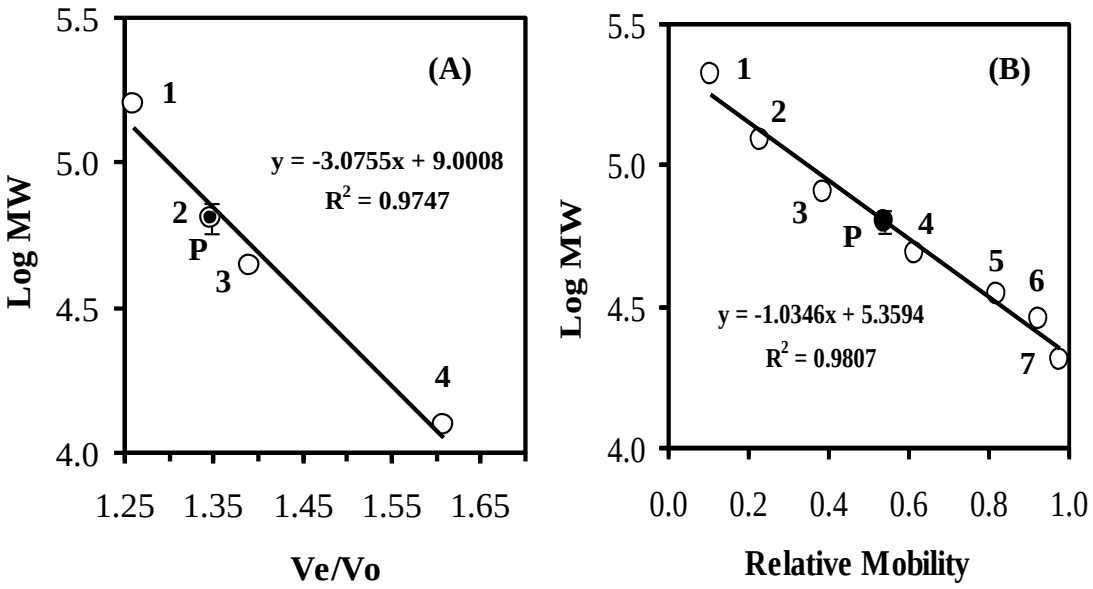


Figure 21. Molecular weight estimation of the broccoli enzyme by gel filtration on a Toyopearl HW 55-s column (A) and by SDS-PAGE (B).

(A) Vo, void volume of the column; Ve, elution volume of the substance; MW, molecular weight in kilo daltons: 1. α-globulin (160 kDa), 2. serum albumin (bovine; 65 kDa), 3. ovalbumin (chicken egg; 44 kDa), and 4. cytchrome c (12.4 kDa); P, purified enzyme.

(B) MW, molecular weight in kDa: 1. myosin (209 kDa), 2. β-galactosidase (124 kDa), 3. serum albumin (bovine; 80 kDa), 4. ovalbumin (chicken egg; 49.1 kDa), 5. carbonic anhydrase (34.8 kDa), 6. soybean trypsin inhibitor (28.9 kDa), and 7. lysozyme (20.6 kDa); P, purified enzyme.

Table 12. Substrate specificities of broccoli PPO

substrates	specific activity (unit/mg protein)
phloroglucinol[1]	2875 ± 359
phloroglucinol carboxylic acid[1]	1232 ± 39
pyrogallol[2]	0 ± 0
gallic acid[2]	0 ± 0
catechol[2]	0 ± 0
chlorogenic acid[2]	0 ± 0
D,L-Dopa[2]	0 ± 0
dopamine[2]	0 ± 0
resorcinol[2]	0 ± 0

[1]Measured by the spectrophotometric method based on differences in spectra[32]
[2]Measured by the colorimetric method[7]

and vegetables, mostly they oxidized *o*-diphenols such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methycatechol. These results indicate that the broccoli PPO we purified belongs to a new type PPO namely "phloroglucinol oxidase (PhO)".

The Michaelis constant (K_m) of the broccoli PPO for oxidation of phloroglucinol was 11 mM (**Figure 22 A**). The K_m value of broccoli PhO was smaller than turnip PhO (0.67 mM),[18] cabbage PhO F-IA (6.4 mM),[19] cabbage PhO F-IB (8.5 mM),[19] and cabbage PhO F-II (1.3 mM).[20] Gawlik-Dziki et al[11] reported larger K_m value in broccoli florest PPO[11] and estimated its K_m value to be about 12.3 mM for catechol and 21.0 mM for 4-methylcatechol.

The purified broccoli enzyme preparation was red in color with an absorption maxima at 405, 490, and 630 nm. Similar results had been obtained using purified PPOs of Satsuma mandarin,[17] turnip,[18] and cabbage,[19,20] which enzymes contain iron. Soybean, Satsuma mandarin, cabbage, and turnip enzymes had dual PPO and POD activities. In the present study, the purified PPO from broccoli gave a single band of protein on SDS-PAGE (**Figure 20**). These results show that the purified broccoli enzyme has a dual activity of PhO and POD, just like the enzymes from Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] At the final step of purification, the broccoli POD activity increased to 33 ± 2.9 fold with a recovery rate of 11 ± 0.9% (**Table 11**). The K_m value of the broccoli POD for the oxidation of guaiacol was 2.9 mM (**Figure 22 B**). The K_m value of three broccoli peroxidase isoenzymes had been reported by Thongsook and Barrett:[31] acidic POD 0.31 mM, neutral POD 0.71 mM, and basic POD 8.79 mM.

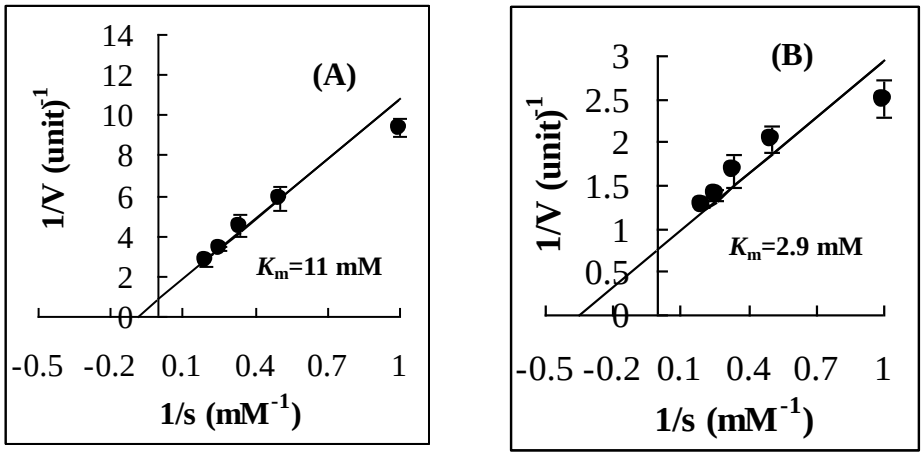


Figure 22. Lineweaver-Burk plots of phloroglucinol (A) and guaiacol (B) oxidation by the broccoli enzyme.

As shown in **Figure 23 A**, the optimum pH of PhO and POD of broccoli was 8.0 and 7.0, respectively. The different optimum pH for PhO and POD had been found in the enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage .[19,20] The optimum pH for the activity of the broccoli PhO was similar to the PPOs purified from turnip (7.6¬8.0),[18] cabbage F-IB (8.0),[20] and Japanese radish root (8.0) (Chapter 3). However, the optimum pH for our purified broccoli PhO differed from that of broccoli florest PPO[11] measured by using catechol and 4-methylcatechol as substrates (pH 5.7). The difference in the optimum pH may due to the different substrates used. The optimum pH of our purified enzyme for POD (pH 7.0) also differed from that of broccoli POD which showed the optimum at pH 6.0 for both neutral and basic PODs and at pH 4.0 for acidic POD.[31]

As shown in **Figure 23 B**, the activities of PPO and POD from broccoli were stable in the wide pH ranges. Eighty percent of the PPO and POD activities were retained in the pH ranges 5.0−10.0. The ranges of pH stability for both PPO and POD were almost same to the stability of cabbage PhO F-IA,[19] which were stable in the range of pH 5.0−11.0. Different result was reported for Satsuma mandarin PPO[17] and turnip PPO.[18]

Figure 24 shows the effect of temperature on the activity and stability of PhO and POD of the purified broccoli enzyme. The optimum temperature for the activities of PhO and POD were 60°C and 20°C, respectively (**Figure 24 A**). The optimum temperature for broccoli PhO was almost same with that of soybean (50°C).[15] However, it was different from that of banana peel (30°C)[8] and cabbage PhO F-IA (40°C).[19] The optimum temperature for broccoli POD was different

from that of cabbage PhO F-IA (45°C)[19] and Turkish black radish (30°C).[42] **Figure 24 B** shows the thermal stability of broccoli PPO enzyme. The activities of PhO and POD were stable at temperature range of 30–60°C. After heating at 70°C for 10 min, 40% of the PPO activity remained; however, POD activity was almost completely lost.

Table 13 shows the effects of several compounds on the activities of purified PhO and POD. Both the PhO and POD activities were markedly inhibited by the metallic enzyme inhibitors such as sodium diethyldithiocarbamate and potassium cyanide. L-ascorbic acid (reducing agent), chlorogenic acid (*o*-diphenol), and hydroquinone (*p*-diphenol) strongly inhibited both PhO and POD activities. EDTA and $CuSO_4$ inhibited PhO activity, but a little affected POD activity. NaF, NaCl, $MnCl_2$, $BaCl_2$, $ZnSO_4$, and resorcinol (*m*-diphenol) a little affected both PhO and POD activities. On the other hand, the strong activation of PhO was induced by $MnCl_2$. Similar results were obtained for the PhOs from Satsuma mandarin,[17] turnip,[18] cabbage,[19,20] and cauliflower in chapter 4.

Table 14 shows the inhibitory action of seven compounds on the activity of PhO and POD from broccoli. Sodium diethyldithiocarbamate, EDTA, $CuSO_4$, L-ascorbic acid, chlorogenic acid, and hydroquinone inhibited PhO and POD activities in a competitive manner. On the other hand, KCN inhibited the activities of PhO and POD in a different manner: KCN inhibited PhO activity in a competitive manner and inhibited POD activity in a noncompetitive manner irrespective of the fact that the activities of PhO and POD appeared in the same peak. These results strongly indicate that PhO and POD have separate active sites.

In addition, KCN inhibited the activities of PhO and POD at 1 and 10 mM. All of these results indicate that the purified polyphenol oxidase from broccoli is a metallic enzyme, quite probably a iron-containing enzyme judging from the *K*i constants recorded (**Table 14**), we can concluded that the activities of PhO and POD were most effectively inhibited by sodium diethyldithiocarbamate which acts as a competitive inhibitor. Almost same result had been found in nettle PPO,[13] however, it was different from broccoli florest PPO[11] and artichoke PPO[14] which were most effectively inhibited by sodium sulphate and dithiothreitol, respectively. The IC_{50} values were also obtained with these compounds, using phloroglucinol and guaicol as substrates for PhO and POD, respectively (**Table 14**).

5.4 Conclusion

The PhO of broccoli was purified to a homogeneous state on SDS–PAGE. The purified broccoli enzyme oxidized 1,3,5-trihdroxybenzenes but not oxidized 1,2,3-trihydroxybenzenes and *o*-diphenols. These results may indicate that this enzyme is a new type of PPO, “phloroglucinol oxidase”, which is similar to the enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] The purified broccoli enzyme showed a dual activity of PhO and POD. The activity of PhO and POD was affected in a different manner by pH, temperature, various compounds exaimined, and inhibitors tested. Based on these results, we conclude that the purified broccoli enzyme has separate active sites for PhO and POD, just like the enzymatic characteristics of cabbage,[19,20] Japanese radish root (Chapter 3), and cauliflower (Chapter 4). The biochemical properties of broccoli PPO it found

were different from those obtained by other researches on the broccoli florest PPO[11] and broccoli POD.[31] The difference may due to the different of substrates and broccoli varieties used, and/or due to the different PPO isoenzyme purified.

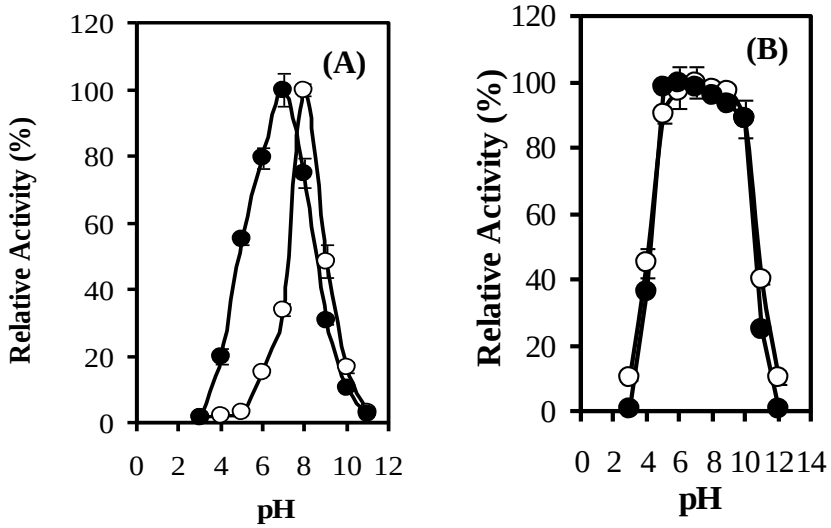


Figure 23. Effect of pH on the activity (A) and stability (B) of the broccoli enzyme. (○) PhO activity; (●) POD activity.

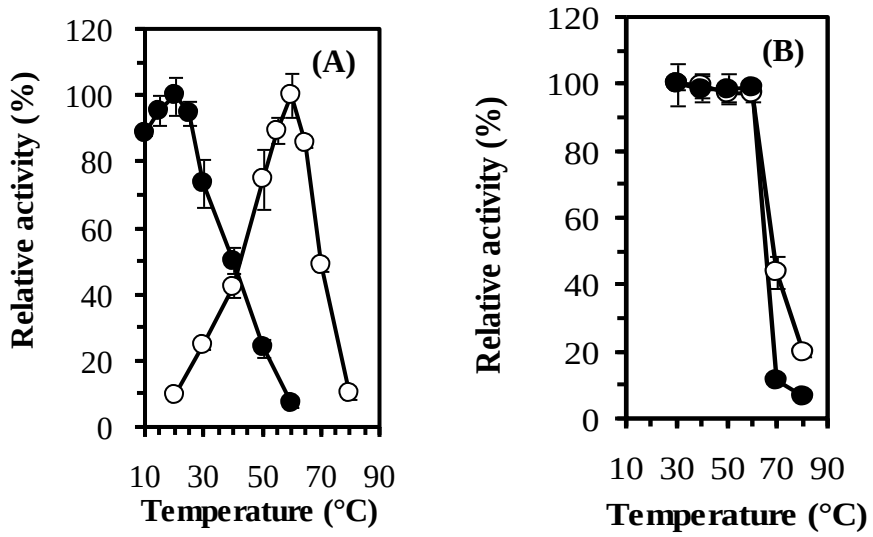


Figure 24. Effect of temperature on the Activity (A) and stability (B) of the broccoli enzyme. (○) PhO Activity; (●) POD Activity.

Table 13. Effects of various compounds on the activities of PhO and POD of broccoli

compounds	relative activity (%)			
	PhO		POD	
	1 mM[a]	10 mM[a]	1 mM[a]	10 mM[a]
none	100 ± 0.20	100 ± 0.20	100 ± 1.29	100 ± 1.29
$NaS_2CN(C_2H_5)_2$ [b]	9 ± 1.23	6 ± 0.10	0 ± 0.05	0 ± 0.11
KCN	54 ± 1.50	29 ± 1.21	0.8 ± 0.09	0 ± 0.89
EDTA	74 ± 2.65	68 ± 2.31	107 ± 2.01	98 ± 1.43
NaF	103 ± 5.40	114 ± 5.10	91 ± 1.56	113 ± 0.99
NaCl	93 ± 0.93	103 ± 0.93	109 ± 2.10	112 ± 1.23
$MnCl_2$	347 ± 4.13	351 ± 4.90	131 ± 5.34	142 ± 1.43
$CuSO_4$	13 ± 0.89	10 ± 0.22	100 ± 0.21	98 ± 0.98
$BaCl_2$	103 ± 1.22	105 ± 1.32	110 ± 3.98	112 ± 1.45
$ZnSO_4$	109 ± 0.79	116 ± 0.89	107 ± 1.72	111 ± 1.67
L-ascorbic acid	0 ± 0.34	0 ± 0.11	0 ± 0.12	0 ± 0.15
chlorogenic acid	14 ± 0.65	13 ± 0.90	57 ± 2.34	0 ± 0.79
resorcinol	98 ± 1.43	108 ± 1.65	116 ± 4.54	116 ± 1.45
hydroquinone	37 ± 1.54	17 ± 0.79	37 ± 1.01	0.7 ± 0.02

[a]Final concentration of compound, [b]sodium diethyldithiocarbamate.

Table 14. Inhibition of some compounds on the acivities PhO and POD of broccoli

compounds	PhO			POD		
	Ki (mM)[a]	IC_{50} (mM)	type of Inhibition	*Ki* (mM)[a]	IC_{50} (mM)	type of Inhibition
$NaS_2CN(C_2H_5)_2$	2.4×10^{-4} [b] ± 0.5×10^{-4}	2.6×10^{-4} ± 0.4×10^{-4}	competitive	0.23 ± 0.02	0.31 ± 0.04	competitive
KCN	1.65 ± 0.12	1.8 ± 0.02	competitive	0.59 ± 0.12	0.80 ± 0.09	non competitive
EDTA	11.0 [c] ± 1.03	24.8 ± 3.57	competitive		not measured[d]	
$CuSO_4$	2.7×10^{-4} [b] ± 0.9×10^{-4}	2.8×10^{-4} ± 0.4×10^{-4}	competitive		not measured[d]	
L-ascorbic acid	0.38 ± 0.07	0.41 ± 0.06	competitive	0.27 ± 0.09	0.36 ± 0.05	competitive
chlorogenic acid	0.23 ± 0.05	0.25 ± 0.03	competitive	3.57 ± 0.14	4.80 ± 0.12	competitive
hydroquinone	0.28 ± 0.09	0.31 ± 0.02	competitive	0.80 ± 0.08	1.08 ± 0.08	competitive

[a]Final concentration of compound (1 mM), [b]final concentration (0.01 mM), [c]final concentration (15 mM), [d] slightly inhibited

CHAPTER 6
GENERAL DISCUSSION

Polyphenol oxidase oxidizes phenolic compounds to produce undesirable browning in damaged tissues of many fruits and vegetables. Their quality and marketability decrease because of this type browning during storage and processing. To prevent such browning, PPO has been widely investigated in many fruits and vegetables. Most of PPO studied strongly oxidized *o*-diphenols such as chlorogenic acid, dopamine, and catechol.[4–14] The distribution of PPO in fruits and vegetables vary for different plant sources. As summarized in **Table 15**, the PPOs of garland chrysanthemum,[5] Japanese butterbur,[34] and edible yam[35] strongly oxidized chlorogenic acid. In addition, banana PPOs[7,8] strongly oxidized dopamine and broccoli florest,[11] butter lettuce[12] and cherry pulp[37] strongly oxidized catechol. A novel type of PPO, which only oxidize 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid in citrus fruits such as Satsuma mandarin[17] and in cruciferae vegetables such as turnip[18] and cabbage.[19,20] The phloroglucinol oxidizing enzyme (PhO) also has strong POD activity.[17–20] On the other hand, the purified PPOs of soybean[15] and edible burdock[16] oxidized not only 1,3,5-trihydroxybenzene but also 1,2,3-trihydroxybenzene, such as pyrogallol and gallic acid, but not oxidized *o*-diphenols. These enzymes also have POD activity. The PPO and POD play important roles in the determination of color and flavor. Therefore, determination of PPO and POD is important to identify its biochemical properties and function and, in turn, to understand how to prevent its deteriorative action during storage

and processing.

As described above, a novel type of PPO, which only oxidize 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid in cruciferae vegetables such as turnip[18] and cabbage.[19,20] The distribution of PhO in the cruciferae vegetables was firstly investigated. **Table 16** shows substrate specificities of crude enzyme from some cruciferae vegetables. Cabbage, turnip, broccoli, and cauliflower PPOs strongly oxidized 1,3,5-trihydroxybenzenes, but not oxidized 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. On the other hand, PPOs of mizuna, takana, and komatsuona strongly oxidized 1,3,5-trihydroxybenzenes, but have pyrogallol oxidizing activity. In addition, leaf mustard, katsuona, and Japanese radish root PPO have small activity toward pyrogallol. **Table 1** also shows that all the cruciferae vegetables PPOs tested did not show activity toward *o*-diphenol. On the other hand, as listed in **Table 15**, most of PPO studied strongly oxidized *o*-diphenol, however, the amount of activity for each compound was different from those of plant sources. For example, garland chrysanthemum and Japanese butterbur strongly oxidized chlorogenic acid; banana pulp, banana peel, and edible yam strongly oxidize dopamine; red Swiss chard leaves strongly oxidized L-DOPA. Catechol was found the main substrate in broccoli florest,[11] butter lettuce,[12] and cherry pulp.[37] The main substrate of fruits and vegetables are different from plant source. According to Yoruk and Marshall,[1] the PPO is active to those substrates with the high preference to the enzyme.

As shown in chapter 2 (**Table 3**), the PPOs from cruciferae vegetables also

had strong POD activity. PPO and POD in broccoli (*Brassica oleracea* L.) had been purified and characterized separately.[11,31] The purified broccoli florest PPO strongly oxidized catechol and 4-methylcatechol but not showed POD activity.[11] On the other hand, the purified PPO from turnip and cabbage only oxidized 1,3,5-trihydroxybenzene and also have POD activity. As shown in chapter 2, we found that the crude enzyme from Japanese radish, cauliflower, and broccoli had strong PhO and POD activities. However, no other detailed information is available on these PPOs. Hence in the chapter 3, 4, and 5, we purified the PPO of broccoli using phloroglucinol as a substrate and characterized the activity of the purified enzymes.

PPOs from Japanese radish root (chapter 3), cauliflower (chapter 4), and broccoli (chapter 5) have been purified by ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. As shown in chapter 3, 4, and 5, the purified enzymes tested showed a sharp single peak on the final gel filtration column and gave a single band of protein on SDS-PAGE (**Figure 8, 14, and 20**). Dual activity of PhO and POD was found in those purified enzymes. Characteristics of these enzymes for PhO and POD were summarized in **Table 16**. In this table, characteristics of purified enzymes of Satuma mandarin, turnip, and cabbage (F-IA, F-IB and F-II) were also listed. To compare the enzyme properties of *o*-diphenol oxidase, characteristics of the “chlorogenic acid oxidase” which were determinated in our laboratory, were also listed in **Table 17**.

The all purified enzymes tested appeared as a single band on SDS-PAGE.

The molecular weight of these purified enzymes were estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE for Japanese radish; about 60 kDa by SDS−PAGE and 54 kDa by gel filtration for cauliflower; about 63 kDa by SDS-PAGE and 65 kDa by gel filtration for broccoli. These results indicate that the purified enzymes are monomeric. While the molecular weight of the purified Japanese radish root PPO was similar to soybean PPO (47 kDa)[15] and leaf lettuce PPO (46 kDa),[40] but it differed from those of edible burdock (40kDa),[6] broccoli florest (57 kDa),[11] butter lettuce (60 kDa),[12] turnip (27kDa),[18] cabbage F-IA(40kDa),[19] cabbage F-IB (43kDa),[20] Japanese pear (56 kDa),[41] black radish (66 kDa),[42] cauliflower (60 kDa) (Chapter 4), and broccoli (63 kDa) (Chapter 5). These results indicated that the difference in the estimated molecular weights may due to the different PPO isoenzyme and/or different plant resource.

Purified PPO of Japanese radish root strongly oxidized phloroglucinol, and also oxidized 1,2,3-trihydroxybenzenes, such as pyrogallol and gallic acid; however, it not oxidized *o*-diphenols, such as catechol, chlorogenic acid, and dopamine, or *p*-diphenols, such as resorcinol. Similar substrate specificity was found in purified soybean PPO[15] and edible burdock PPO.[16] However, as shown in **Table 16**, the substrate specificities of these three enzymes were different from the PPOs of cauliflower, broccoli, turnip, and cabbage which only oxidized 1,3,5-trihydroxybenzenes. As described above, in case of the other fruits and vegetables, most of PPO oxidized *o*-diphenols such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methycatechol. These results indicate that the Japanese radish, cauliflower, and broccoli PPOs belong to a group of a new type

of PPO that is “phloroglucinol oxidase (PhO)”.

Purified Japanese radish root, cauliflower, and broccoli PPO strongly oxidizes phloroglucinol. The difference of Michaelis constants (K_m) of the enzymes for the oxidation of phloroglucinol were found in the enzyme resource (**Table 17**). The values of each enzyme were calculated for Japanese radish as 2.0 mM; for cauliflower as 3.3 mM; and for broccoli as 11.0 mM. These values also differed from that of other plant PPOs, specifically, Satsuma mandarin PPO (0.67 mM),[17] cabbage PPO F-IA (6.4 mM),[19] and cabbage PPO F-IB (8.5 mM).[20] The value of K_m indicated the affinity of the enzyme for enzyme-substrate complex formation. The K_m value of the Japanese radish root enzyme was larger than cauliflower and broccoli enzyme. It is indicated that Japanese radish root PPO has the greater affinity than cauliflower and broccoli PhO, and cauliflower has a greater affinity than broccoli PhO. The K_m value of cauliflower enzyme was also larger than turnip PhO,[18] but smaller than cabbage PhO.[19,20] It was indicated that cauliflower PhO has the greater affinity compared to cabbage PhO, but it reverses to turnip PhO. As shown in **Table 18**, in the case of *o*-diphenolase, the values of K_m vary for different substrate used. For example, the K_m values from garland chrsanthemum[5] were 2.0 and 10.0 mM for chlorogenic acid and (-)-epicatechin, respectively.

The purified Japanese radish root, cauliflower, and broccoli enzyme preparations were red in color with an absorption maxima at 405,490, and 630 nm, which was similar to that of soybean PPO.[15] Similar results were obtained using purified PPOs of Satsuma mandarin,[17] turnip,[18] and cabbage[19,20] which contain

iron. Soybean,[15] Satsuma mandarin,[17] turnip,[18] and cabbage,[19,20] enzymes had dual PPO and POD activities. As shown in **Figure 7**, **Figure 13**, and **Figure 19** activities of both PPO and POD were detected in the same peak fraction with the final purification of the enzymes tested. Furthermore, the purified enzymes were visualized as a single protein band on SDS-PAGE (**Figure 8, Figure 14, and Figure 20**). Therefore, we reasoned that these three enzymes have dual PPO and POD activities, similar to the soybean,[15] turnip,[18] and cabbage[19,20] enzymes. Further characterization of the PPO and POD activities of the enzymatic preparation was conducted.

The optimum pHs of Japanese radish root, cauliflower, and broccoli enzymes for PPO activity were 8.0. However, optimum pHs for POD activity were different each other: that was Japanese radish, 5.0; cauliflower, 4.0; broccoli, 7.0. The different optimum pH for PhO and POD had been also found in the enzymes of Satsuma mandarin, turnip, and cabbage. The optimum pHs for the activity of the three purified PhOs were similar to the PPOs purified from turnip (7.6¬8.0) and cabbage F-IB (8.0). However, the optimum pH for our three purified PhOs differed from that of broccoli florest PPO[11] measured by using catechol and 4-methylcatechol as substrates (pH 5.7). The difference in the optimum pH may due to the different substrates used. The optimum pH of our purified broccoli enzyme for POD (pH 7.0) also differed from that of broccoli POD which showed the optimum at pH 6.0 for both neutral and basic PODs and at pH 4.0 for acidic POD.[31] In the case of "chlorogenic acid oxidase", the pH optima from various fruits and vegetables PPO by using *o*-diphenol such ChO and EPO

as a substrate showed widely in the optimum pH range of 4.0–8.0 (**Table 18**).

As shown in **Table 17**, eighty percent of the PPO and POD activities of Japanese radish enzyme were retained in the pH ranges 3.0¬10.0 and pH 3.0¬11.0, respectively. The ranges of pH stability for both PPO and POD activities differed slightly from those of cabbage F-IA PPO[19] in which both activities were stable in the range of pH 5.0–11.0. Similar results were found for Satsuma mandarin PPO[17] and turnip PPO.[18] On the other hand, the activity of PhO of cauliflower enzyme was more stable than POD in the wide range of pH. Over 80% of PhO activity remained in the pH range of 3.0–11.0. However, the POD activity was stable at pH 5.0–8.0 and declined rapidly at the pH below 8.0 after 20 h incubation at 5°C. The stability of PhO and POD of cauliflower was slightly different from that of cabbage PhO F-IA[19] in the range of pH 5.0–11.0. Köksal and Gülçin showed that the pH stability of cauliflower buds[45] by using several substrates in the range of pH 8.0–9.0. In the present result, it was found close pH stability from that of cauliflower buds by using guaiacol as substrate that is pH 8.5. In addition, the activities of PPO and POD from broccoli enzyme were stable in the wide pH ranges. Eighty percent of the PPO and POD activities were retained in the pH ranges 5.0–10.0. The ranges of pH stability for both PPO and POD were almost same to the stability of cabbage PhO F-IA which were stable in the range of pH 5.0 –11.0. Differed result was reported for Satsuma mandarin[17] and turnip[18] PhOs.

The PhO enzyme from Satsuma mandarin,[17] turnip,[18] and cabbage[19,20] had a very high thermal stability. After heating at 80°C for 10 min, only 20% of the Japanese radish PhO activity remained; however, POD activity was almost

completely lost. The thermal stability of this preparation of PPO was lower than that of the above enzymes, specifically that of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] In contrast, the soybean enzyme[15] had a similar thermal stability of PPO and POD activities as the Japanese radish root enzyme. On the other hand, the purified enzyme of cauliflower showed a relatively low thermal stability. The activity of PhO and POD was stable at the temperature range of 20–50°C. At the higher temperature than 50°C, the activity of POD was almost lost. Contrarily, the PhO was more heat-stable and 60% of its activity remained after heating at 80°C for 10 min (**Figure 18 B**). The POD activity was close with that of found by Rayan et al.[44] in which showed that the heat inactivation kinetic for POD cauliflower was at temperature range from 65–85°C. It indicated that the POD activity will be inactivated at temperature above 65°C. In addition, the activities of PhO and POD of broccoli enzyme were stable at temperature range of 30–60°C. After heating at 70°C for 10 min, 40% of the PPO activity remained; however, POD activity was almost completely lost.

The optimum temperature of PhO and POD activities of cauliflower enzyme were 55°C and 20°C, respectively. The optimum temperature for cauliflower PhO was almost same with that of soybean (50°C).[15] However, it was different from that of banana peel (30°C)[8] and cabbage F-IA (40°C).[19] The optimum temperature of cauliflower POD was different from that of cabbage F-IA (45°C) [19] and slightly different from that of Turkish black radish (30°C).[42] In addition the optimum temperature for the activities of PhO and POD of the broccoli enzyme were 60°C and 20°C, respectively. The optimum temperature for broccoli PhO was almost

same with that of soybean (50°C).[15] However, it was different from that of banana peel (30°C)[8] and cabbage PhO F-IA (40°C).[19] The optimum temperature for broccoli POD was different from that of cabbage PhO F-IA (45°C)[19] and Turkish black radish (30°C).[42]

Table 17 also shows the effect of different compounds on the PPO and POD activities of the purified enzymes. Almost same effect of these compounds were found in all purified enzyme tested. Both the PPO and POD activities of all purified enzymes were markedly inhibited by metallic enzyme inhibitors such as sodium diethyldithiocarbamate and potassium cyanide when used at a final concentration of 10 mM. These results indicate that all purified enzyme are metallic enzyme, and the metal is seemed to be iron as mentioned previously. Additionally, L-ascorbic acid inhibited both PPO and POD activities at 10 mM. $CuSO_4$ inhibited PPO activity, but little inhibited POD activity. $MnCl_2$ was found to remarkable activator of PPO in cauliflower, broccoli, edible burdock,[16] turnip,[18] and cabbage;[19,20] however, a little activation was found for Japanese radish root PPO. Almost same result had been found in nettle PPO,[13] however, it was different from broccoli florest PPO[11] and artichoke PPO[14] which were most effectively inhibited by sodium sulphate and dithiothreitol, respectively. The activities of PhO and POD of all purified enzymes tested were markedly inhibited by chlorogenic acid (*o*-diphenols) and hydroquinone (*p*-diphenol) just like the enzymes of Satsuma mandarin[17] and cabbage.[19,20] As shown in **Table 10** and **Table 14**, Sodium diethyldithiocarbamate, $CuSO_4$, L-ascorbic acid, and hydroquinone inhibited both of PhO and POD activities in a competitive manner. On the other

hand in cauliflower, chlorogenic acid inhibited PhO activity in a noncompetitive manner and inhibited POD activity in a competitive manner (**Table 10**). And in broccoli enzyme (**Table 14**), KCN inhibited PhO activity in a competitive manner and inhibited POD activity in a noncompetitive manner. It is interesting that, chlorogenic acid and KCN inhibited the activities of PhO and POD in a different manner irrespective of the fact that the activities of PhO and POD appears in the same peak. These results strongly indicate that PhO and POD have separate active sites.

Table 18 also shows the effect of inhibitors to various fruit and vegetables PPO using *o*-diphenol such as chlorogenic acid and epicatechin as a substrate. The results showed that KCN and L-ascorbic acid strongly inhibited all enzymes ChO and EPO. In addition, sodium diethyldithiocarbamate and $MnCl_2$ also strongly inhibited apple,[4] garland chrysanthemum,[5] edible burdock,[6] Japanese butterbur,[34] and leaf lettuce,[40] but little inhibited Japanese pear[41] and eggplant[46] ChOs and EPOs.

In conclusion, the PPOs of Japanese radish, cauliflower, and broccoli were purified to a homogeneous state on SDS–PAGE. The purified enzymes tested oxidized 1,3,5-trihdroxybenzenes but not oxidized 1,2,3-trihydroxybenzenes and *o*-diphenols. These results may indicate that these enzymes are a group of a new type of PPO, namely “phloroglucinol oxidase”, which is similar to the enzymes of Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] All purified enzymes showed a dual activity of PhO and POD. The activity of PhO and POD was affected in a different manner by pH, temperature, various compounds, and type of inhibition

tested. The biochemical properties of broccoli PPO we found were different from those obtained by other researches on the broccoli florest PPO[11] and difference from broccoli POD.[31] The difference may due to the different of substrates and broccoli varieties used, and/or due to the different PPO isoenzyme purified. Based on these results, we speculate that all purified enzymes tested have separate active sites for PhO and POD. The results of biochemical properties of all enzymes were also beneficial in an attempt to inhibit or control PhO and POD activities in Japanese radish, cauliflower, and broccoli during storage and processing.

Table 15. Substrate specificity of PPO from fruits and vegetables

substrates	relative activity (%)								
	garland chrysanthemum[5]	banana pulp[7]	banana peel[8]	broccoli florets[11]	butter lettuce[12]	Japanese butterbur[34]	edible yam[35]	red Swiss chard leaves[36]	cherry pulp[37]
***o*-diphenols**									
catechol	76	54	34.0	100	100	39	35	86	100
4-methylcatechol				62.76	88.50				
chlorogenic acid	100	24.5	5.3	1.89	53.40	100	6.5	80	
DL-DOPA	72	12.3	8.0			2	6.5		
L-DOPA								100	
dopamine	74	100	100			9	100		
resorcinol	0	0	0			0	0		0
caffeic acid		2.0	0.7	5.92	2.30	42			
D-catechin	70	35.6	11.5			37			
epicatechin	100	22.7	9.3			94	20		
p-dimethylphenol								10	
m-dimethylphenol								11	
ferulic acid				1.10	0.20				
1,2,3-trihydroxybenzenes:									
pyrogallol	70	5.5	1.4			0	15		
gallic acid	72	0	0			0	0	73	70.1
1,3,5-trihydroxybenzenes:									
Phloroglucinol	0	0	0	0.16		0	0		0

Table 16. Substrate specificity of crude PPO from various cruciferae vegetables

substrates	relative activity (%)									
	mizuna	takana	cabbage	turnip	broccoli	leaf mustard	katsuona	komatsuna	cauliflower	Japanese radish root
1,3,5-trihydroxybenzenes:										
phloroglucinol	100	100	100	100	100	100	100	100	100	100
phloroglucinol carboxylic acid	45	99	71	47	86	34	25	40	42	46
1,2,3-trihydroxybenzenes:										
pyrogallol	15	18	0	0	0	4	2	26	0	2.5
gallic acid	0	0	0	0	0	0	0	0	0	0.4
***o*-diphenols**										
catechol	0	0	0	0	0	0	0	0	0	0
chlorogenic acid	0	0	0	0	0	0	0	0	0	0
DL-DOPA	0	0	0	0	0	0	0	0	0	0
dopamine	0	0	0	0	0	0	0	0	0	0
***m*-diphenols**										
resorcinol	0	0	0	0	0	0	0	0	0	0

Above data were averages of three trials

Table 17. Characterization of PhO from fruits and vegetables

origin	Japanese radish root		cauliflower		broccoli		Satsuma mandarin[17]		turnip[18]		cabbage F-IA[19]		cabbage F-IB[20]		cabbage F-II[20]	
Molecular weight																
SDS-PAGE (kDa)	45.7		60		63		–		–		40		43		32	
gel filtration (kDa)	44.0		54		65		27		27		39		45		33	
Substrate specificities (%)																
phloroglucinol	100		100		100		100		100		100		100		100	
pyrogallol	2.5		0		0		0		0		0		0		0	
chlorogenic acid	0		0		0		0		0		0		0		0	
catechol	0		0		0		0		0		0		0		0	
	PhO	POD	PhO	POD	PhO	POD	PhO	POD	PhO	POD	PhO	POD	PhO	POD	PhO	POD
K_m value (mM)	2.0		3.3		11.0	2.9	0.67		0.67		6.4		8.5		1.3	
optimum pH	8.0	5.0	8.0	4.0	8.0	7.0	7.6-8.0	5.0	7.6-8.0	5.0	7.6	6.4	8.0	6.7	7.4	6.7
pH stability	3-10[b]	3-11[b]	3-11[b]	5-8[b]	5-10[b]	5-10[b]	6-10[a]	–	–	–	5.0-11.0[a]	5.0-11.0[a]	6.0-11.0[a]	6.0-11.0[a]	6.0-11.0[a]	6.0-10.0[a]
optimum temperature (°C)	–	–	55	20	60	20	–	–	–	–	40	45	–	–	–	–
thermal stability (°C)	20-40[a]	20-40[b]	20-50[a]	20-50[a]	30-60[a]	30-60[a]	40-100[a]	–	40-100[b]	40-60[a]	20-60[b]	20-40[a]	62[c]	57[c]	65[c]	53[c]
Effector[d]																
sodium diethyldithiocarbamate	0	33	11	14	6	0	0[e]	77[e]	0[e]	15[e]	0	0	0	0	0	0
KCN	32	0	40	0	29	0	10[e]	38[e]	10[e]	80[e]	36	0	41	0	28	0
EDTA	94	92	68	119	68	98	–	–	36[e]	95[e]	10	84	0	77	10	92
$MnCl_2$	132	90	278	127	351	142	453[e]	110[e]	423[e]	103[e]	278	10	425	10	481	45
$CuSO_4$	13	96	21	92	10	98	0[e]	101[e]	0[e]	97[e]	0	65	0	50	0	71
L-ascorbic acid	0	1	0	0	0	0	0[e]	82[e]	0[e]	0[e]	0	0	0	0	0	0
chlorogenic acid	16	1	6	0	13	0	–	–	–	–	0	27	–	–	–	–
hydroquinone	26	1	46	1	17	0.7	–	–	–	–	2	1	–	–	–	–

[a]Above 90% remaining activity, [b]above 80% remaining activity, [c]above 50% remaining activity, [d]final concentration of compound (10 mM), [e]final concentration of compound (0.5 mM), [17,18,19,20] references number.

Table 18. Characterization of PPO from fruits and vegetables

origin	apple[4]	garland chrysanthemum[5]		edible burdock[6]		Japanese butterbur[34]		leaf lettuce[40]		Japanese pear[41]	eggplant[46]
molecular weight (kDa)											
SDS-PAGE	65	45		40		25		46		-	-
gel filtration	–	47		41		26		45		56	-
Substrate specificities (%)											
chlorogenic acid	100	100		100		100		100		100	100
(–) – epicatechin	–	100		80		94		54		–	–
	ChO	ChO	EPO	ChO	EPO	ChO	EPO	ChO	EPO	ChO	ChO
K_m value (mM)	122 μM	2.0	10.0	0.4	2.7	0.14	0.7	1.0	1.4	0.6	0.5
optimum pH	4	4.0	8.0	5.0	8.0	5.0	8.0	5.0	8.0	4	4
pH stability	6-8	5-11[b]	5-10[b]	5-7[c]	5-7[c]	4-9[d]	4-9[d]	5-8[d]	5-8[d]	3.5-5.0[d]	4-7[a]
optimum temperature (°C)	–	30	40	20	20	30	30	30	30	35	-
thermal stability (°C)	0-30[d]	20-50[a]	20-50[a]	45[d]	45[d]	30-60[d]	30-60[d]	30-70[d]	30-70[d]	40-70[a]	30-50[a]
Effector[e]											
sodium diethyldithiocarbamate	0	25	0	0	0	0	0	0	0	98[f]	79[g]
KCN	24.1	5	0	0	0	0	0	0	0	5[f]	42[g]
EDTA	83.4	21	11	12	63	62	94	94	39	102[f]	100[g]
$MnCl_2$	27.5	0	5	-	-	25	-	0	-	78[f]	70[g]
$CuSO_4$	–	11	100	-	-	19	-	11	0	101[f]	100[g]
L-ascorbic acid	–	0	0	0	0	0	0	0	0	-	-

[a]Above 90% remaining activity, [b]above 80% remaining activity, [c]above 70% remaining activity, [d]above 50% remaining activity, [e]final concentration of compound (10 mM), [f]final concentration of compound (1 mM), [g]final concentration of compound (0.1 mM), [4,5,6,34,40,41,46] references number.

CHAPTER 7
CONCLUSION

Polyphenol oxidase is a group of copper protein that oxidizes phenolic compounds (PPs) to produce undesirable browning in many fruits and vegetables.[4–20] Undesirable browning occurred, because of damaging tissues of the fruits and vegetables including PP which have been associated with flavor and color of them as results the food quality and economics of fruits and vegetables decrease. To prevent such browning, PPOs have been purified and characterized in many fruits and vegetables.[4–20] The cruciferae vegetables such as cabbage, Japanese radish, cauliflower, and broccoli widely consumed all over the world and have economically important, because of these vegetables contain high level of antioxidant are potentially protective factors against cancer and heart diseases. In addition, Cartea et al.[28] reported that the high antioxidant activity of cruciferae vegetables was due to the presence of PP. A new type PPO, which only oxdized 1,3,5-trihydroxybenzenes such as phloroglucinol (Phl) have been found in turnip[18] and cabbage[19,20] which belong to cruciferae vegetables. The Phl oxidizing enzymes (PhO) also have POD activity. PPO and POD play important roles in deterioration of color and flavor. However, little is known about the PhO from cruciferae vegetables except for turnip and cabbage PPOs. The objectives of these studies are: to investigate the distribution of PhO in other cruciferae vegetabels, and to purify and characterize the PhO from Japanese radish root, cauliflower, and broccoli. The research results were as follows.

In **chapter 2**, the distribution of the enzyme in cruciferae vegetables were

investigated. The crude enzymes of turnip, cabbage, broccoli, cauliflower, nabana, shingensai strongly oxidized 1,3,5-trihydroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid, but not oxidized 1,2,3-trihydroxybenzenes such as pyrogallol and gallic acid. Japanese radish root PPO has small activity toward 1,2,3-trihydroxybenzens such as pyrogallol and gallic acid. In addition, PPOs of mizuna, takana, leaf mustard, katsuona, and komatsuona also have small activity toward pyrogallol. All enzymes tested not oxidized *o*-diphenols such as catechol, chlorogenic acid, DL-dopa, and dopamine. All the enzymes solution showed dual activities of phloroglucinol oxidase (PhO) and peroxidase (POD). However, the level content of the activity were different from those plant source. Japanese radish had the highest activity toward PhO compared to other cruciferae vegetables PPO tested, that is 127 unit/mg protein. The level activity of PhO from various cruciferae vegetables in order as follows: Japanese radish root > turnip > cabbage > broccoli > cauliflower > nabana > Chinese cabbage > shingensai > mizuna > takana > komatsuna > katsuona. On the other hand, turnip PPO showed the highest activity toward POD compared to others PPO, that is 432.0 unit/mg protein. The activity level of POD from cruciferae vegetables in order as follows: turnip > cabbage > Japanese radish root > broccoli > cauliflower > nabana > mizuna > Chinese cabbage > shingensai > takana > komatsuna > katsuona. These results indicate that all of the PPOs distributed in cruciferae vegetables tested are a new type enzyme “phlorogrucinol oxidase”.

In **chapter 3** the purification and characterization of PPO from Japanese radish root was performed. The enzyme was purified about 192-fold with a

recovery rate of 15% by ammonium sulfate fractionation, ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. The purified enzyme appeared as a single band on SDS-PAGE. Molecular weight of the purified enzyme was estimated to be about 44 kDa by gel filtration and 45.7 kDa by SDS-PAGE. These results indicate that the purified enzyme is monomeric. The purified enzyme quickly oxidized 1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid, and quickly oxidized 1,2,3-trihydroxybenzenes, such as pyrogallol; however, it not oxidized *o*-diphenols, such as chlorogenic acid and dopamine, which was similar to soybean PPO[15] and edible burdock PPO.[16] The Michaelis constant (K_m) of the enzyme for the oxidation of phloroglucinol was 2 mM. Furthermore, the substrate specificities of these enzymes differed from those of Satsuma mandarin PPO,[17] turnip PPO,[18] cabbage PPO,[19,20] which only oxidized 1,3,5-trihydroxybenzene. POD activity was also found in the purified Japanese radish root PPO. The final preparation of the purified enzyme showed a 259-fold increase in POD activity and had a recovery rate of 20% for POD. The optimal pH for PPO and POD activities was 8.0 and 5.0, respectively. Both PPO and POD activities of the Japanese radish root enzyme were stable in wide pH ranges. Eighty percent of the PPO and POD activities were retained in the pH ranges 3.0¬10.0 and pH 3.0¬11.0, respectively. After heating at 80°C for 10 min, only 20% of the PPO activity remained; however, POD activity was almost completely lost. These results indicated that the effect of pH, temperature, and various compound for PhO activity were differed from those POD activity. Due to the

different effect of pH, temperature, and various compounds on PPO and POD activities of the purified Japanese radish root enzyme, it is probable that this enzyme has separate active sites for PPO and POD activities, and this may also be the case for soybean,[15] turnip,[18] and cabbage[19,20] PPOs.

PPO of cauliflower was purified and characterized in **chapter 4**. The enzyme of cauliflower was also purified by ion exchange chromatography, hydrophobic chromatography, and gel filtration using phloroglucinol as a substrate. The PPO was purified up to 282-fold purification with a recovery rate 8.1 % as compared with the crude extract. The purified enzyme of cauliflower produced a single band on SDS–PAGE. The molecular weight of the enzyme was estimated to be 60 kDa by SDS–PAGE and 54 kDa by gel filtration. The purified cauliflower PPO oxidized 1,3,5-tryhdroxybenzenes such as phloroglucinol and phloroglucinol carboxylic acid but not oxidized *o*-diphenols. The Michaelis constant (K_m) value of phloroglucinol oxidation of the purified cauliflower enzyme was 3.3 mM. Similar substrate specificity of cauliflower PPO had been found in the purified enzymes of Satsuma mandarin,[17] turnip ,[18] cabbage F-IA ,[19] and cabbage F-IB.[20] In addition, the substrate specificity of cauliflower enzyme differed from the specificity of the PPOs of soybean,[15] edible burdock,[16] and Japanese radish root (Chapter 3) which oxidized both 1,3,5-trihydroxybenzenes and 1,2,3-trihydroxybenzenes but not oxidized *o*-diphenols. These results indicate that the cauliflower PPO is a group of a new type of PPO that is “phloroglucinol oxidase (PhO)”. The purified cauliflower enzyme has a dual activity of PhO and POD, just like the enzymes from Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20]

At the final step of purification, the POD activity of cauliflower enzyme increased to 110-fold with a recovery rate of 3.2 %. The optimum pH of PhO and POD of cauliflower was 8.0 and 4.0, respectively. The activity of PhO of cauliflower enzyme was more stable than POD in the wide range of pH. Over 80% of PhO activity remained in the pH range of 3.0 – 11.0. However, the POD activity was stable at pH 5.0–8.0 and declined rapidly at the pH below 8.0 after 20 h incubation at 5°C. The optimum temperature of PhO and POD was 55°C and 20°C, respectively. The purified enzyme of cauliflower showed a relatively low thermal stability. The activity of PhO and POD was stable at the temperature range of 20–50°C. At the higher temperature than 50°C, the activity of POD was almost lost. Contrarily, the PhO was more heat-stable and 60% of its activity remained after heating at 80°C for 10 min. The effect of 13 kinds of compounds on the activity of PhO and POD of purified cauliflower enzyme were determined. Sodium diethyldithiocarbamate inhibited PhO (IC_{50} 0.64; *Ki* 0.15 mM) and POD (IC_{50} 6.61; *Ki* 1.64 mM) activities of the enzyme competitively. The inhibition rate for PhO was higher than that for POD at 1 mM. Competitive inhibitor of sodium diethyldithiocarbamate was also found in the PPO of nettle (*Ki* 1.79×10^{-9} mM).[13] KCN completely inhibited POD activity (IC_{50} 0.03; *Ki* 29 μM), and also strongly inhibited PhO activity at 10 mM (IC_{50} 7.88; *Ki* 1.95 mM). KCN inhibited both PhO and POD activities at 1 mM. These results indicate that the purified enzyme is metallic enzyme, and the metal is seemed to be iron as mentioned previously. L-ascorbic acid competitively inhibited the activity of PhO and POD at 10 mM but not at 1 mM. The IC_{50} for PhO and POD activities were 3.55 ± 0.07

mM and 3.67 ± 0.66 mM, respectively. $CuSO_4$ noncompetitively inhibited PhO activity (IC_{50} 0.56 ± 0.05) and slightly inhibited POD activity at 1 mM and 10 mM. EDTA inhibited PhO and slightly activated POD of cauliflower. $BaCl_2$ and $ZnSO_4$ showed a weak activation of PhO and a weak inhibition of POD. However, the strong activation of PhO was induced by $MnCl_2$. These results were similar from the PhOs of turnip[18] and cabbage,[19,20] which showed a remarkable activation by $MnCl_2$. The activity of PhO and POD of cauliflower was markedly inhibited by chlorogenic acid (*o*-diphenols) and hydroquinone (*p*-diphenol) in 1 mM and 10 mM just like the enzymes of Satsuma mandarin[17] and cabbage.[19,20] Chlorogenic acid showed noncompetitive inhibition for PhO activity and competitive inhibition for POD activity. It is indicated that both PhO and POD have separate active sites. Hydroquinone competitively inhibited both PhO and POD activities of the purified enzyme. It is interesting that the type of inhibition of chlorogenic acid to the cauliflower enzyme for both PhO and POD activities were different.

In **chapter 5**, we purified the PPO of broccoli using phloroglucinol as substrate, and characterized the activity of the purified enzyme. The broccoli PPO was purified by precipitating with 80% ammonium sulfate, ion exchange chromatography, hydrophobic chromatography, and gel filtration. Finally, the PPO was purified up to 80-fold with a recovery rate 26 % as compared with the crude enzyme. The purified enzyme produced a single band on SDS-PAGE, and the molecular weight of purified enzyme was estimated to be about 63 kDa by SDS-PAGE and 65 kDa by gel filtration. These results suggest that the purified PPO is a monomer protein. The purified broccoli PPO strongly oxidized

1,3,5-trihydroxybenzenes, such as phloroglucinol and phloroglucinol carboxylic acid but not oxidized 1,2,3-trihydroxybenzenes and *o*-diphenols. In case of the PPOs of fruits and vegetables, mostly they oxidized *o*-diphenols such as catechol, chlorogenic acid, dopamine, DL-dopa, and 4-methycatechol. These results indicate that the broccoli PPO we purified belongs to a new type PPO namely "phloroglucinol oxidase (PhO)". The Michaelis constant (K_m) of the broccoli PPO for the oxidation of phloroglucinol was 11 mM. The purified broccoli enzyme has a dual activity of PhO and POD, just like the enzymes from Satsuma mandarin,[17] turnip,[18] and cabbage.[19,20] At the final step of purification, the broccoli POD activity increased to 33-fold with a recovery rate of 11 %. The K_m value of the broccoli POD for the oxidation of guaiacol was 2.9 mM. The optimum pH of PhO and POD of broccoli was 8.0 and 7.0, respectively. The optimum pH of our purified enzyme for POD (pH 7.0) also differed from that of broccoli POD[31] which showed the optimum at pH 6.0 for both neutral and basic PODs and at pH 4.0 for acidic POD. Both PPO and POD activities of broccoli enzyme were stable in the wide pH ranges. Eighty percent of the PPO and POD activities were retained in the pH ranges 5.0–10.0. The effect of temperature on the activity and stability of PhO and POD of the purified broccoli enzyme were also determined. The optimum temperature for the activities of PhO and POD were 60°C and 20°C, respectively. The activities of PhO and POD were stable at temperature range of 30–60°C. After heating at 70°C for 10 min, 40% of the PPO activity remained; however, POD activity was almost completely lost. Both the PhO and POD activities were markedly inhibited by the metallic enzyme inhibitors such as

sodium diethyldithiocarbamate and potassium cyanide. L-ascorbic acid (reducing agent), chlorogenic acid (*o*-diphenol), and hydroqinone (*p*-diphenol) strongly inhibited both PhO and POD activities. EDTA and CuSO4 inhibited PhO activity, but a little affected POD activity. NaF, NaCl, $MnCl_2$, $BaCl_2$, $ZnSO_4$, and resorcinol (*m*-diphenol) a little affected both PhO and POD activities. On the other hand, the strong activation of PhO was induced by $MnCl_2$. Similar results were obtained for the PhOs from Satsuma mandarin,[17] turnip,[18] cabbage,[19,20] and cauliflower in chapter 4. Sodium diethyldithiocarbamate, EDTA, $CuSO_4$, L-ascorbic acid, chlorogenic acid, and hydroquinone inhibited the PhO and POD activities in a competitive manner. On the other hand, KCN inhibited the activities of PhO and POD in a different manner: KCN inhibited PhO activity in a competitive manner and inhibited the POD activity in a noncompetitive manner. These results strongly indicate that PhO and POD have separate active sites. All of these results also indicate that the purified polyphenol oxidase from broccoli is a metallic enzyme, quite probably an iron-containing enzyme.

In **chapter 6**, we compared the characterics of PPO (PhO) from cruciferae vegetables with those of *o*-diphenolase from other fruits and vegetables, and then carried out general discussion.

Cruciferae vegetables widely consumed all over the world and economically important, because of these vegetables contain high level of antioxidant are potentially protective factors against cancer and heart diseases. The browning of these vegetables caused decrease their quality and marketability. To prevent such as browning of cruciferae vegetables, purification and characterization of PPO and

POD which is responsible for browning in these vegetables are important to prevent its deteriorative action during storage and processing. We hope that the results of this study can be used to control the enzymatic browning of these vegetables.

LITERATURE CITED

(1) Yoruk, R.; Marshall, M.R. Physicochemical properties and function of plant polyphenoloxidase: a review. *J. Food Biochem*. **2003**, 27, 361–422.

(2) Queiroz,C.; Mendes Lopes, M.L.; Fialho, E.; Valente-Mesquita, V.L. Polyphenol oxidase: characteristics and mechanisms of browning control. *Food reviews international*, 24; Taylor and Francis: Mortimer street, London, **2008**; 4, 361–375.

(3) Ramírez, E.C.; Whitaker, J.R.; Virador, V.M. Polyphenol oxidase. *Handbook of food enzymology*, Marcel dekker, Inc.: Madison avenue, New York, **2003**.

(4) Murata, M.; Kurokami, C.; Homma, S. Purification and properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Biosci. Biotechnol. Biochem.* **1992**, 56, 1705–1710.

(5) Nkya, E.; Kouno, C.; Li, Y. J.; Yang, C,-P.; Hayashi, N; Fujita S. Purification and characterization of polyphenol oxidase from garland chrysanthemum (*Chrysanthemum coronarium* L.). *J. Agric. Food Chem*. **2003**, 51, 5467–5471.

(6) Han, Y.-Z.; Ayumu, M.; Eline, N.; Hayashi, N.; Fujita, S. Purification and characterization of chlorogenic acid oxidase from edible burdock (*Arctium lappa* L.). *Food Preser. Sci.* **2006**, 32, 275–281.

(7) Yang, C.-P.; Fujita, S.; Ashrafuzzaman, Md.; Nakamura, N.; Hayashi, N. Purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) pulp. *J. Agric. Food Chem*. **2000**, 48, 2732–2735.

(8) Yang, C.-P.; Fujita, S.; Kohno, K.; Kusubayashi, A.; Ashrafuzzaman, Md.; Hayashi, N. Partial purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) peel. *J. Agric. Food Chem*. **2001**, 49, 1146–1149.

(9) Wang, J.; Jiang, W.; Wang, B.; Liu, S.; Gong, Z.; Luo, Y. Partial properties of polyphenol oxidase in mango (*Mangifera indica* L. CV. “Tainong”) pulp. *J. Food Biochem*. **2007**, 31, 45–55.

(10) Palma-Orozco, G.; Ortiz-Moreno, A.; Dorantes-Àlvarez, L.; Sampedro, J.G.; Nàjera, H. Purification and partial biochemical characterization of polyphenol oxidase from mamey (*Pouteria sapota*). *Phytochem*istry **2011**, 72, 82–88.

(11) Gawlik-Dziki, U.; Szymanowska, U.; Baraniak, B. Characterization of polyphenol oxidase from broccoli (*Brassica oleraceae* var. *botrytis italica*) florest. *Food Chem*. **2007**, 105, 1047¬1053.

(12) Gawlik-Dziki, U.; Złotek, U.; Świeca, M. Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. *capitata* L.). *Food Chem*. **2008**, 107, 129¬135.

(13) Gülçin, İ.; Küfrevioğlu, Ö.İ.; Oktay, M. Purification and characterization of polyphenol oxidase from nettle (*Urtica dioica* L.) and inhibitory effects of some chemicals on enzyme activity. *J. Enzym. Inhib. Med. Chem*. **2005**, 20, 297–302.

(14) Aydemir, T. Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads. *Food Chem.* **2004**, 87, 59¬67.

(15) Toiguchi, S.; Hayashi, K.; Adachi, Y.; Motoki, M.; Haraguchi, K. Purification and characterization of soybean oxidase. *Nippon Shokuhin Kogyo Gakkaishi* **1989**, 36, 597−602.

(16) Murao, S.; Oyama, H.; Nomura, Y.; Tono, T.; Shin, T. Purification and characterization of Arctium lappa L. (edible burdock) polyphenol oxidase. *Biosci. Biotechnol. Biochem*. **1993**, 57, 177−180.

(17) Fujita, S.; Tono, T. Peroxidase activity of phloroglucinoloxidase from Satsuma mandarin fruits and effect of metal ions on the enzyme activity. *Nippon Nogeikagaku Kaishi* **1980**, 54, 201–208.

(18) Fujita, S.; Tono, T. Purification of phloroglucinoloxidase from turnip and its properties. *Nippon Nogeikagaku Kaishi* **1980**, 54, 429–435.

(19) Fujita, S.; Saari, N.; Maegawa, M.; Tetsuka, T.; Hayashi, N.; Tono, T. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). *J. Agric. Food Chem*. **1995**, 43, 1138–1142.

(20) Fujita, S.; Saari, N.; Maegawa, M.; Tetsuka, T.; Hayashi, N.; Tono, T. Isolation and characterization of two phloroglucinol oxidase from cabbage (*Brassica oleracea* L.). *J. Agric. Food Chem*. **1997**, 45, 59–63.

(21) Lozano, J.E. Fruit manufacturing (scientific basis, engineering properties, and deteriorative reactions of technological importance). Springer **2006**, chapter 8.

(22) Chutintrasri, B.; Noomhorm, A. Thermal inactivation of polyphenoloxidase in pineapple puree. *Food Sci.Technol.* **2006**, 39, 492 – 495.

(23) Lado, B.H.: Yousef, A.E. Alternative food-preservation technologies: efficacy and mechanisms. *Microbes and Infection* **2002**, 4, 433¬440.

(24) Plaza, L.; Muñoz, M.; Ancos, B.d; Cano, M.P. Effect of combined treatments of high-pressure, citric acid and sodium chloride on quality parameters of tomato puree. *Eur. Food Res. and Technol*. **2003**,216, 514 – 519.

(25) Fang, L.; Jiang, B.; Zhang, T. Effect oc combined high pressure and thermal treatment on kiwifruit peroxidase. *Food Chem.* **2008**, 109, 802–807.

(26) Chen, L.; Mehta, A.; Berenbaum, M.; Zangerl, A.R.; Engeseth, N.J. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. *J. Agric. Food Chem*. **2000**, 10, 4997 – 5000.

(27) Liu, X.; Gao, Y.; Peng, X.; Yang, B.;Xu, H.; Zhao, J. Inacivaion of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris* L.) exract with high pressure carbon dioxide. *Innovative Food Sci. and Emerging Technol.* **2008**, 9, 24–31.

(28) Cartea, M.E.; Francisco, M.; Soengas, P.; Velasco, P. Phenolic compounds in *Brassica* vegetables. *Molecules* **2011**,16, 251–280.

(29) Warwick, S.I. Brassicaceae in agriculure. *Genetics and genomics of the brassicaceae*, R. Schmidt, I. Bancraft, Eds.; Springer science: Canada, **2011**.

(30) Soengas, P.; Sotelo, T.; Velasco, P.; Cartea, M.E. Antioxidant properties of brassica vegeables. *Functional sci. and biotechnol.* **2011**, 5, 43–55.

(31) Thongsook, T.; Barrett, D.M. Purification and partial characterization of broccoli (*Brassica oleraceae* var. *italica*) peroxidases. J. Agric. Food Chem. 2005, 53, 3206– 3214.

(32) Fujita, S.; Kawahara, H.; Nazamid, S.; Tono, T. Spectrophotometric determination of phloroglucinoloxidase activity based on difference spectra. *Bull. Fac. Agric. Saga Univ*. **1993**, 74, 81–88.

(33) Hartree, E.F. Determination of protein: a modification of Lowry method that gives a linear photometric response. *Anal. Biochem.* **1972**, 48, 422–427.

(34) Han, Y.; Zhao, F.; Ogawa, T.; Ohta, M.; Fujita, S. Purification and characterizaion of polyphenol oxidase from Japanese butterbur (*Petasies japonicus*). *Food Preser. Sci.* **2009**, 35, 179–186.

(35) Fujita, S.; Han, Y-Z.; Kouno, C.; Matsuo, T.; Yamashita, M.; Haraguchi, Y.; Li, Y-J.; Hayashi, N.; Yang, C-P. Purification and characterization of polyphenol oxidase from edible yam (*Dioscorea opposita* Thunb.). *Food Sci. Technol. Res.* **2006**, 12, 235–239.

(36) Gao, Z-J.; Han, X-H.; Xiao, X-G. Purification and characterizaion of

polyphenol oxidase from red Swiss chard (*Beta vulgaris* subspecies *cicla*) leaves. *Food Chem*. **2009**, 117, 342–348.

(37) Jia, G.; Baogang, W.; Xiaoyuan, F.; Haoru, T.; Wensheng, L.; Kaichun, Z. Partial properties of polyphenol oxidase in sour cherry (*Prunus cerasus* L. CV. CAB) pulp. *World J. Agric. Sci.* **2011**, 7, 444–449.

(38) Andrews, P. The gel-filtration behavior of protein related to their molecular weight over wide range. *Biochem. J.* **1965**, 96, 595–605.

(39) Weber, K.; Osborn, M. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem*. **1969**, 244, 4406–4412.

(40) Han, Y.; Hayashi, N.; Fujita, S. Purification and properties of polyphenol oxidase from leaf lettuce (*Lactuca sativa*). *Food Preser. Sci.* **2005**, 31, 295¬301.

(41) Tono, T.; Fujita, S.; Kawasaki, H.; Li, Z. Purification and high L-epicatechin oxidase activity in polyphenol oxidase of Japanese pear. *Nippon Nogeikagaku Kaishi* **1986**, 60, 705–712.

(42) Şişecioğlu, M.; Gűlçin, I.; Çankaya, M.; Atasever, A.; Şehitoğlu, M.H.; Kaya, H.B.; Őzdemir, H. Purification and characterization of peroxidase from Turkish black radish (*Raphanus sativus* L.). *J. Med. Plant Res*. **2010**, 4, 1187–1196

(43) Waliszewski, K.N.; Marquez, O.; Pardio, V.T. Quantification and characterizaion of polyphenol oxidase vanilla bean. *Food Chem*. **2009**, 117, 196–203.

(44) Rayan, A.M.M.; Gab-Alla, A.A.; Shatta, A.A.; El-Shamei, Z.A.S. Thermal inactivation kinetics of quality-related enzymes in cauliflower (*Brassica oleracea* var. *botrytis*). *Eur. Food Res. Technol.* **2011**, 232, 319–326.

(45) Köksal, E.; Gülçin, İ. Purification and characterization of peroxidase from cauliflower (*Brassica oleracea* L. var. *botrytis*) buds. *Protein Peptide Letters* **2008**, 15, 320–326.

(46) Fujita, S.; Tono, T. Purification and some properties of polyphenol oxidase in eggplant (*Solanum melongena*). *J. Sci. Food Agric.* **1988**, 46, 115–123.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude and appreciation to my advisor, Dr. Shuji Fujita, Professor of Food Science, Saga University, for his continuous support, constructive guidance, suggestions and encouragement during all phases of my research work. I consider myself very fortune to have had the opportunity to work under his supervising. He is a great advisor and excellent motivator. I also would like to express my sincere thanks to Dr. Nobuyuki Hayashi, Professor of Food Science, Saga University, and Dr. Toshihiko Suganuma, Professor of Food Science, Kagoshima University, for their support, time for discussions, valuable ideas, and suggestions to improve this work.

My sincere appreciation also extends to the members of my doctoral committees: Dr. Fumio Yagi, Professor of Food Science, Kagoshima University, and Dr. Koji Wada, Professor of Food Science, Ryukyu University, for their comments, valuable ideas, and suggestions that helped improve this work significantly.

I would like to thank 'Hasanuddin University Indonesia' for granting me study leave for this study. I would like to acknowledge to Directorate General for Higher Education (DIKTI) for provided me with the scholarship during my study.

I would like to express the gratitude from my heart to my parents for giving me the invaluable and endless support in my life. A special thank to my husband, who supported me throughout my study, and to my lovely son, for his happiness.

Finally, I would like to thank all of my lab mates for their supports. I would

also like to express my gratitude to all Indonesian's friends and international students in Saga for making my Japan life more comfortable and enjoyable.