CONTROL OF POLYPHENOL OXIDASE AND PECTIN METHYLESTERASE ACTIVITIES BY ULTRA HIGH PRESSURE

By

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MIYUKI MIYAWAKI find it satisfactory and recommended that it be accepted.

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Chair

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Abstract

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Chair: Barry G. Swanson

Fruit and vegetable processing aims for preservation of foods with acceptable quality and extended shelf life by minimizing the changes in natural color, texture, and flavor. Development of rapid determination methods of intrinsic enzyme activities to evaluate the adequacy of processing methods as well as selections of processing methods to control enzyme activities are of technological and economical importance to the food industry.

Polyphenol oxidase (PPO) catalyzes oxidation of phenolic compounds into *o*-quinones resulting in discoloration of fruits and vegetables. A rapid fluorescence oxygen probe method for the determination of PPO activity was developed and its performance compared to the conventional spectrophotometric method. The fluorescence oxygen probe method enables the direct determination of diphenol oxidase activity of PPO in fruit homogenates in a reasonably short time.

Nonthermal ultra high pressure (UHP) processing potentially inhibits intrinsic PPO activity. Combined exogenous cysteine proteases papain, bromelain, and ficin, and UHP treatments, involving the addition of EDTA and cysteine as activators for the proteases, were evaluated to inhibit Granny Smith apple PPO activity. Papain inhibited PPO activity in apple homogenates in the presence of cysteine during incubation at 25 °C. PPO activity in apple homogenates containing papain and cysteine was inhibited by UHP treatment at 600 MPa and an initial temperature of 25 °C. UHP treated apple homogenates containing only cysteine exhibited little discoloration during storage. The combined method adding papain and cysteine to apple homogenates followed by UHP treatment is a potential application for color preservation of apple products.

Pectin methylesterase (PME) catalyzes deesterification of pectins resulting in increased firmness of fruit and vegetable tissues. PME exhibits stability under combined pressures and temperatures compared to individual pressures or temperature treatments. Combined mild heat and UHP treatments of strawberries were examined to assess potential increases in fruit firmness attributed to endogenous PME activity and exogenous $CaCl_2$. The addition of $CaCl_2$ to syrups in packaged strawberries enhanced the firmness of strawberries treated with UHP at ambient temperature.

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Introduction

A description by Hutchings (2003) regarding expectations of people toward food is summarized as follows: “Food evaluation always starts with visual inspections. We consciously or unconsciously examine food in the first place by appearance including color, visual structure, and surface texture. Temporal properties such as climate, place, and physical condition can affect the evaluation as well as individual background. In the next step, we identify safety of the food followed by assessment of flavor and texture. The final step of food evaluation before eating is to guess pleasant and satisfaction brought to us by eating the food.”

There is an increasing consumer trend to select food demonstrating good quality as described by good color, texture, and flavor, in addition to high nutritional value, microbial safety, and convenience. To respond to the demand for quality, fruit and vegetable processors expend considerable efforts to improve product quality from the beginning to the end of the preservation process.

First of all, proper handling of raw materials after harvest is critical. Fruit and vegetables are classified based on their peculiar morphology, indicating that physical, chemical, and biological characteristics vary among plant sources.

Respiration during the postharvest period also changes physiological properties of cultivars. For example, climacteric fruits such as apples and bananas exhibit sudden and rapid increases of respiration during storage resulting in maturation observed as tissue softening and brown discoloration. Maturation of cultivars is controlled by the manipulation of storage temperature, relative humidity, and atmosphere as well as packaging materials in the fresh market and the processing industry. Physical damage to fruit and vegetables promotes cell decompartmentalization followed by the induction of chemical reactions involving intrinsic enzymes and substrates.

Fruit and vegetable processing aims for preservation of foods with high quality and extended shelf life by minimizing the changes in natural color, texture, and flavor, or even improving these quality factors during processing. Processing methods to control the growth of pathogenic or spoilage microorganisms as well as the activity of intrinsic enzymes play the key role in the processes because the failure to achieve enzyme and microbial inactivation leads directly to inadequate safety and quality of food products. Intrinsic enzymes affecting the quality of fruits and vegetables are represented by polyphenol oxidase (PPO), pectic enzymes pectin methylesterase (PME) and polygalacturonase (PG), peroxidase (POD), and lipoxygenase (LOX) responsible for biochemical changes in color, texture, and flavor. These enzymes are widely characterized in various plants. The enzyme characterizations include purification, molecular investigations, and kinetic examinations of enzyme

activity and stability. In addition, development of rapid and precise methods to determine enzyme activity is of technological importance to the food industry.

Unlike pathogenic and spoilage microorganisms, intrinsic plant enzymes are not always detrimental, but may also be beneficial and improve the quality of food. PPO, for instance, contributes to the development of dark color pigments in tea, coffee, and cocoa during the fermentation process. PME and PG are commercially applied to reduce the viscosity of plant extracts or disrupt cellular plant tissues to improve juice yield. A fresh-like fruit or vegetable juice product rich in aldehyde and alcohol compounds is produced by controlling LOX activity. However, complete inactivation of detrimental microorganisms together with intrinsic plant enzymes in the final food is preferable to avoid quality changes during storage and distribution. "Control" of intrinsic plant enzyme activities during fruit and vegetable processing gives rise to preserved foods with desirable quality and extended shelf life.

Heating is the most conventionally applied processing method to control microbial populations and enzyme activity to extend the shelf lives of fruits and vegetables. The disadvantages of selected thermal processes are the large energy consumption and the accompanying changes in flavor, texture, and color of foods attributed to the exposure of food constituents and ingredients to heat for an appreciable time. Alternatives to heat are nonthermal processing methods such as combined methods, ultra high pressure (UHP), pulsed electric fields (PEF), high intensity light, ultrasonics, or ionizing irradiation. Nonthermal UHP processing has

evolved in the food industry over the past 10 years. The expectation to utilize UHP varies depending on the target foods. UHP potentially preserves fruits and vegetables while retaining fresh-like flavors, textures and colors desired by consumers, as well as providing adequate microbial safety and extended acceptable shelf lives.

In 1990, the first UHP preserved fruit preserves were available on the retail market in Japan. Unfortunately, UHP fruit preserves were not successful in the market because of unacceptably short shelf lives under refrigerated conditions resulting from insufficient inactivation of detrimental enzymes during the pressure (345 MPa) process. A new commercial UHP system marketed by Avure Technologie (Kent, WA) equipped with 215 L vessel capacity enables pressurization of 600 MPa and may provide adequate inactivation of detrimental enzymes. NC Hyperbaric (Burgos, Spain) also produces an UHP system with 300 L vessel capacity and the maximum working pressure of 600 MPa. Research applying ultra high pressures and adequate treatment times to inactivate detrimental microorganisms and enzymes as well as providing safety and fresh-like quality may improve commercially available fruit and vegetable foods. In this dissertation, three objectives are addressed. The contribution of this research to effective processing resulting in desirable fresh-like quality of preserved fruits is explained.

In chapter 3, a rapid and precise fluorescence oxygen probe method for the determination of polyphenol oxidase (PPO) activity is developed as an alternative to the conventional spectrophotometric method. The fluorescence oxygen probe method

provides an opportunity to determine PPO activity in fruit and vegetable materials and confirm the adequacy of processing methods accurately and economically. Due to the direct use of fruit homogenates as the enzyme source, the fluorescence oxygen probe method enables the determination of PPO activity in the entire tissue in a reasonably short time.

In chapter 4, combined protease treatments together with protease activators followed by UHP treatment to inhibit Granny Smith apple PPO activity is evaluated. The threshold concentrations and requirement of papain, cysteine, and EDTA are explored by determining residual apple PPO activity in homogenates during incubation as a treatment prior to UHP treatments. Endogenous PPO activity and exogenous papain activity are monitored during incubation and UHP treatment of the apple homogenates treated with papain, cysteine, EDTA, or combinations of these constituents. After the shelf life study to determine discoloration of apple homogenates during storage, contributions of the method combining incubation and UHP treatment are evaluated in relation to PPO inactivation and color retention depending on the combination of constituents. The uses of proteases, cysteine, and EDTA combined with UHP technology and potentially extend the shelf lives of fruit products is evaluated

Chapter 5 focuses on the alteration of endogenous PME activity by UHP together with mild thermal blanching to improve the firmness of preserved strawberries in the presence of $CaCl_2$. UHP treatment is considered as a tool to

activate intrinsic PME in strawberries under elevated temperature conditions. The effectiveness of the combined mild thermal blanching and UHP treatments to improve strawberry tissue firmness is compared to the methods applying little or no heating treatment.

Development of successful UHP processing technologies and combined methods to retain or improve the quality of fruit and vegetables flavor, texture, and color may potentially provide great benefits to both consumers and processing manufacturers.

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Hutchings JB. 2003. Expectations, color and appearance of food. In: *Expectations and the food industry, the impact of color and appearance*. Kluwer Academic/Plenum Publishers, New York, NY. pp 141-175.

CHAPTER 2

Literature Review: Inhibition of Polyphenol Oxidase, Pectin Methylesterase, and Polygalacturonase

PART I: POLYPHENOL OXIDASE (PPO)

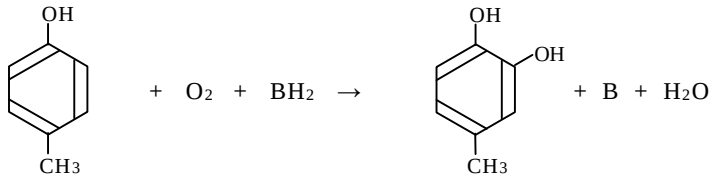
A. Overall features

Polyphenol oxidase (PPO), monophenol dihydroxy phenylalanine: oxygen oxidoreductase (E.C. 1.14.18.1), widely distributed in the plant kingdom, is the enzyme responsible for catalyzing the discoloration of fruits and vegetables (Mayer and Harel 1979). Plant PPO is located in the chloroplast bound to thylakoid membranes (Tolbert 1973, Golbeck and Cammarata 1981, Martinez and Whitaker 1995). Latent PPO is activated following release into the cytosol when plant tissues undergo physical damage such as bruising, cutting, ripening, or senescence. PPO catalyzes two different oxidative reactions in combination with molecular oxygen: 1) the hydroxylation of monophenols into *o*-diphenols (monophenol oxidation) and 2) the oxidation of diphenols into *o*-quinones (diphenol oxidation). Oxidative reaction models using *p*-cresol as a monophenol substrate and catechol as a diphenol substrate are presented in Figure 1 (Whitaker 1994). In Figure 1, BH_2 represents any compound

that acts as a proton donor. Subsequent polymerization of *o*-quinones, amino acids, and proteins results in tissue discoloration and loss of nutrients from fruits and vegetables (Whitaker 1994).

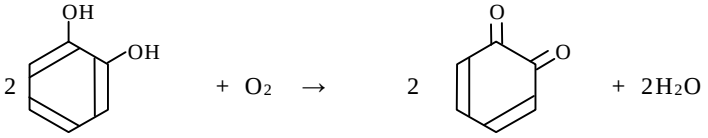
Figure 1: Oxidative reactions of monophenol and diphenol by PPO.

1) Monophenol oxidation



p-Cresol Proton donor 4-Methyl catechol

2) Diphenol oxidation

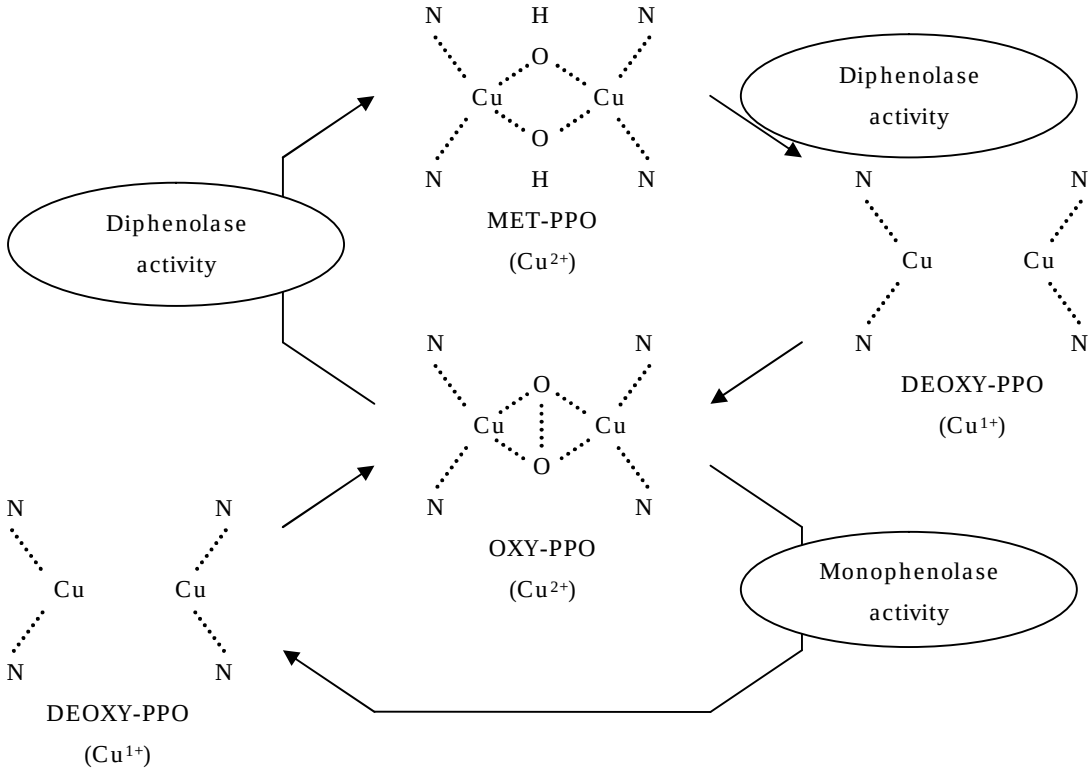


Catechol *o*-Benzoquinone

Active PPO molecules contain two copper atoms in the active site (Gaykema 1984). Lerch (1983) proposed a change in PPO molecules during the catalysis of mono- and diphenol oxidation reactions. There are three recognized molecular forms of PPO, met- (Cu^{2+}), oxy- (Cu^{2+}), and deoxy-(Cu^{1+}) dependent on the charge of the copper ions and surrounding oxygen atoms (Figure 2). Changing of PPO molecular configurations as well as the PPO active site results in two different catalytic activities attributed to PPO. While diphenol oxidase of PPO from many plant sources were

characterized in a number of literature, less intensive studies on monophenol oxidase activity of PPO were reported because of the difficulty to detect the activity in assays.

Figure 2: Proposed mechanisms and active sites for monophenol and diphenol oxidase activities of PPO. (Lerch 1983)



B. PPO from plant sources

It is estimated that nearly 50% of harvested tropical fruits in the world are discarded because of the quality defects resulted from tissue discoloration by the catalytic action of PPO (Whitaker 1996). A number of intensive research studies of PPO were conducted to control PPO activity and to prevent loss of fruit and vegetable

crops for the fresh market and food processing industry. PPO has been partially purified and characterized from many plant sources.

- Extraction

During the PPO extraction and purification process, release of PPO from chloroplast membranes and removal of intrinsic phenolic compounds are critical to achieve high purity and yield.

Detergents, such as Triton X-100 and sodium dodecyl sulfate (SDS) are added to suspensions or homogenates to solubilize PPO in extracts. Buffers containing Triton X-100 detergent are used for extraction of PPO from peaches (Wissemann and Montgomery 1985) and bean sprouts (Nagai and Suzuki 2003). Triton X-100 was added to suspension of precipitate obtained from centrifugation of homogenate for purification of PPO from strawberry (Serradell and others 2000) and Chinese cabbage (Nagai and Suzuki 2001). Acetone precipitation followed by extraction using a Triton X-100 detergent is effective for the partial purification of PPO from wild rice (Owusu-Ansah 1989), Indian tea leaves (Halder and others 1998), or mangos (Sharma and others 2001). The addition of Triton X-100 to extracting buffer did not increase PPO activity from loquat fruit PPO (Ding and others 1998). The combination of acetone precipitation and extraction using SDS was applied for characterization of PPO from yam tubers (Anosike and Ayaebene 1981).

The addition of insoluble polyvinylpyrrolidone (PVP) or polyvinyl-polypyrolidone (PVPP) was carried out to prevent the reaction between PPO and

phenolic compounds during the extraction of litchi (Jiang and others 1997), longan fruit (Jiang 1999), field beans (Paul and others 2000), or coffee (Mazzafera and Robinson 2000; Goulart and others 2003). Gonzalez and others (2000) investigated the influence of buffer composition on extraction of PPO from blackberries, yielding high activity by adding 4% PVP and 0.5% Triton X-100 in the extracting buffer. Broothaerts and others (2000) observed 44 and 74% increases in PPO activity in the extracts of tobacco and apple leaves, respectively, following the addition of Triton X-100 during the extraction process.

Bordier (1981) and Sanchez-Ferrer (1989) proposed an effective extraction method as an alternative to acetone, and ammonium sulphate precipitation of PPO as well as the addition of PVP in extracting buffers. The addition of the detergent Triton X-114 to homogenates resulted in two phase partitioning between hydrophilic and hydrophobic fractions depending on temperature. PPO and endogenous phenolic compounds were separated in hydrophilic and hydrophobic solvents, respectively. This method was successfully applied to extraction of PPO from spinach (Sanchez-Ferrer and others 1989), peach, plum, almond, cherry, apricot (Fraignier and others 1995), iceberg lettuce (Chazarra and others 1996), bananas (Sojo and others 1998a), orchid aerial roots (Ho 1999), truffles (Perez-Gilabert and others 2001), medlar (Dincer and others 2002), and persimmons (Nunez-Delicado and others 2003).

- Characterization

The examinations of substrate specificity and inhibition kinetics were assessed to characterize partially purified PPO from selected plant sources. The determinations of activity of purified PPO at selected pH and temperature were also achieved to establish adequate handling and processing methods preventing PPO-catalyzed discoloration of fruits and vegetables. Selected Properties of PPO from selected plant sources including substrate, K_m, and optimum pH and temperature are summarized in Table 1.

The presence of both mono- and diphenol oxidase activities are well documented in kiwifruit (Park and Luh 1985), bananas (Thomas and Janave 1986; Sojo and others 1998b), cherimoya (Martinez-Cayuela and others 1988), Airen grapes (Calero and others 1988), Monastrell grapes (Sanchez-Ferrer and others 1988), basil (Baritaux and others 1991), olives (Stefano and Sciancalepore 1993), potatoes (Sanchez-Ferrer and others 1993; Lee and others 2002), pears (Espin and others 1996a; Espin and others 1996b), strawberries (Espin and others 1997a), avocados (Espin and others 1997b), artichoke heads (Espin and others 1997c), apples (Espin and others 1998), raspberries (Gonzalez and others 1999), lettuce (Chazarra and others 1999; Hisaminato and others 2001), eggplant (Perez-Gilabert and Garcia-Carmona 2000), mangoes (Sharma and others 2001), peaches (Laveda and others 2001), field beans (Gowda and Paul 2002), sorghum (Dicko and others 2002), persimmons (Nunez-Delicado and others 2003), and quince fruit (Orenes-Pinero and

Table 1: Properties of partially purified PPO from selected fruits and vegetables.

Source	Substrate	Km (mM)	Optimum pH	Optimum T (°C)	Reference
Yam tubers	Catechol	9.1			Anosike and Ayaebene
	Pyrogallol	6.3			(1981)
	DL-DOPA	2.2			
Grape	4-MC		3.5~4.5	25~45	Valero and others (1988)
Wild rice	Catechol		7.8	25	Owusu-Ansah (1989)
Plum	Catechol	20	6.0	20	Siddiq and others (1992)
Sunflower seed	Gallic acid	1.11	7.9		Raymond and others (1993)
Mango	4-MC		5.8	30	Robinson and others (1993)
Apple	Catechol	34		18	Oktay and others (1995)
Almond	4-MC		5.0		Fraignier and others (1995)
Plum	4-MC		4.5 ~ 5.5		Fraignier and others (1995)
Peach	4-MC		5.0		Fraignier and others (1995)
Cherry	4-MC		4.5		Fraignier and others (1995)
Apricot	4-MC		5.0 ~ 5.5		Fraignier and others (1995)
Iceberg lettuce	CA	0.8 ~ 1.4			Chazarra and others (1996)
	TBC	1.0 ~ 1.2			
Litchi	4-MC	10	7.0	70	Jiang and others (1997)
Herb	Catechol	25	7.5		Arslan and others (1997)
Pineapple			6.0 ~ 7.0		Das and others (1997)
Loquat	CA	0.105	4.5	30	Ding and others (1998)
Indian tea leaf	Catechin	0.49			Halder and others (1998)
	Epicatechin	0.81			
	Catechol	12.52			
	Pyrogallol	17.81			
	Gallic acid	19.33			
Raspberry	Catechol		8.0, 5.5		Gonzalez and others (1999)
Longan	4-MC		6.6	35	Jiang (1999)
Orchid aerial	4-MC	3.7 ~4.0	7.0		Ho (1999)
root	(+)-Catechin	2.1 ~ 5.5			
Blackberry					Gonzalez and others (2000)
Wild	Catechol	17.3			
Thornless	Catechol	196.4			
Coffee					Mazzafera and Robinson
Leaves	CA	0.882	6.0 ~ 7.0	30	(2000)
Endosperm	CA	2.27	6.0 ~ 7.0	30	

4-MC; 4-methylcatechol, L-DOPA; L-3,4-dihydroxyphenylalanine, DHPPA; 3-(3,4-dihydroxyphenol) propionic acid, CA; chlorogenic acid, TBC; tert-butylcatechol

Table 1: Properties of partially purified PPO from selected fruits and vegetables. *(Continued)*

Source	Substrate	K_m (mM)	Optimum pH	Optimum T (°C)	Reference
Field bean	Catechol	10.5			Paul and Gowda (2000)
	4-MC	4.0			
	L-DOPA	1.18			
	Pyrogallol	12.5			
Strawberry	Catechol	11.2	7.2	50	Serradell and others (2000)
Banana					
Pulp	Dopamine	2.8	6.5	30	Yang and others (2000)
Peel	Dopamine	3.9	6.5	30	Yang and others (2001)
Chinese cabbage	Catechol	682.5	5.0	50	Nagai and Suzuki (2001)
	Pyrogallol	15.4			
	Dopamine	62.0			
Peppermint	Catechol	6.25	7.0	30	Kavrayan and Aydemir
	L-DOPA	9.00	7.5	55	(2001)
	DL-DOPA	7.93	7.5	50	
Medlar	4-MC	7.5	6.5		Dincer and others (2002)
	Catechol	88.0			
	DHPPA	7.2			
	L-DOPA	76.2			
	Epicatechin	60.6			
Persimmon	TBC		3.5		Nunez-Delicado and others (2003)
Bean sprouts	Catechol	71.0	9.0	40	Nagai and Suzuki (2003)
	Pyrogallol	84.6			
	Dopamine	4.5			
Garland	CA	2.0	4.0	30	Nkya and others (2003)
chrysanthemum	(-)-epicatechin	10.0	8.0	40	
Artichoke	Catechol	10.2	6.0		Aydemir (2004)
	4-MC	12.4	6.0		
	DL-DOPA	36.3	8.0		
	L-DOPA	37.7	8.0		
	Pyrogallol	14.3	6.5		
	Callic acid	43.6	6.0		

4-MC; 4-methylcatechol, L-DOPA; L-3,4-dihydroxyphenylalanine, DHPPA; 3-(3,4-dihydroxyphenol) propionic acid, CA; chlorogenic acid, TBC; tert-butylcatechol

others 2005). On the other hand, no monophenol oxidase activity was reported in dates (Hasegawa and Maier 1980), Muscat grapes (Interesse and others 1984), sunflower seeds (Raymond and others 1993), pineapple (Das and others 1997), or longan fruit (Jiang 1999).

C. Assays of PPO activity

Development of a rapid, sensitive, and accurate method to determine PPO activity is of technological importance, providing adequate handling and controlling the acceptable quality of raw plant materials for both the fresh market and processing industry. The most common quantitative assay for extracted PPO determines the rate of *o*-quinone formation by spectrophotometrically assaying the increase in absorbance at 380 ~ 420 nm, depending on the selected substrate. For example, *o*-quinones formed from (+)-catechin, 4-methylcatechol, and chlorogenic acid substrates exhibit λ max at 385, 400, and 420 nm, respectively (Rouet-Mayer and others 1990). In the plot of absorbance over reaction time, the increase in absorbance is linear for only ~ 30 to 90 s and a decrease in absorbance is observed later. The decrease in absorbance is a result of the formation of insoluble dark colored polymers called melanins. While reacting with *o*-diphenol, PPO is inactivated by reacting with intermediate *o*-semibenzoquinone free radicals that act on copper atoms in the PPO active site (Whitaker 1994).

Several other chromogenic assays coupled with the addition of amino acids such as cysteine (Gauillard and others 1993; Penalver and others 2002), or glutathione (Penalver and others 2002) to reaction mixtures were proposed to determine diphenol oxidase activity by assaying corresponding quinone-amino acid or quinone-thiol adducts exhibiting greater molar absorptivity (ε) than *o*-quinones.

Moreover, Rodriguez-Lopez and others (1994) reported that 3-methyl-2-bezothiazoline hydrazone (MBTH) is a good nucleophile, forming MBTH-adducts with *o*-benzoquinone to increase the sensitivity of the spectrophotometric determination of PPO activity. Based on the V_{max} for catechol oxidation, the assay sensitivity to determine MBTH adduct was greater by 12.5 fold than the sensitivity to determine *o*-benzoquinone attributed to the large molar absorptivity of MBTH adduct. Espin and others (1996) confirmed that the method to add MBTH to reaction mixtures is also effective to determine monophenol oxidase activity of PPO extracted from pears by demonstrating large molar absorptivity of MBTH adduct as well as short lag periods.

Another approach to measurement of PPO activity is the determination of changes in oxygen concentration in reaction mixtures containing extracted PPO and selected substrate with an oxygen-sensitive electrode. Unlike the plot of absorbance of reaction mixtures containing PPO and substrates, the plot of oxygen concentration in a reaction mixture decreases over reaction time as oxygen molecules are consumed by PPO-related reactions (Mayer and others 1966). The polarographic method was used

for the determination of PPO from apples (Goodenough and others 1983), pears (Wissemann and Montgomery 1985) and coffee (Mazzafera and Robinson 2000).

HPLC (high performance liquid chromatography) methods can also be applied to determine PPO activity by quantifying the amounts of degraded substrates or *o*-quinones. Labuza and others (1990) determined commercial tyrosinase activity expressed as residual concentrations of tyrosine substrate.

D. Apple PPO

PPO from apples is more intensively studied than any other PPO source because of the large commercial production of apple foods around the world. Controlling PPO activity in apple tissues is one of the critical points providing high quality processed apple foods.

■ Postharvest physiology and control of apple PPO

The predominant phenolic compounds in apple cortex tissue are (+)-catechin, chlorogenic acid, (-)-epicatechin, procyanidin B_2, and phloridzin (Burda and others 1990; Mayr and others 1995; Russell and others 2002). In addition to these compounds, quercetin glycosides linked to rhamnose, rutinose, xylose, glucose plus galactose, and arabinose are detected in apple peels (McRae and Lidster 1990). The studies of apple PPO include the examination of relationships between endogenous phenolic compounds and PPO activity in apples at selected storage conditions

providing information on the browning of apple cultivars during distribution and preparation for processing.

The decrease of apple PPO activity during fruit maturation before harvest or storage after harvest is documented. Janovitz-Klapp and others (1989) reported that PPO activity in apples cv. Red Delicious decreased during one month of postharvest storage. Burda and others (1990) observed constant concentrations of (-)-epicatechin in extracts of apples cv. Golden Delicious, Empire, and Rhode Island Greening, and described tissue browning potential as the decrease in Hunter L* values of cut surfaces during storage of the cultivars. Amiot and others (1992) examined bruised apple tissue of twelve cultivars in relation to phenolic compound degradation and brown color formation. The degree of tissue browning was significantly correlated to the degradation of flavan-3-ols represented by epicatechin, or hydroxycinnamic derivatives represented by chlorogenic acid. Murata and others (1995) compared concentrations of phenolic compounds in apple cv. Fuji homogenates to the concentrations in original tissues as an index of PPO activity toward intrinsic substrates. Using apples six months after flowering, nearly 50% of phenolic compounds were degraded after apple homogenate preparation. Higher percentages of phenolic compounds in immature apples were degraded during homogenization, suggesting the decrease in PPO activity as apple fruit matured before harvesting.

During postharvest handling of raw apple fruit, minimizing metabolic respiration of crops by means of controlled atmosphere and temperatures leads to

maintenance of cell compartmentalization and retardation of PPO-catalyzed discoloration reactions in cells. During nine months storage at 0 °C in air, concentrations of phenolic compounds including phloridzin, chlorogenic acid, and quercetin rhamnoside were relatively stable in peels of apples cv. Granny Smith, Crofton, or Lady Williams (Golding and others 2001). Lattanzio and others (2001) reported that concentrations of chlorogenic acid, phloridzin, and quercetin-glycosides in apples cv. Golden Delicious increased during the first 60 d and decreased gradually for longer periods of cold storage at 2 °C. Lattanzio and others (2001) observed that larger concentrations of phenolic compounds and PPO activity in rotten apple tissues during the storage later than 105 d compared to the concentrations phenolic compounds and PPO activity in healthy tissues. In addition, a potential protective metabolism of apples against fungal attack was considered based on the fact that the presence of oxidized phloridzin and chlorogenic acid slowed spore germination of a fungi *Phlyctaena vagabunda*.

- Biochemical studies

Apple PPO exhibits both mono and diphenol oxidase activities. Goodenough and others (1983) partially purified apple cv. Dabinett PPO with a series of procedures consisting of acetone precipitation, ammonium sulfate precipitation, and column chromatography with Sephadex G15. The selected substrates used to determine K_m values for oxidation by the partially purified apple PPO were catechin (4.7 mM),

epicatechin (5.7 mM), chlorogenic acid (6.0 mM), L-Dopa (2.7 mM), and 4-methyl catechol (3.2 mM).

Espin and others (1995) established a sensitive and reliable spectrophotometric method to determine apple monophenolase activity. In the study, Triton X-114, (*p*-hyroxyphenyl) propionic acid (PHPPA), and MBTH were used as detergents for PPO extraction, a monophenol substrate, and a nucleophile forming MBTH-adducts, respectively. Espin and others (1998) conducted an atomic level investigation by NMR (nuclear magnetic resonance) coupled with a PPO kinetics approach using twelve substrates provided preferable substrates for apple cv. Verdedoncella PPO; i.e., monophenol 4-hydroxyanisole exhibiting high electric density at the carbon atom in the 4-position of the aromatic ring and diphenol catechol exhibiting no other ring substituents were adequate substrates for apple PPO reactivity.

Rocha and Morais (2001) compared substrate specificity of apple cv. 'Jonagored' PPO to five diphenol and two monophenol substrates. Substrate specificity of apple PPO, expressed as V_{max}/K_m, for *p*-cresol was the greatest, followed by L-dopa, 4-methyl catechol, (+)-catechin, dopamine, catechol, and L-tyrosine.

According to Janovitz-Klapp and others (1989), an effective buffer for apple PPO extraction included 0.5% Triton X-100 and 15 mM ascorbic acid adjusted to a pH of 7.2. After the extraction of apple cv. Red Delicious PPO with the buffer, a series of purification procedures consisting of 30-80% ammonium sulphate precipitation followed by hydrophobic chromatography with Phenyl Sepharose CL4B column

resulted in 120 fold of purification factor with 12% activity. The molecular weight of purified apple PPO was 46 kDa with a pI (isoelectric point) at 4.5 ~ 4.8. Maximum PPO activity was observed at pH of 4.5 ~ 5.0. The examination of enzyme kinetics using 4-methylcatechol, chlorogenic acid, and (+)-catechin resulted in similar K_m values ranging from 4.25 to 6.2 mM at pH 5.0. Zhou and others (1993) reported partially purified PPO from cv. Monroe apple peels exhibited maximum activity at pH of 4.5 ~ 5.0 and 30°C by using 4-methylcatechol, catechol, and chlorogenic acid as substrates.

Janovitz-Klappand others (1989) also determined and compared PPO activities in the peels and cortex (arbitrary units/kg of lyophilized tissue) of twelve apple cultivars obtained in France. The greatest PPO activity was exhibited by cv. Red Delicious and smallest activity by cv. Elstar in both peel and cortex extracts (Table 2).

Table 2: PPO activity (arbitrary units/mg lyophilized powder) in peel and cortex of 12 apple cultivars. (Janovitz-Klapp and others 1989)

Cultivar	Peel	Cortex	Cultivar	Peel	cortex
Red Delicious	3.04	3.10	Canada	1.46	2.73
MacIntosh	1.40	2.48	Granny Smith	1.30	2.26
Fuji	1.74	2.20	Mutsu	2.17	1.67
Gala	0.90	1.49	Jonagold	1.30	1.33
Florina	1.27	1.24	Charden	0.93	1.21
Golden Delicious	0.99	0.93	Elstar	0.31	0.62

- Controlled or modified atmosphere

Due to the loss of cell compartmentalization, fresh-cut fruits are considered more susceptible to enzymatic reactions than original whole fruits (Rolle and others 1987). The storage methods to apply modified and controlled atmosphere were investigated to increase shelf life stability of fresh-cut ready-to-eat apples such as slices, wedges, or cubes.

Soliva-Fortuny and others (2001) concluded that the shelf life of diced apples cv. 'Golden Delicious' stored at 4°C was extended by decreasing PPO activity with a vacuum in plastic pouches possessing oxygen permeability of 30 $cm^3/cm^2 \cdot bar \cdot 24$ hr under an initial atmosphere composed of 2.5% O_2 and 7% CO_2. Lanchiotti and others (1999) applied 15 mM hexanal to 100 g of fresh apple cv. Granny Smith slices packed in plastic bags and stored in modified atmospheres consisted of 70% N_2 and 30% CO_2. The little change in hue angle ($\tan^{-1}(*b/*a)$), an index of browning observed during storage at 4 or 15 °C for 18 d was attributed to the atmosphere containing volatile hexanal.

Rocha and Morais (2001) observed that PPO activity in diced apples cv. 'Jonagored' is inhibited by controlled atmosphere storage with large concentrations of CO_2 (~ 12%) resulting in the retention of apple cut surface color. Gunes and others (2001) concluded that storage of apple cv. Delicious slices under positive CO_2 pressure at 15 kPa at 5°C did not retard the color change of the apple slices during examination for 3 weeks.

Purified apple cv. Red Delicious PPO exhibited little activity in an environment with small oxygen concentrations as demonstrated in Lineweaver-Burk plots using 4-methyl catechol (Janovitz-Klapp and others 1990).

■ Thermal inactivation

Thermal processing is the most commonly utilized method to inactivate detrimental enzymes in fruits and vegetables for preserved foods. Thermokinetic studies of apple PPO are summarized in Table 3, demonstrating that z-values for thermal inactivation of apple PPO range around 10°C in the temperature range from 55 to 78 °C regardless of cultivar.

Table 3: z-values (°C) for thermal inactivation of PPO extracted from apples.

Reference	Wakayama (1995)		Yemenicioglu and others (1997)
Temperature range	55.0 ~ 67.5		68 ~ 78
Medium pH	3.8		6.8
Cultivar	z-value	Cultivar	z-value
Golden Delicious	11.8	Golden Delicious	9.9
Fuji	11.8	Starking Delicious	9.5
Jonathan	13.0	Granny Smith	9.5
Ohrin	11.4	Gloster	10.0
Red Delicious	10.5	Amasya	8.9
Tsugaru	11.4	Starcrimson	7.1

Zhou and others (1993) reported 13 and 94% inactivation of PPO activity in apple cv. Monroe peels at pH 5.0 after a 5 min heat treatment of 50 and 70°C, respectively. Chen and others (2004) suggested inactivation of PPO activity as an

index of pasteurization for adequate inactivation of pathogenic microorganisms in apple cider. Although further study is required examining cider composition, pH, and Brix, 50% PPO inactivation corresponded closely to the standard 5-log reduction in *E. coli* O157:H7 or *L. monocytogenes* required in apple juice with pH 3.7 and 12.5 °Brix.

- Antibrowning agents

According to Janovitz-Klapp and others (1990), PPO antibrowning agents that inhibit PPO activity are largely classified into two groups; reducing agents and enzyme inhibitors. Additionally, the enzyme inhibitors are divided into competitive and non-competitive compounds.

Sulphites. Sulphiting agents consist of sulfur dioxide (SO_2), sulphite (SO_3^-), bisulphite (HSO_3^-), and metabisulphite ($S_2O_5^{2-}$) depending on the pH of the environment. Sulphiting agents are reducing agents that reduce *o*-quinones to corresponding colorless diphenols. In spite of the effectiveness of sulphiting agents to prevent browning of foods (Molnar-Perl and Friedman 1990a, Molnar-Perl and Friedman 1990b), federal laws and public health concerns surrounding the ingestion of the sulphiting agents restrict the use of sulphites in the food industry. One of the objectives of PPO-related studies is to find alternatives to sulphites applicable in the food industry.

Sulfur-containing compounds. Molnar-Perl and Fiedman (1990a) reported the capabilities of L-cysteine, reduced glutathione, or N-acetyl-L-cysteine to inhibit browning of fresh apple cv. Golden Delicious. More than 80% inhibition was observed

with 1.7 mM concentrations of cysteine based on Hunter L* value change ((ΔL control – ΔL treated) x 100 / (ΔL control)) for 24 h at 1.7 mM concentrations. However, 1.7 mM concentrations of L-cysteine resulted in production of an undesirable odor surrounding the apple juice, whereas 1.7 mM concentrations of other the two compounds did not. Molnar-Perl and Fiedman (1990b) also regarded the use of reduced glutathione or N-acetyl-L-cysteine in dipping solutions as potential alternatives for sodium sulphite to prevent browning of sliced apples cv. Golden Delicious and Red Delicious. Son and others (2001) pointed out that methionine and histidine in addition to L-cysteine, reduced glutathione, and N-acetyl-L-cysteine were effective inhibitors of browning in apple cv. Liberty slices at equivalent concentrations in dipping solutions.

The mode of L-cysteine inhibition of brown color development is different from other reducing agents. Richard and others (1991) identified colorless 'cysteine addition compounds' (cysteine-quinone adducts) as reaction products formed by the addition of L-cysteine during oxidation of diphenols such as 4-methylcatechol, chlorogenic acid, (-)-epicatechin, or (+)-catechin by apple PPO. Moreover, Richard-Forget and others (1992) pointed out that partially purified cysteine-quinone adducts, formed in the reaction mixture containing apple cv. Red Delicious PPO, 4-methylcatechol,and L-cysteine, inhibited apple cv. Red Delicious PPO activity, potentially due to the role of cysteine-quinone adducts as substrate analogs. In the

presence of small concentrations of L-cysteine, the cysteine-quinone adducts were oxidized by apple PPO resulting in formation of dark colored pigments.

Ascorbic acid and its derivatives. Ascorbic acid is an antibrowning agent reducing *o*-quinones to *o*-diphenols. The addition of ascorbic acid to apple cv. Golden Delicious homogenates reduces not only *o*-quinones but also some polymerized pigments to *o*-diphenol and *o*-quinone, respectively, resulting in the removal of brown discoloration of the apple homogenates (Rouet-Mayer and others 1990). Ascorbic acid 2-phosphate, an ascorbic acid derivative, is more effective than ascorbic acid or erythorbic acid for prevention of browning of apple cv. Liberty slices (Son and others 2001).

Acidulants. PPO activity is affected by pH of the medium potentially due to conformation and configuration changes of the PPO molecules. Acidulants exert an inhibitory effect by reducing the pH and inhibiting PPO activity. Son and others (2001) compared the effectiveness of 12 acidulants at equivalent concentrations in dipping solutions, and listed oxalic, oxalacetic, malonic, tartaric, pyruvic, citric, malic, and lactic acids in order of capability to inhibit browning of apple cv. Liberty slices.

Halide salts. The inhibitory mode of halide salts on PPO activity is considered bilateral; one is the chelating action of anions on copper atoms in PPO molecules, and another is the ability to alter ionic environment for PPO molecules resulting in conformational changes. Stability and activity of PPO may vary with salt concentrations as well as pH, changing ionic strength of the medium. Rouet-Mayer and Philippon (1986) demonstrated that inhibition of apple cv. Golden Delicious PPO

by sodium chloride was pH dependent. More than 80% inhibition of PPO activity was achieved with 200 mM sodium chloride in solutions with a pH less than 5. Fan and others (2005) reported a different behavior between crude and purified PPO from apples cv. Fuji resulting from the addition of sodium chloride. A large concentration of salt at 5.1 M activated crude PPO and inactivated purified PPO. The study with UV spectra scanning demonstrated considerable stability of purified apple PPO in the presence of salt at concentrations more than 3.4 M during storage for three weeks. Other halide salts such as calcium chloride also contribute to prevention of undesirable color development in apple products. The presence of 1% calcium chloride in dipping solution was equivalent to 1% ascorbic acid or 1% sodium chloride in the inhibition of browning in apple cv. Delicious slices (Son and others 2001).

Sugars and Maillard reaction products. Billaud and others (2003) reported the inhibitory effects of sugars and Maillard reaction products on apple cv. Red Delicious PPO activity. The inhibition of apple PPO activity in the presence of 2 M glucose or fructose was attributed to the reduction of water activity. Glucose or fructose powders heated at 90°C for 24 h exhibited more significant inhibitory effects on apple PPO activity. Furthermore, Maillard reaction products formed from glucose/cysteine or fructose/cysteine by prolonged heating at 90°C exhibited even greater inhibition of apple PPO activity. Although the inhibition mechanisms were not clear, the results suggested possible functionality of caramelized sugars and Maillard reaction products as antibrowning agents.

Phenolic acids and substrate analogs. Son and others (2001) demonstrated the larger effectiveness of kojic acid to inhibit PPO activity in apple cv. Delicious slices compared to other six phenolic acids; *p*-coumalic acid, ferulic acid, cinnamic acid, gallic acid, chlorogenic acid, or caffeic acid. According to Chen and others (1991), kojic acid exhibits additional functionality as a reducing agent, while other phenolic acids play a role as substrate competitors. 4-Hexylresorcinol, a substituted resorcinol, is also regarded as a substrate competitor to inhibit PPO activity (Monsalve-Gonzalez A and others 1993). 4-Hexylresorcinol was characterized by an inhibitory effect at relatively low concentrations equivalent to five fold sulphite concentration on PPO activity in apple cv. Delicious slices (Son and others 2001).

Other sources. Lozano-De-Gonzalez and others (1993) analyzed the inhibitory effects of ten pineapple juice fractions on PPO activity in fresh apple cv. Red Delicious rings. Although not identified, the presence of small molecular weight proteins (< 10 kDa) may potentially contribute to the inhibition of browning in addition to the ascorbic acid present in pineapple juice. Chen and others (2000) concluded that the inhibition of PPO activity and browning of apple cv. Red Delicious homogenate after the addition of honey from five sources is attributed to the inhibition to the presence of antioxidants in the honeys. Additional inhibitory effects of honey were observed when combined with metabisulphite or ascorbic acid. Yoruk and others (2003) reported that the inhibitory activity of a new natural apple PPO inhibitor(s) extracted from

houseflies was highly pH dependent and extremely tolerant of physical treatments such as heating and irradiation.

Combination use of antibrowning agents. The combined use of anti-browning agents often demonstrates additional or synergistic effects to inhibit discoloration of fruits and vegetables. Pizzocaro and others (1993) pointed out two effective methods to inhibit PPO activity in diced apples cv. Golden Delicious. Comparing to individual use of ascorbic acid, the combination of ascorbic acid with citric acid or sodium chloride dissolved in dipping solutions resulted in 90 ~ 100% inhibition of the PPO activity. Ozoglu and Bayindirli (2002) investigated effective concentrations of three antibrowning agents for combined use to inhibit brown color development of apple cv. Golden Delicious juice. The addition of 0.49 mM ascorbic acid, 0.42 mM L-cysteine, and 0.05 mM cinnamic acid to the apple juice resulted in 99 and 106% inhibition of color development after incubation at 25 °C for 2 h represented as lightness (*L) and absorbance at 420 nm. Iyidogan and Bayindirli (2004) conducted a similar experiment using apple cv. Amasha juice and observed nearly 90% browning inhibition after incubation at 25 °C for 24 h, attributed to the addition of 3.96 mM L-cysteine, 2.78 mM kojic acid, and 2.34 mM 4-hexylresorcinol to the apple juice.

E. Plant cysteine proteases and PPO activity

Plant cysteine proteases are commercially applied in food industry to tenderize meat, clarify brews, and change texture of baked goods (Cowan 1983).

Properties of representative plant cysteine proteases; papain, ficin and bromelain are summarized in Table 4 (Wong 1995; Bankus and Bonds 2001).

Table 4: Properties of papain, ficin, and bromelain. (Wong 1995, Bankus and Bonds 2001)

	Papain	Ficin	Bromelain
EC	3.4.22.2	3.4.22.3	3.4.22.4
Source	Papaya	Fig latex	Pineapple stem
Molecular weight	21 kDa	26 kDa	33 kDa
Amino acid	159		
Cysteine bridge	3		3
pI	8.5 ~ 9.0		
Optimum pH	3.0 ~ 8.0	3.5 ~ 9.0	6.0 ~ 8.0
Optimum temperature (°C)		50 ~ 60	
Inactivation temperature		80	
Inhibitors	Heavy metal ions	Oxidants	Hg^{2+}
	Carbonyl derivatives	Heavy metals	
	Ascorbic acid	10^{-4} Sorbic acid	
Substrate specificity	Nonspecific	Phe-COOH	Lys-COOH
		Tyr-COOH	Arg-COOH
			Phe-COOH
			Tyr-COOH

Frozen fresh pineapple juice potentially containing bromelain activity as a dipping solution exhibits an equivalent inhibitory effect on browning of fresh apple cv. Golden Delicious slices with a 0.7% commercial solution containing sucrose, ascorbic acid, citric acid, and calcium phosphate (Lozano-de-Gonzalez and others 1993). However, the inhibitory effect of fresh frozen pineapple juice was less than canned pineapple juice presumably absence protease activity after dipping treatments of sliced apples for 18 h.

Labuza and others (1990) focused on the investigation of proteolytic enzymes to inhibit enzymatic browning in foods. First, an inhibition of 500 Sigma units commercial mushroom tyrosinase activity was achieved with 5 mg of ficin during incubation at 4 or 25°C. Tyrosinase activity was assessed by determining residual tyrosine substrate concentration or absorbance at 475 nm of reaction mixtures containing mushroom tyrosinase and tyrosine substrate. In a study using shrimp, 2% ficin in the dipping solution resulted in better inhibition of black spot formation than 0.5% sulfite solution after storage at 4°C for 72 h. In addition, the use of 2% papain in dipping solution was as effective as 0.5% sulfite solution to inhibit browning of apple cv. Red Delicious slices at 4 or 24°C for 45 h, while ficin or bromelain solution at equivalent concentrations were not as effective. Moreover, dipping treatments of potato pieces in 2% ficin solution and in 0.5% sulfite solution resulted in the equivalent browning inhibition after incubation at 4°C for 96 h. Labuza and others (1990) concluded that papain, ficin, and bromelain exhibited selective browning inhibitory effects resulting from particular specificity to PPO from selected plant sources. Marshall and others (FDA 2000) described the high costs of proteolytic enzymes as a reason for lack of utilization in the fruit and vegetable industry.

When crude proteolytic enzyme preparations are applied, there are additional inhibitory effects expected for browning inhibition of fruits and vegetables. For example, Richard-Forget and others (1998) purified and identified an inhibitor of endive PPO from papaya tree. The inhibitor was a dipeptide γ-Glu-Cys that reacted

with quinone to form peptide-quinone adducts. Rigal and others (2001) pointed out the presence of an inhibitor of PPO in papaya tree that reacted with active sites in PPO molecules.

F. Ultra high pressure (UHP) inhibition of PPO activity

■ UHP technology in the food industry

With the increase in consumers' trend toward high quality; acceptable appearance, flavor, and nutritional value in addition to microbial safety, the introduction of nonthermal processing technologies are of great interest to the food industry (Hoover 1997, Butz and others 2002). UHP processing is considered one of the potential nonthermal technologies to extend shelf life and retain or modify sensory attributes (Pothakamury and others 1995; Tewari and others 1999).

Research utilizing UHP technology was carried out to inactivate pathogenic and spoilage microorganisms as well as intrinsic enzymes to impart extended safety and shelf life to food products (San Martin and others 2002). Structural changes of proteins resulting from UHP treatment play a critical role in the inactivation of both microorganisms and enzymes. Like any other chemical reactions, the principle of Le Chatelier is applied to UHP technology (Balny and Masson 1993). Under pressurized conditions, the decrease in molar volume results in equilibrium shifts toward the disruption of selected chemical bonds. However, not all chemical bonds in protein molecules are affected by UHP. Non-covalent bonds such as ionic bonds, hydrogen

bonds, and hydrophobic bonds (affinities) contributing to secondary, tertiary, and quaternary structures may be disrupted resulting in structural opening of proteins (Heremans 1982).

- UHP treatment of PPO extracted from plant sources

Seyderhelm and others (1996) investigated sensitivity of eight commercially available enzymes to UHP treatment, and listed the barotolerance based on inhibition kinetic constants (*k*) at selected pressure and temperature as follows; peroxidase > polyphenol oxidase > catalase > phosphatase > lipase > pectin esterase > lactoperoxidase > lipoxygenase. PPO is relatively less sensitive to UHP compared to other enzymes.

Few research experiments report complete inactivation of PPO by UHP treatment at ambient temperatures (Table 5). Gomes and Ledward (1996) reported inactivation of commercial purified tyrosinase from mushrooms by UHP treatment at 800 MPa for at least 5 min, or by mild heat treatment at 60°C for 30 min. The HPLC profile of UHP treated tyrosinase differed from the profile of heat treated tyrosinase, suggesting protein aggregation during UHP treatment. Gomes and Ledward (1996) also pointed out that partially purified mushroom PPO was more pressure resistant than commercial purified mushroom PPO.

The activation of PPO activity after the treatment at relatively low pressure around 400 MPa was proposed after the report of Asaka and Hayashi (1991) that PPO in Bartlett pears was activated by UHP treatment of 400 ~ 500 MPa for 10 min.

Table 5: Inactivation of PPO from plant sources by UHP treatment at ambient temperature.

Reference (Year) Source, Media	Pressure (MPa)	Time (min)	Effect
Asaka and Hayashi (1991)			
Pear cv. Bartlett	400 ~ 500	10	Activation
Rovere and others (1994)			
Apricot cv. not mentioned			
Puree	300	2.5	42% activation
	500	5	30% activation
	900	2.5	80% inactivation
Anese and others (1995)			
Apple, cv. not described			
Crude juice pH 4.5	0 ~ 900	1	Activation at 200 MPa
Crude juice pH 5.4	0 ~ 900	1	Activation at 200 MPa
Crude juice pH 7.0	0 ~ 900	1	99% inactivation at 900 MPa
Gomes and Ledward (1996)			
Mushroom			
Purified, commercial	800	5	100% inactivation
Partially purified	400	10	40% activation
	800	10	40% inactivation
Potato			
Partially purified	800	10	60% inactivation
Weemaes and others (1998)			
Apple cv. Golden Delicious			
Purified pH at 6.0	800	30	84% inactivation
Avocado cv. South African			
Purified pH at 7.0	800	180	73% inactivation
White grape cv. Italian			
Purified pH at 6.0	800	75	87% inactivation
Pear cv. Durondean			
Purified pH at 6.0	900	180	58% inactivation
Plum cv. Red Beauty			
Purified pH at 7.0	800		Not inactivated

Table 5: Inactivation of PPO from plant sources by UHP treatment at ambient temperature. *(Continued)*

Reference Source, Media	Pressure (MPa)	Time (min)	Effect
Palou and others (1999)			
Banana			
Puree (Blanched)	0 ~ 689	10	Activation at 517 MPa 21% inactivation at 689 MPa
Lopez-Malo and others (1999)			
Avocado			
Puree pH at 4.1	345 ~ 689	10 ~ 30	75, 78, 84% inactivation at 689 MPa for 10, 20, 30 min
Guerrero-Beltran and others (2004)			
Peach			
Puree	103 ~ 517	0 ~ 25	99% inactivation at 517 MPa for 25 min
Garcia-Palazon and others (2004)			
Red Raspberry			
Homogenate	400 ~ 800	0 ~ 15	Not inactivated
Strawberry			
Homogenate	400 ~ 800	0 ~ 15	99% inactivation at 600 MPa

Rovere and others (1994) observed that apricot PPO was activated by UHP treatment at 300 MPa for 2.5 min or 500 MPa for 5 min. Remarkable inactivation of apricot PPO was achieved when pressurized at 900 MPa. Weemaes and others (1998) reported that the inactivation of PPO activity by UHP treatment at higher than 600 MPa follows first-order kinetics. Conducting kinetic studies of PPO inactivation by UHP treatment, Weemaes and others (1998) listed PPO fruit sources in order of decreasing pressure tolerance; plums, pears, avocadoes, grapes, and apples. Garcia-Palazon and others

(2004) demonstrated a difference of pressure stability of PPO between red raspberries and strawberries; the former PPO was extremely stable at 400 ~ 800 MPa for 15 min while the latter PPO was 99% inactivated at 600 MPa for 15 min.

- Combined UHP and heat processing

Rovere and others (1994) compared UHP inactivation of apricot PPO at 25 and 50 °C. While residual PPO activity was still detected after UHP treatment at 900 MPa and 25 °C for 10 min, complete inactivation of PPO was achieved with UHP treatment at 700 MPa for 5 min or at 900 MPa for 2.5 min by elevating temperature to 50 °C.

Weemaes and others (1998) examined temperature dependence of kinetic constant (*k*) values for inactivation of plum PPO at atmospheric pressure and 900 MPa. A stabilizing effect of UHP treatment at 900 MPa on plum PPO was observed by increasing temperature to 57.5 °C or more, compared to thermal treatments at equivalent temperatures and atmospheric pressure.

Rapeanu and others (2005) conducted UHP treatments at selected pressure and temperature to inactivate white grape var. Victoria PPO. Plots of ln (*k*) values against selected temperatures and pressures were used to calculate E_a and V_a, respectively (Table 6). Comparing V_a values in relation to equivalent pressure ranges at selected temperatures, grape PPO was less sensitive to the change in pressure as temperature increased. The *k* values for grape PPO inactivation at selected pressures and temperatures are summarized in Table 7. A stabilizing effect of UHP treatment at

100 ~ 600 MPa on grape PPO activity was observed by combining pressure with temperature at 45 °C or greater. At 52.5°C, for example, *k* values for white grape PPO inactivation at 300 and 600 MPa were 0.052 and 0.168, respectively, whereas PPO was more rapidly inactivated (*k* = 0.532) at ambient pressure.

Table 6: Parameters (E_a and V_a) for thermal and combined pressure- temperature inactivation of Victoria grape PPO. (Rapeanu and others 2005)

Temperature		Pressure		
Range	E_a	Temperature	Range	V_a
°C	(kJ/mol)	°C	MPa	(cm^3/mol)
45 ~ 55	202.91 ± 6.15	10	600 ~ 800	-28.0 ± 1.8
		20	600 ~ 800	-25.1 ± 1.9
		30	500 ~ 800	-30.2 ± 1.3
		40	500 ~ 800	-23.2 ± 2.1
		45	400 ~ 600	-17.2 ± 6.4
		47.5	400 ~ 600	-15.8 ± 5.3
		50	400 ~ 600	-12.7 ± 8.6

Table 7: First-order kinetic constants *k* ($\times 10^{-2}$) for combined pressure-temperature inactivation of Victoria grape PPO. (Rapeanu and others 2005)

	Temperature (°C)				
Pressure (MPa)	45	47.5	50	52.5	55
0.1	8.61	17.89	29.47	53.24	92.92
100	4.15	8.36	14.39	20.72	
300	1.27	2.18	3.91	5.18	
600	8.87	10.36	15.19	16.80	

■ Combined UHP and antibrowning agents

In addition to the effects of pressure, time, and temperature, the use of antibrowning agents is intensively studied in relation to the efficacy, improvement,

and optimization of UHP treatment to inactivate PPO. For example, potato cv. Desiree PPO activity was more significantly inhibited by UHP treatment at 400 MPa for 15 min as citric acid concentration in dipping solutions increased (Eshtiaghi and Knorr 1993). Combined with UHP treatment, the roles of antibrowning agents are classified into a sensitization effect; described as additive or synergistic inactivation together with UHP; no effect; described as no change in efficacy of UHP treatment by adding antibrowning agents; and stabilizing effect, described as inhibition of pressure inactivation. The effects of selected antibrowning agents combined with UHP treatment to inactivate PPO activity are summarized in Table 8.

Weemaes and others (1997) determined stability of commercial mushroom tyrosinase to pressure and heat treatment in the absence and presence of antibrowning agents. EDTA did not alter pressure stability of mushroom tyrosinase, while stabilizing the tyrosinase toward heat treatment. The presence of benzoic acid or glutathione decreased pressure stability of PPO. Unlike the response to pressure, the presence of benzoic acid increased heat stability of mushroom tyrosinase. Weemaes and others (1999) lists selected concentrations of antibrowning agents capable of inhibiting avocado PPO activity by 25 ~ 50% at ambient pressure. Among five antibrowning agents tested, only EDTA was demonstrated to assist avocado PPO inactivation by UHP, whereas the addition of NaCl, 4-hexylresorcinol, glutathione, or benzoate resulted in larger V_a values than controls, indicating a stabilizing effect on avocado PPO during UHP treatments.

Table 8: PPO inactivation by UHP treatment with antibrowning agents.

Agent	Concentration	Pressure (MPa)	Time (min)	Effect
Mushroom, purified (Weemaes and others 1997)				
EDTA	5 mM	800		No effect
Benzoic acid	50 mM	800		Sensitization
Glutathione	5 mM	800		Sensitization
Avocado, partially purified (Weemaes and others 1999)				
EDTA	10 mM	575 ~ 700	0 ~ 180	Sensitization
NaCl	10 mM	700 ~ 825	0 ~ 180	Stabilization
4-Hexylresorcinol	0.05 mM	700 ~ 825	0 ~ 180	Stabilization
Glutathione	0.05 mM	700 ~ 800	0 ~ 180	Stabilization
Benzoate	10 mM	700 ~ 800		Stabilization
Apple, diced in brine (Ibarz and others 2000)				
Ascorbic acid	50 ppm	138 ~ 690	1 ~ 30	No effect
Citric acid	50 ppm	138 ~ 690	1 ~ 30	No effect
4-Hexylresorcinol	50 ppm	138 ~ 690	1 ~ 30	Sensitization
Peach, puree (Guerrero-Beltran and others 2004)				
Ascorbic acid	300 ppm	103 ~ 310	0 ~ 25	Stabilization
		414 ~ 517	0 ~ 25	No effect
Cysteine	300 ppm	310 ~ 517	0 ~ 25	Sensitization

Ibarz and others (2000) reported PPO in apple cv. Golden Delicious or Granny Smith slices is significantly sensitized by UHP treatment when the apple slices are dipped in 50 ppm 4-hexylresocinol solution. Addition of ascorbic acid or citric acid to brines did not alter the pressure stability of apple PPO.

Guerrero-Beltran and others (2004) concluded that the effect of adding ascorbic acid or cysteine on UHP inactivation of peach PPO was pressure dependent. The presence of ascorbic acid stabilized peach PPO during UHP treatments at 103 ~

310 MPa, while no effect was observed during UHP treatments at 414 ~ 517 MPa. The addition of cysteine promoted pressure inactivation of peach PPO by UHP at 310 MPa or greater pressures.

- Color stability of UHP treated fruit products during storage

Extended shelf life of fruit and vegetable products by UHP treatment is expected to be attributed to the inhibition of intrinsic PPO, resulting in small color change during storage.

Lopez-Malo and others (1999) investigated pressure and pH effects on inhibition of avocado PPO and color stability during storage. Avocado PPO was significantly inhibited by lowering initial pH of the puree and increasing the pressure. The color change of UHP treated avocado puree during storage was dependent on residual PPO activity after UHP treatment and storage temperature.

Palou and others (1999) examined inhibition of PPO activity in banana puree subjected to short-time steam blanching followed by UHP treatment expressed as color change potential during storage. Processing methods including longer blanching times and higher pressure treatments resulted in smaller residual activity of banana PPO. The residual banana PPO activity determined immediately after UHP treatment was negatively correlated to the browning of banana puree during an induction period and positively correlated to browning rate during storage at 25 °C.

Palou and others (2000) observed color changes of guacamole treated with continuous or oscillatory UHP for selected times. Even though PPO inactivation by

86% was achieved with four cycles of UHP for 5 min each, no difference in initial color parameters was detected compared to guacamoles containing larger residual PPO activity. The increase in Hunter a* value of guacamole during storage was dependent on storage time, temperature, and the number of pressurization cycles.

Guerrero-Beltran and others (2004) examined the discoloration of UHP treated peach puree or UHP treated peach puree containing anti-browning agents during storage. Regardless of the addition of antibrowning agents, the trend of less discoloration of peach puree treated with higher pressure for longer time was observed as long as the puree was stored at refrigerated temperature.

PART II: PECTIN METHYLESTERASE (PME) AND POLYGALACTURONASE (PG)

A. Overall features of pectic substances and pectic enzymes

Pectic substances, the main cell wall components of fruits and vegetables together with cellulose and hemicellulose, are located in the middle lamella playing a role of “glue” to adhere one cell wall to another. Pectic substances include two groups, pectic acid and pectin. Pectic acid is homogeneous galacturonic acid residues linked by α-1,4 glycoside bonds. Pectin is a heterogeneous polysaccharide complex naturally present in fruits and vegetables. The main components of pectin are galacturonic acid residues linked by α-1,4 glycoside bonds with methylesterified carboxyl groups in variable quantity. Pectin is also characterized by the presence of other natural sugars

such as galactose, glucose, rhamnose, arabinose, and xylose in both main and side chains. The degree of methyl esterification in pectin molecules varies depending on plant source and maturity.

Pectin methylesterase (PME) is an endogenous enzyme in plants that catalyzes deesterification of carboxyl groups in pectin molecules yielding galacturonic acid and methanol. The resulting galacturonic units in pectin molecules are potentially substrates for polygalacturonase (PG). PG is also an endogenous enzyme that catalyzes hydrolytic cleavage of α-1,4 glycoside bonds between galacturonic acid residues, resulting in tissue softening of fruits and vegetables.

The contributions of PME and PG to tissue softening vary depending on source of fruits and vegetables. Barrett and Gonzalez (1994) reported that softening of cherries cv. 'Royal Anne' and cv. 'Bada' before or after harvest accompanied rapid increases in PME and PG activities together with increases in weight, volume, and soluble solid concentration of cherries. In the study, cherry PME activity appeared one week prior to PG activity. Wegrzyn and MacRae (1992) pointed out that an increase in kiwi cv. 'Hayward' PG activity was associated with the decrease in kiwi firmness. The activity of kiwi PME after harvest gradually decreased during storage under atmospheric condition, while separate PME activity was induced during ethylene treatment to promote ripening of kiwi fruits. Ketsa and Daenglanit (1999) and Imsabai and others (2002) investigated PG and PME activities during the ripening of durian fruit at 27°C after harvest in relation to pectin composition. A rapid increase

in durian PG activity paralleled tissue softening and an increase in water soluble pectin, whereas PME activity did not change dramatically. Tijskens and others (1998) observed the simultaneous increase in PG activity in peaches cv. Red Haven and the decrease in fruit firmness were dependent on storage temperature.

B. Control of PME and PG activities

■ Isozymes of PG and PME

Pathak and Sanwal (1998) separated three banana PGs (PG I, II, and III) exhibiting different molecular weights, optimum pH, thermostability, and response to the addition of Ca^{2+}. As endo-PGs that randomly hydrolyze α-1,4 glycoside bonds between galacturonic residues, decrease in PG I activity and increase in PG III activity were observed during ripening of bananas. Pathak and others (2000) demonstrated that purified banana PG III, the largest molecular weight PG, consisted of an approximate 90 kDa main unit and a 29 kDa subunit. Tomatoes also contain two different forms of PG, PG I and II. PG I is composed of a subunit of PG II tightly bound with a 37 ~ 39 kDa glycoprotein. PG II is a single polypeptide containing the catalytic active site of polygalacturonase (Zheng and others 1992). Pressey (1986) established a rapid method to extract tomato PG I and II separately applying selected concentrations of sodium chloride in the extraction buffer. After insolublization of PG at pH 3, PG I and II were extracted at pH 6.5 in 1.2 or 0.5 M sodium chloride solutions, respectively.

Isozymes of PME and PG are often detected by identified specific stability during heat or pressure treatment. The presence of thermotolerant or barotolerant PME resulted in the residual activity after a prolonged pressure or heat treatment time, leading to a fractional conversion model in the plot of residual activity versus treatment time.

- Kinetic data analysis

Inactivation of an enzyme is generally described by first order kinetics following the equation (Whitaker 1994), where A_0 and A_t represent the initial and residual activity at treatment time t.

$$\text{Ln}\ (A_t / A_0) = -k \bullet t$$

The kinetic constant *k* is available as far as Ln (A_t / A_0) follows a linear regression curve against the treatment time. When both labile and resistant fractions are present, pressure or heat treatment can result in a horizontal curve exhibiting residual activity after prolonged treatment times because of little inactivation of the resistant fraction. A fractional conversion model is applied to explain the inhibition of the labile fraction using A_∞ as a residual activity after prolonged treatment times (Broeck and others 1999).

$$\text{Ln}\ \{(A_t - A_\infty)/(A_0 - A_\infty)\} = -k \bullet t$$

■ Thermal inactivation of PME and PG

The studies on thermal inactivation of PME and PG include the investigation of dependence on media pH or tissue status, and the comparison of thermostability among isozymes or between PME and PG from the selected plant source (Table 9).

Table 9: z-values (°C) for thermal inactivation of PME and PG from plant sources.

Source	Forms	Range (°C)	z-value (°C)	Reference
PME				
Tomato	Purified	62 ~ 70	4.6	Fachin and others (2002a)
	Juice	62 ~ 80	6.2	
	Juice	70 ~	4.8 ~ 5.2	Anthon and others (2002)
Carrot	Purified	50 ~ 56	6.5 (pH 4.5)	Balogh and others (2004)
		50 ~ 60	6.1 (pH 5.5)	
		50 ~ 60	5.4 (pH 6.0)	
	Juice	50 ~ 60	5.7 (pH 6.0)	
	Pieces	66 ~ 74	4.1 (pH 6.0)	
Papaya	Purified	55 ~ 70	7.8 (pH 4.0)	Fayyaz and others (1995)
		60 ~ 75	8.4 (pH 7.5)	
Soursop	Purified (pH 7.5)	45 ~ 70	8.5 for PE I 8.6 for PE II	Arbaisah and others (1997b)
Mango	Partially purified	70 ~ 85	18.5	Labib and others (1995)
Acerola	Partially purified	98 ~ 106	4.7	Assis and others (2000)
PG				
Tomato	Juice	90 ~ 70 ~	7.1 ~ 7.7 (PG I) 10.4 ~ 10.8 (PG II)	Anthon and others (2002)
Mango		70 ~ 80	12.3	Labib and others (1995)

Media pH. Fayyaz and others (1995) demonstrated that purified papaya PME was more heat stable at pH 7.5 than at pH 4.0. Complete inactivation of purified papaya PME was achieved with heat treatment at 70 °C and pH 4.0 for 5 min or at 75 °C and pH 7.5 for 5 min. Balogh and others (2004) examined pH dependence of purified carrot PME stability during heat treatment. As media pH increased from 4.5 to 6.0, z-values for the inactivation of purified carrot PME decreased.

Tissue status. Fachin and others (2002a) determined heat stability of purified tomato PME to the stability of PME in crude juice. The greater heat stability of purified PME was demonstrated with larger D-values in selected temperature ranges compared to D-values of PME in juice. Balogh and others (2004) compared heat stability of carrot PME in three different tissue states. At pH 6.0, carrot PME was more heat resistant in carrot pieces compared to purified PME or carrot PME in juice.

Isozymes. Arbaisah and others (1997a) purified soursop PME I and PME II separately, exhibiting molecular weights of 29 kDa and 24 kDa, respectively. Arbaisah and others (1997b) concluded that PME I is more heat stable than PME II as demonstrated by a larger D-value at 65 °C and z-values in a temperature range of 45 to 75 °C. Anthon and others (2002) demonstrated greater heat stability of tomato PG I than PG II. D-values for inactivation of tomato PG I at 90 °C and PG II at 70 °C were 15.1 to 16.1, and 2.6 to 4.0, respectively.

Comparison between PG and PME. Labib and El-Ashwah (1995) determined heat stability of PG and PME extracted from mangoes. Setting the reference temperature at

70 °C, the less heat stability of PG with z-value of 12.25 °C was demonstrated compared to PME with z-value of 18.5 °C. According to Anthon and others (2002), D-values for inactivation of tomato PG II and tomato PME at 70 °C were 2.6 to 4.0 min and 7.2 to 10.4 min, respectively, suggesting greater heat stability of tomato PME compared to PG II.

Comparison to other sources. Assis and others (2000) observed that acerola PME was inactivated by heat treatment at 98 °C for 110 min. The heat stability of acerola PME was substantially greater than citrus PME inactivated by heat treatment at 90 °C for 1 min. The heat treatment of acerola PME at 98, 102, or 106 °C resulted in a z-value of 4.71 °C.

- UHP inactivation of PME and PG

The presence of a pressure stable fraction as well as a heat stable fraction of PME was reported for selected fruits and vegetables. A fractional conversion model was applied for the study of inactivation kinetics. Compared to thermal inactivation at atmospheric pressure, application of UHP at elevated temperatures often resulted in an antagonistic effect on PME inactivation. UHP treatment at 600 MPa combined with increased temperatures of 70 °C inhibited tomato PME or a stable fraction of orange PME very little. In addition, unique behavior of heat stable tomato PG toward UHP treatment was demonstrated. A number of studies on UHP inactivation of PME extracted from oranges, carrots, bananas, and tomatoes are documented as follows.

Orange PME. Substantial PME activity in citrus juice results in cloud loss of products during storage. UHP processed orange juice was evaluated in relation to residual PME activity and cloud loss. Parish (1998) concluded that pressure treatment at 700 MPa and ambient temperature for 90 s or treatment at 500 MPa and 60 °C for 90 s did not produce orange juice possessing good cloud stability during storage at 4 or 8 °C for 16 weeks when compared to thermal treatment at 98 °C for 10 s. Goodner and others (1999) applied pressures of 700 MPa or more for 1 min to orange juice, resulting in the production of the juice exhibiting 90 d of refrigerated shelf life judged by cloud stability as well as microbial safety. Nienaber and Shellhammer (2001a) produced an orange juice composed of orange juice treated with UHP at 800 MPa and 25 °C for 1 min and thermally pasteurized pulp exhibiting good cloud stability during storage at 4 or 37 °C for more than 2 months.

Basak and Ramaswamy (1996) examined the effects of pH and soluble solids concentration on pressure inactivation of orange PME. Endogenous orange PME in orange juice was inactivated more rapidly at pH 3.2 than at pH 3.7. Using pasteurized orange concentrates and exogenous orange PME, baroprotective effect of media containing large concentrations of soluble solids on PME activity was demonstrated. Broeck and others (1999) examined the effects of media pH and presence of sucrose and $CaCl_2$ on orange PME inactivation by thermal and pressure treatments. Lowering media pH or soluble solids concentration in the media resulted in the acceleration of orange PME inactivation by thermal or pressure treatment. The addition of 1.5 M

$CaCl_2$ to the media increased the inactivation rate constants for thermal treatments. A stabilizing effect of equivalent concentrations of $CaCl_2$ on orange PME was observed during pressure treatment at 800 MPa or greater.

Nienaber and Shellhammer (2001b) investigated the effect of combined pressure and temperature treatment on orange PME activity in labile and stable fractions separately. Labile orange PME was effectively inactivated as pressure and temperature increased within a pressure range of 400 to 600 MPa and a temperature range of 25 to 50°C, resulting in greatest inactivation kinetic constants obtained from treatment at 600 MPa and 50 °C. However, little inhibition of the orange PME stable fraction was achieved with pressure treatment at 900 MPa and 25 °C.

Broeck and others (2000) applied combined heat and pressure treatment to inactivate the heat stable fraction of orange PME that accounted for approximately 5% of total activity and was inhibited at 73 °C or more. The inhibition of the heat stable orange PME was suppressed by pressure of 100 MPa or greater combined with treatment temperatures of 70 or 80 °C. The application of pressure at 600 MPa combined with temperatures of 70 or 80 °C provided barostability to orange PME.

Carrot PME. Ly-Nguyen and others (2002a) described the presence of both heat and pressure stable fractions in purified carrot PME. Thermal or pressure inactivation of purified carrot PME followed a fractional conversion model within the temperature range of 48 to 60 °C or within the pressure range of 600 to 700 MPa, respectively. Ly-Nguyen (2003a) also pointed out decreased inactivation of carrot PME when pressures

of 100 to 300 MPa were applied at temperatures greater than 50 °C. The smallest V_a value for carrot PME inhibition was obtained by UHP treatment at 600 to 815 MPa and at 50 °C (Table 10).

Table 10: Thermal and/or pressure inactivation of PME extracted from plant sources.

PME Source (Reference)				
Temperature		Pressure		
Range	E_a (kJ/mol)	Temperature	Range	V_a (cm^3/mol)
Carrot, purified (Ly-Nguyen and others 2002a)				
48 ~ 60°C	289.2	10°C	600 ~ 850 MPa	-54.7
Carrot, purified (Ly-Nguyen and others 2003a)				
48 ~ 60°C	274.5	10°C	700 ~ 810 MPa	-18.6
60 ~ 66°C	291.2	20°C	700 ~ 820 MPa	-30.0
		30°C	600 ~ 825 MPa	-44.9
		40°C	600 ~ 815 MPa	-46.0
		50°C	600 ~ 815 MPa	-47.7
		54°C	500 ~ 815 MPa	-39.6
		60°C	600 ~ 750 MPa	-32.1
		65°C	500 ~ 700 MPa	-25.0
Strawberry, purified (Ly-Nguyen and others 2002b)				
54 ~ 63°C	206.7	10°C	850 ~ 1000 MPa	-10.8
Banana, partially purified (Ly-Nguyen and others 2002c)				
65 ~ 72.5°C	379.4	10°C	600 ~ 700 MPa	-59.2
Banana, purified (Ly-Nguyen and others 2003b)				
64 ~ 76°C	379.2	30°C	800 ~ 900 MPa	-33.7
		40°C	700 ~ 900 MPa	-41.5
		50°C	800 ~ 900 MPa	-36.7
		55°C	800 ~ 900 MPa	-37.7
		60°C	700 ~ 900 MPa	-39.5
		64°C	700 ~ 800 MPa	-45.6
		70°C	500 ~ 800 MPa	-34.2
		73°C	400 ~ 800 MPa	-25.6
		76°C	300 ~ 800 MPa	-21.6

Banana PME. Approximately 8% of purified banana PME activity remained after UHP treatment at 600 to 700 MPa at 10 °C for prolonged times accounting for the presence of a pressure stable fraction of PME in bananas, while more than 90% of activity of the PME was lost by heat treatment at 75 °C for 5 min (Ly-Nguyen 2002c). The application of UHP of 800 MPa at 73 or 76 °C less effectively inactivated purified banana PME compared to equivalent heat treatments at atmospheric pressure (Ly-Nguyen 2003b). Banana PME was sensitive to pressure increases ranging from 700 to 800 MPa at 64 °C (Table 10).

Tomato PME and PG. Relatively large numbers of studies on pressure inactivation of PME and PG in tomato products are reported. Shook and others (2001) examined stability of PME and PG in diced tomatoes during pressure treatment at 400 to 800 MPa. Tomato PG is effectively inactivated at 45 °C by pressure treatment at 600 MPa or more for 5 min. Tomato PME is activated during pressure treatment at 400 MPa and 45 °C and exhibited high stability to UHP treatment at 600 and 800 MPa. Crelier (2001) compared heat inactivation and combined pressure-heat inactivation of PME and PG in tomato juice. Tomato PME and PG were inactivated by heat treatment at atmospheric pressure following first order kinetics at temperatures of 60 to 75 °C and 80 to 105 °C, respectively. Tomato PG activity was inactivated completely by UHP treatment at 600 MPa and 30 °C for 5 min. Stabilization of tomato PME was observed at temperatures of 60 to 75 °C under pressure conditions at 100 to 600 MPa as presented in Table 11.

Table 11: First-order kinetic constants k ($\times 10^{-6} \cdot s^{-1}$) for combined pressure-temperature inactivation of tomato PME. (Crelier and others 2001)

Pressure	Temperature (°C)			
(MPa)	60	65	70	75
0.1	161	872	6250	36800
100	7.58	163	883	574
300	9.78	112	51.4	18.3
600	43.8	44.3	70.2	52.5
800	1500	2330	2920	2710

Further studies of tomato PG inactivation using heat and pressure conducted by Fachin and others (2002b) are summarized in Table 12. Heat inactivation of tomato PG at temperature range from 58 to 65 °C was characterized by fraction conversion model, while biphasic inactivation of tomato PG was observed at higher temperatures. Combined pressure and temperature treatments of tomato PG resulted in the potential reduction of pressure treatment time by increasing temperature to achieve equivalent PG inactivation. Fachin and others (2003) also pointed out the absence of pressure resistant PG fraction in tomato juice with pressure treatments ranging from 350 to 500 MPa.

The stabilities of two PG isoforms to heat or pressure treatments were elucidated by Peeters and others (2004). PG I was completely inactivated by exposing the enzyme to temperature at 65 °C for 5 min. PG II activity remained after heat treatment at 90 °C for 5 min. UHP treatments of 500 MPa and 25 °C for 15 min led to significant inactivation of both PG I and PG II below the detection limit. Similar

stability of PG I and II after equivalent pressure treatments were attributed to rapid dissociation of β-subunit from PG I by increasing pressure.

Table 12: Thermal and pressure inactivation of tomato PG. (Fachin and others 2002b)

Temperature		Pressure		
Range	E_a (kJ/mol)	Temperature	Range	V_a (cm^3/mol)
58 ~ 65°C	457.1 ± 47.8	5°C	500 ~ 600 MPa	-44.8 ± 5.1
70 ~ 90°C	347.9 ± 18.0	10°C	450 ~ 600 MPa	-40.1 ± 7.6
		15°C	450 ~ 550 MPa	-35.2 ± 4.9
		20°C	350 ~ 550 MPa	-43.6 ± 6.5
		25°C	350 ~ 500 MPa	-58.8 ± 6.1
		30°C	350 ~ 450 MPa	-76.9 ± 5.4
		35°C	350 ~ 450 MPa	-84.0 ± 10.4
		40°C	350 ~ 400 MPa	-89.7 ± 7.5

C. Utilization of PME to alter texture of fruits and vegetables

In fruit and vegetable processing, the actions of intrinsic PME and PG to degrade pectin molecules result in the decrease of firmness of solid tissues or viscosity of homogenates. The common methods to produce tomato juice or paste are defined as “cold break” and “hot break” processing to produce tomato products exhibiting selected viscosities (Anthon and others 2002). Crushed tomatoes are incubated at around 60 °C potentially activating intrinsic PME and PG activities during the cold break processing of tomatoes. The hot break processing of tomatoes involves crushing tomatoes at temperatures of approximately 95 °C or heating crushed tomatoes immediately to 95 °C to inactivate intrinsic tomato PME and PG activities.

The deesterifying action of PME on pectin provides not only substrates for PG, but also substrates for subsequent cross-linking in the presence of divalent cations. Carboxyl groups located at the carbon-6 position of galacturonic acid residues bind with each other between adjacent pectin chains by way of divalent cations such as calcium to form calcium bridges, resulting in an increase in tissue firmness (Whitaker 1996).

Bartolome and Hoff (1972) demonstrated the increase in potato firmness after mild heat treatment in water, and hypothesized the mechanism as follows; mild heat treatment maintained or activated PME activity and changed cell membrane function allowing intrinsic divalent cations to permeate through the cell membranes to cell walls, resulting in the formation of calcium cross-linking of pectins defined generally as 'calcium bridges'. Alonso and others (1997) observed that thermal pretreatment of cherries at 50 °C for 10 min or 70 °C for 2 min decreased the degree of esterification in pectin molecules and increased the activity of PME bound to cell walls, as well as increasing concentrations of divalent cations in cell walls.

In addition to the utilization of intrinsic divalent cations, the processing techniques to expose fruits and vegetables to calcium at mild temperatures are being explored to improve plant tissue firmness. Alonso and others (1997) reported that the immersion of cherries in 100 mM $CaCl_2$ solutions following thermal treatment at 50 °C for 10 min effectively retained the firmness of thawed cherries. Anthon and others (2005) examined preheating temperatures and times as well as calcium chloride

concentrations in dipping solutions to increase diced tomato firmness. 'Heinz 9665' tomato PME was activated either by increasing preheating temperature to 50 ~ 75°C, or by the addition of calcium chloride up to 2% in dipping solutions. After pasteurization at 100°C for 5 min, the texture of diced tomatoes pretreated in 0.5% calcium chloride at 70°C for 5 min prior to pasteurization was 2.5 times as firm as diced tomatoes pretreated in 0.5% calcium chloride at room temperature.

D. Strawberry PG and PME

- Ripening and softening of strawberries

Because of marked perishability associated with ripening, it is important to elucidate the mechanism of strawberry fruit softening. However, the contributions of pectin degradation involving PG and PME to strawberry tissue softening are not well documented. Koh and others (2002) demonstrated the decrease in cell wall pectin (CWP) occurring in both cortical and pith tissues of strawberry fruit as the ripening stage proceeded. The degradation of CWP in cortical tissue was attributable to the solubilization of pectic substances.

Some researchers were unable to detect apparent PG activity in strawberries (Huber 1984, Abeles and Takeda 1990). Nogata and others (1993) separated three PG isozymes, PG I, PG II, and PG III from strawberry fruit with cation exchange chromatography. PG I exhibited both endo- and exo-PG activities, while PG II and PG III were exo-PG. The predominant strawberry exo-PG activity decreased as fruit

ripened from small green to over-ripe. Nogata and others (1993) concluded that endo-PG activity was extremely low in strawberries compared to other plant sources as demonstrated in the colorimetrical detection of PG activity requiring 18 h incubation at 37°C. In addition, Redondo-Nevado and others (2001) reported that a transcript level expression of endo-PG genes was detected in strawberry fruit only at very beginning of ripening stage immediately after elongation stages, suggesting that direct action of PG on pectic substances had little effect on softening of strawberry fruit.

Instead of the action of PG, the presence of pectate lyase was suggested as an important contributor to cell wall pectin degradation of strawberries. Medina-Escobar and others (1997) reported the expression of a pectate lyase mRNA during ripening of strawberries. Jimenez-Bermudez and others (2002) observed that transgenic strawberries, possessing 30% lower expression of pectate lyase gene than non-transgenic strawberries, exhibited slow softening during ripening stages from white to red. In addition to pectate lyase, involvement of other enzymes such as β-galactosidase (Trainotti and others 2001), endo-β-(1, 4)-glucanase (Woolley and others 2001; Palomer and others 2004), and β-xylosidase (Martinez and others 2004) were studied in relation to gene expression during ripening to elucidate the degradation of cell wall materials in strawberry fruit.

Lefever and others (2004) characterized the pectic enzyme activities, pectin composition, and firmness of twelve strawberry cultivars. Strawberry PME and PG activities increased similarly with maturity and were well correlated. PME activity was

regarded as a potential indicator of fruit maturity related to fruit firmness and water-soluble pectin concentration. Castillejo and others (2004) described strawberry PME as a contributor to maintain cell wall integrity by producing galacturonic acid residues resulting in the cross-linking by way of intrinsic divalent cations, since strawberry PG activity was small.

- UHP processing of strawberries

UHP processing of strawberries is advantageous from the point of view of nutrient content because small detrimental impacts of UHP treatment on nutrients in strawberries are documented. Sancho and others (1999) reported that naturally occurring vitamin C in strawberry coulis was well preserved for over 30 d after UHP treatment at 400 MPa for 30 min. Zabetakis and others (2000) examined the stability of two predominant anthocyanins, pelargonidin-3- glucoside and pelargonidin-3-rutinoside, in UHP processed strawberries during storage. UHP processing at 200 to 800 MPa did not alter the stability of pelargonidin-3-glucoside, while pelargonidin-3-rutinoside was stabilized by UHP treatment at 200 or 800 MPa. Gimenes and others (2001) pointed out greater degradation of pelargonidin-3- glucoside and pelargonidin-3-rutinoside during thermal processing than pressure processing.

Ly-Nguyen and others (2002b) purified strawberry PME and subjected the PME separately to heat treatments at atmospheric pressures and UHP treatments at ambient temperatures. Pressure stability of strawberry PME was characterized by a fractional conversion model suggesting the presence of both pressure labile and

resistant fractions with prolonged pressure treatment. Heat and pressure stabilities of strawberry PME are presented in Table 13. Strawberry PME was extremely pressure resistant with a smaller *k* value during pressure treatments at 1000 MPa than the *k* value during thermal treatments at 54 °C determined under equivalent assay conditions.

Table 13: First-order kinetic constants *k* ($\times 10^{-6}$•min^{-1}) for thermal or pressure inactivation of strawberry PME. (Ly-Nguyen and others 2002).

Heat inactivation		Pressure inactivation	
Temperature (°C)	*k* (min^{-1})	Pressure (MPa)	*k* (min^{-1})
54	0.0376	850	0.0131
57	0.0555	925	0.0177
60	0.1271	1000	0.0260
63	0.4940		
E_a = 206.7 ± 38.4 kJ/mol		V_a = -10.77 ± 0.67 cm^3/mol	

■ Processing to increase firmness of strawberry fruit

Several studies to utilize endogenous and exogenous PME together with the addition of $CaCl_2$ were conducted to increase the firmness of maturing strawberries and strawberry products.

Garcia and others (1996) observed that strawberries immersed in 1% $CaCl_2$ solution at 45 °C for 15 min exhibited reduction in microbial decay during storage at

18 °C for 3 d. The pretreatment to dip postharvest strawberries in $CaCl_2$ solutions maintained fruit firmness and soluble solid concentrations during the storage.

When exogenous $CaCl_2$ is added to strawberries to increase tissue firmness, it is important to consider the penetration and the distribution of $CaCl_2$ in strawberry tissues. Suutarinen and others (2002a) reported an effective pretreatment of strawberry fruit in 1% $CaCl_2$ solution containing exogenous fungal PME under vacuum pressure at 15 kPa at 37°C to produce frozen strawberries with a firm texture for jam manufacturing. After the pretreatment with $CaCl_2$ and exogenous PME under vacuum pressure, calcium absorption in cell walls of the strawberry tissues was observed visually with a microscope. Strawberries pretreated with only 1% $CaCl_2$ under equivalent vacuum conditions, or pretreated with 1% $CaCl_2$ and exogenous PME at atmospheric pressure did not increase firmness of strawberries when compared to control strawberries dipped in water. Suutarinen and others (2002b) determined pretreatment conditions for strawberries dipped in 1% $CaCl_2$ solution containing 50 to 100 nkat exogenous fungal PME/g (1 nkat was defined as PME activity to release 1 nmol carboxyl group per second under assay conditions) at less than 20°C for 5 to 10 min.

While vacuum was applied (Suutarinen and others 2002a; Suutarinen and others 2002b) to promote penetration of $CaCl_2$, Duvetter and others (2005) reported comparable passive osmotic, vacuum-assisted, or pressure-assisted infusion techniques to investigate strawberry fruit firmness as affected by infusion solutions

containing 0.5% $CaCl_2 \cdot 2H_2O$ and exogenous PME. Among these methods, the vacuum-assisted infusion method applying 10 hPa for 5 min or 15 min effectively increased strawberry firmness. Strawberries immersed in the infusion solution under vacuum-infusion method followed by thermal treatments at 60 to 80 °C or pressure treatments at 400 to 550 MPa for up to 20 min exhibited smaller firmness loss than control strawberries. Above all, strawberries pretreated in the infusion solutions containing 0.5% $CaCl_2 \cdot 2H_2O$ and exogenous PME under vacuum conditions and processed under UHP retained 70% of the firmness of raw strawberries.

PART III: COMMERCIAL UHP CONDITIONS

Meyer and others (2000) estimate production costs for UHP processing in the food industry to reach $0.0455/lb food including labor, maintenance, and depreciation costs. The utilization of UHP equipment with 215 L volume produced by Avure Technologie Corp. (Kent, WA) with 75% load rate, 6 to 8 min cycle time, and 135,000,000 lb annual food production capacity assumes depreciation of the capital costs over 10 years. A new commercial UHP system marketed by NC Hyperbaric (Burgos, Spain) equipped with 300 L capacity vessel enables pressurization to 600 MPa. The company recommends cycle times of 6 to 8 min for industrial food applications. Research applications to utilize pressure, temperature, and treatment time to improve fruit product quality is realistic.

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CHAPTER 3

Rapid Assay of Polyphenol Oxidase with Fluorescence Oxygen Probe

ABSTRACT

A prototype instrument equipped with a fluorescence oxygen probe was developed for rapid assay of polyphenol oxidase (PPO) as an alternative to the conventional spectrophotometric method. Oxygen consumption rate and increasing rate of absorbance during monophenol (*p*-cresol) or diphenol (catechol) oxidation catalyzed by selected activity of commercial tyrosinase were determined with the fluorescence oxygen probe method and the spectrophotometric method, respectively. Monophenol oxidase and diphenol oxidase activities of Granny Smith apple homogenates determined with the fluorescence oxygen probe method were compared to the activities determined with the spectrophotometric method. Precise assay of monophenol oxidase activity with the fluorescence oxygen probe method is unlikely due to the small *p*-cresol oxidase activity in apples and the large standard deviation in the measured activity. Catechol oxidase activity of Granny Smith apples determined with the fluorescence oxygen probe method is more than four times larger than the activity determined with the spectrophotometric method. The greater catechol oxidase

activity in Granny Smith apple homogenates determined with fluorescence oxygen probe method is attributed to determination of oxygen consumption and assay by both soluble and insoluble PPO fractions. The fluorescence oxygen probe method to assay apple homogenates reduced assay time by 70 to 80% compared to the spectrophotometric method.

INTRODUCTION

Polyphenol oxidase (PPO), monophenol dihydroxy phenylalanine: oxygen oxidoreductase (E.C. 1.14.18.1), widely distributed in the plant kingdom, is an enzyme responsible for catalyzing the discoloration of fruits such as avocados (Lopez-Malo and others 1999), bananas (Palou and others 1999), apples (Ibarz and others 2000), and peaches (Guerrero-Beltran and others 2004) and also results in the discoloration of seafood such as shrimp (Chen and others 1997). PPO catalyzes two different oxidative reactions in combination with molecular oxygen: 1) the hydroxylation of monophenols into *o*-diphenols (monophenol oxidation) and 2) the oxidation of diphenols into *o*-quinones (diphenol oxidation). Subsequent polymerization of *o*-quinones, amino acids, and proteins results in tissue discoloration of fruits and vegetables (Whitaker 1994). Development of a rapid and sensitive method to determine PPO activity is of technological importance to the food industry.

The most common quantitative assay of extracted PPO, including monophenol and diphenol oxidase activities, determines the rate of *o*-quinone

formation by spectrophotometrically assaying an increase in absorbance at approximately 400 nm depending on the selected substrate. For example, *o*-quinones formed from (+)-catechin, 4-methylcatechol, and chlorogenic acid substrates exhibit λ max at 385, 400, and 420 nm, respectively (Rouret-Mayer and others 1990). Several other chromogenic assays coupled with the addition of amino acids such as cysteine (Gauillard and others 1993; Penalver and others 2002) to reaction mixtures containing extracted PPO and selected substrate are proposed to estimate diphenol oxidase activity of PPO by determining absorbance due to quinone-amino acid adducts. Moreover, 3-methyl-2-bezothiazoline hydrazone (MBTH) is a good nucleophile forming MBTH-quinone adducts from oxidized phenolic substrates to increase the sensitivity of the spectrophotometric determination of diphenol oxidase activity of PPO compared to the *o*-quinone determination (Rodriguez-Lopez and others 1994). The application of the method using MBTH to determine monophenol oxidase activity of PPO is demonstrated using PPO extracted from pears (Espin and others 1996). However, these spectrophotometric methods to determine absorbance require cleanup of pulps or particles with centrifugation and filtration to obtain optically clear PPO extracts. For example, centrifugation of apple homogenates and subsequent filtration of the supernatant requires at least 20 min per extract (Guerrero-Beltran and others 2004).

In fruits and vegetables, PPO is located in the chloroplasts bound to thylakoid membranes (Tolbert 1973, Martinez and Whitaker 1995). Latent PPO is activated following release into the cytosol when plant tissues undergo physical damage such as

bruising, cutting, ripening, or senescence. For the extraction of PPO from plant sources, the utilization of hydrophilic buffers for the extraction may result in low yield of PPO activity in optically clear extracts, while some PPO activity remains in insoluble fractions. Detergents such as Triton X-100 were added to suspensions or homogenates to increase solubility of PPO extracted from blackberries (Gonzalez and others 2000), tobacco and apple leaves (Broothaerts and others 2000). The addition of Triton X-100 to extracting buffer did not improve activity yield of loquat fruit PPO (Ding and others 1998). The selection of appropriate concentrations of Triton X-100 to extracting buffers is dependent on plant sources and may increase yields of PPO activity determined with the spectrophotometric method.

A rapid alternative method for quantitative determination of PPO activity is the assay for consumption of dissolved oxygen molecules during PPO-catalyzed reactions. The method using a fluorescence oxygen probe is more advantageous than the spectrophotometric method because crude plant tissue homogenates or extracts can be used as PPO sources to avoid loss of PPO activity yield and improve the sensitivity to determine PPO activity of plant tissues. Reyes De Corcuera and others (2003) developed a prototype instrument equipped with a fluorescence oxygen probe to determine lipoxygenase activity as an alternative to the spectrophotometric method.

The objectives of this research were to develop a prototype instrument equipped with a fluorescence oxygen probe for rapid quantitative determination of oxygen consumption dependent on PPO activity, and to compare the fluorescence

oxygen probe and the conventional spectrophotometric methods in relation to accuracy, precision, and reproducibility.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase (T-3824), catechol (C-9510), and *p*-cresol were purchased from Sigma Chemical Co. (St. Louis, MO). Disodium phosphate and citric acid to prepare McIlvaine buffer solutions at selected pHs were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Deionized water was used for the preparation of solutions. 'Granny Smith' apples were purchased at a local market in Pullman, WA.

Standard comparison using commercial tyrosinase

A standard comparison between the fluorescence oxygen probe method and the spectrophotometric method to determine PPO activity used purified tyrosinase at selected concentrations. The mushroom tyrosinase (nominal activity, 3,960 Sigma units/mg lyophilized powder) was dissolved in McIlvaine buffer solution at pH 6.5 to prepare a dilution of 1,000 Sigma units/mL and subjected to selected experiments. One Sigma unit of tyrosinase activity is defined by Sigma-Aldrich as the enzyme activity necessary to increase absorbance at 280 nm (A 280 nm) of 0.001 per min at pH 6.5 and 25 ˚C in a 3 mL reaction mixture containing L-tyrosine. Each determination of enzyme activity was conducted in triplicate.

Assay with fluorescence oxygen probe instrument. The prototype instrument consisted of a tubular reaction cell with a tapered top and a bottom modified to contain two syringes (Figure 1). The dissolved oxygen concentration in the reaction mixture in the tubular reaction cell was determined with the fluorescence oxygen probe (Catalog No. FOXY-R, Ocean Optics, Dunedin, FL). System calibration was carried out using oxygen-saturated water (280 µM O_2) and oxygen-free water (0 µM O_2) obtained from the continuous flow of air or nitrogen gas through deionized water for 20 min, respectively. For the assay of monophenol oxidase activity of tyrosinase, 8.5 mL of McIlvaine buffer as the enzyme blank or selected dilutions of tyrosinase not exceeding 80 Sigma units/mL in McIlvaine buffer pH at 6.5, and 8.5 mL of 1 mM *p*-cresol substrate solution in McIlvaine buffer were injected simultaneously into the reaction cell, allowing overflow of the mixture. Fluorescence of the reaction mixture at 610 nm was determined for 30 s intervals for 30 min at 23 °C. Diphenol oxidase activity of tyrosinase was assessed by using selected dilutions of tyrosinase not exceeding 4 Sigma units/mL in McIlvaine buffer pH at 6.5 and 100 mM catechol substrate solution in the McIlvaine buffer, and by determining the fluorescence of reaction mixtures at 23 °C at 16 s intervals for 5 min. The oxygen concentrations in the reaction mixtures were collected using LabVIEW (National Instruments, Austin, TX) software. Linear portions of oxygen concentration curves exhibiting correlation coefficients $r > 0.990$ over reaction time were obtained with Microsoft Excel software and converted to PPO activity expressed as rate of oxygen consumption in µM/min.

Spectrophotometric assay. Spectrophotometric assays were conducted in an 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) at 23 ˚C. Monophenol oxidase activity of tyrosinase was analyzed by assaying absorbance at 400 nm of 3.5 mL of McIlvaine buffer pH of 6.5 containing selected units of tyrosinase from 5 to 40 Sigma units/mL and 0.5 mM *p*-cresol. Tyrosinase activity in a reaction mixture containing diphenol substrate was determined by monitoring absorbance at 420 nm of 3.5 mL of McIlvaine buffer pH of 6.5 containing selected activity units of tyrosinase from 0.25 to 2 Sigma units/mL and 50 mM catechol. Absorbance was determined at 30 s intervals for 30 min or 16 s intervals for 5 min to assay tyrosinase activity with *p*-cresol substrate or catechol substrate, respectively. Using Microsoft Excel software, linear portions of absorbance curves exhibiting correlation coefficient $r > 0.990$ over reaction time were selected to calculate tyrosinase activity expressed as rate of increases of absorbance at 400 nm or 420 nm per min.

Determination of Granny Smith apple PPO activities

Tyrosinase activities using *p*-cresol and catechol substrates in Granny Smith apple homogenates using the fluorescence oxygen probe were compared to tyrosinase activities in the apple extracts determined by spectrophotometric assays. After washing, apples were peeled, cut into four wedges, cored, and cut into 1 cm cubes. One hundred grams of the cubed apple flesh was blended with an equal weight of cold deionized water at 4 °C in a commercial Waring blender for 1 min. The resulting

homogenate was used as a crude PPO enzyme preparation for the PPO assay with the fluorescence oxygen probe.

Granny Smith apple homogenates were centrifuged in a Sorvall RT 6000B centrifuge (DuPont Co., Newtown, CT) at 1710 × g for 40 min and filtered through Whatman No.1 filler paper to obtain optically clear apple extracts for the determination of PPO activity with the spectrophotometric method. Homogenization, centrifugation, and filtration were carried out at 4˚C in approximately 50 minutes.

p-Cresol oxidase and catechol oxidase activities in Granny Smith apples were determined with the fluorescence oxygen probe and the spectrophotometric methods as described, except that the apple homogenates were diluted with McIlvaine buffer at pH 5.0 and McIlvaine buffer at pH 5.0 was used to prepare substrate solutions. Apple homogenates and supernatants boiled for 20 min were used as blanks to determine the changes in oxygen concentration and absorbance of reaction mixtures with no PPO activity. After examining the linear portions of the plot of oxygen consumption or absorbance over reaction time, *p*-cresol oxidase and catechol oxidase activities in Granny Smith apples were calculated and converted in Sigma units/g fresh weight (f.w.)/mL based on the standard curves of *p*-cresol oxidase and catechol oxidase activities obtained from assays of commercial mushroom tyrosinase with the fluorescence oxygen probe and the spectrophotometric methods.

Statistical analysis

Means, standard deviations, and correlation coefficients were calculated using Microsoft Excel software. Analysis of variance (ANOVA) of the effect of experimental conditions on mushroom and apple PPO activities were performed using Minitab Release 14 software (Minitab Inc., State College, PA). Significant differences are defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

p-Cresol oxidase activity of commercial tyrosinase

Using the fluorescence oxygen probe method, the presence of selected tyrosinase activity from 5 to 40 Sigma units/mL and *p*-cresol substrate in McIlvaine buffer at pH 6.5 resulted in two zones of linear oxygen consumption over the reaction time, exhibited by correlation coefficient $r > 0.990$ (Figure 2). The initial zone ranging from the beginning of the reaction to 50 µM of oxygen consumption and a second zone exhibiting oxygen consumption from 150 to 250 µM were referred to as zone I and zone II, respectively. The time of tyrosinase-catalyzed *p*-cresol oxidation reaction accounting for zone I and II became shorter as tyrosinase activity in the reaction mixture increased. Nearly 280 µM oxygen was consumed during *p*-cresol oxidation involving tyrosinase activity from 5 to 40 Sigma units/mL, indicating the reaction proceeded until depletion of oxygen molecules in the reaction mixtures. The slopes of continuous lines in plots of oxygen consumption over the reaction time in zone I and II

(Figure 2) were separately considered to develop the standard curves (Figure 4-A). The two standard curves presenting the rate of oxygen consumption against tyrosinase activity not exceeding 40 Sigma units/mL exhibit were linearly correlated with correlation coefficients $r^2 = 0.987$ and 0.990 in zone I and zone II, respectively.

The plot of absorbance at 400 nm of McIlvaine buffer pH at 6.5 containing *p*-cresol substrate in the presence of tyrosinase activity of 5 to 40 Sigma units/mL contained a single linear zone, lasting until absorbance at 400 nm increased by approximately 0.230 (Figure 3). At selected tyrosinase activities of 5 to 40 Sigma units/mL, absorbance at 400 nm over reaction time decreased gradually after reaching a maximum value of absorbance at 400 nm. The large tyrosinase activity in reaction mixtures did not change the maximum values of absorbance at 400 nm, but reduced reaction times necessary to reach the maximum values of absorbance at 400 nm. At selected tyrosinase activities of 5 to 40 Sigma units/mL, linear increasing portions of absorbance at 400 nm accounted for correlation coefficients $r > 0.990$ over tyrosinase-catalyzed *p*-cresol oxidation reaction time. The slopes of continuous lines in plots of absorbance at 400 nm over the reaction time (Figure 3) were selected and translated to the standard curve. The increase in absorbance at 400 nm per min and tyrosinase activity within the range examined was linearly correlated in the standard curve exhibiting correlation coefficient $r^2 = 0.994$ (Figure 4-B).

Oxygen was more rapidly consumed in zone II than in zone I in the reaction mixtures containing tyrosinase activities of 5 to 40 Sigma units/mL and 0.5 mM *p*-

cresol substrate (Figure 2). There are three oxygen-consuming reactions: 1) hydroxylation of *p*-cresol to 4-methyl catechol, 2) oxidation of 4-methyl catechol to 4-methyl-*o*-benzoquinone, and 3) nonenzymatic polymerization of 4-methyl-*o*-benzoquinone to form insoluble dark-colored pigments defined as melanins (Whitaker 1994). However, these three reactions may not progress in sequence. Once 4-methyl catechol is formed by hydroxylation of *p*-cresol, co-oxidation of *p*-cresol and 4-methyl catechol can occur resulting in the formation of 4-methyl catechol and 4-methyl benzoquinone, respectively (Whitaker 1994). During the co-oxidation reactions, 4-methyl catechol plays a role as a proton donor to promote hydroxylation of *p*-cresol. The increase in oxygen consumption rate in zone II is attributed to co-oxidation involving *p*-cresol and 4-methyl catechol and promoted polymerization of 4-methyl-*o*-benzoquinone to form melanins.

On the other hand, the linear increases of absorbance at 400 nm were observed upon initiation of *p*-cresol oxidation reaction catalyzed by tyrosinase until oxygen was depleted in the reaction mixtures (Figure 3). Absorbance at 400 nm, dependent on the concentration of 4-methyl benzoquinone detected spectrophotometrically, is attributed to the following three reactions: 4) oxidation of 4-methyl catechol to 4-methyl-*o*-benzoquinone, 5) co-oxidation of *p*-cresol and 4-methyl catechol to 4-methyl catechol and 4-methyl-*o*-benzoquinone, and 6) nonenzymatic polymerization of 4-methyl-*o*-benzoquinone to form insoluble dark-colored pigments defined as melanins. The first two reactions (4) and 5)) contribute to the increasing

rate of absorbance at 400 nm, while the third reaction (6)) results in decreases in the absorbance due to insolubility of melanins.

Predominant reactions initiated by the presence of tyrosinase and *p*-cresol substrate were proposed to account for the differences in increasing patterns between oxygen consumption and absorbance at 400 nm (Figure 5). Once *p*-cresol oxidation reaction is initiated, 4-methyl catechol oxidation catalyzed by tyrosinase occurs in sequence in zone I, resulting in constant increases in both oxygen consumption and absorbance at 400 nm. Oxygen consumption is accelerated by promoted *p*-cresol oxidation involving 4-methyl catechol as a proton donor and polymerization of 4-methyl-o-benzoquinone to melanins in zone II. The constant increase of absorbance at 400 nm in appearance until oxygen depletion in reaction mixtures is attributed to the balance between formation and degradation of 4-methyl-*o*-benzoquinone in zone II.

Catechol oxidase activity of commercial tyrosinase

The fluorescence oxygen probe assay for catechol oxidation catalyzed by tyrosinase activity of 0.25, 0.50 or 0.75 Sigma units/mL resulted in linear oxygen consumption curves ($r > 0.990$) for 5 min (Figure 6). The linear increases ($r > 0.990$) in absorbance at 420 nm attributed to catechol oxidation catalyzed by equivalent activity of tyrosinase were observed for less than 2 min (Figure 7). Greater tyrosinase activity in the reaction mixtures resulted in shorter duration of reaction times exhibiting linear increases in both oxygen consumption and absorbance at 420 nm.

After the linear increase in oxygen consumption, oxygen is consumed continuously in the reaction mixtures containing tyrosinase and catechol. For example, tyrosinase activity of 2.0 Sigma units/mL with catechol substrate resulted in a constant oxygen consumption rate for the first 1.8 min of the reaction, consuming approximately 150 μM oxygen. During the following 3.2 min of the reaction, the oxygen consumption rate gradually decreased and approximately 50 μM oxygen was consumed. An inhibition of tyrosinase activity may occur during the last 3.2 min of the reaction before the depletion of available oxygen or substrate.

On the other hand, the equivalent reaction mixture consisting tyrosinase activity of 2.0 Sigma units/mL and catechol substrate exhibits an increase in absorbance at 420 nm at a constant rate for a short time of 48 s, followed by a gradual decrease observed in the spectrophotometric assay. The apparent decrease in absorbance at 420 nm and the concentration of *o*-benzoquinone indicates that a larger quantity of *o*-benzoquinone is involved in polymerization to form water insoluble dark pigments than the quantity of *o*-benzoquinone formed by catechol oxidation.

According to Whitaker (1994), an intermediate identified as semi-*o*-benzo-quinone can inhibit PPO activity in oxidation of catechol. Since oxygen is involved in the polymerization to form dark pigments (Figure 5), the fluorescence oxygen probe determines oxygen consumed as far as catechol oxidation proceeds by tyrosinase depending on the activity. Therefore, the decrease in rate of oxygen consumption reflects the inhibition of tyrosinase activity by semi-*o*-benzoquinone. Determination of

absorbance at 420 nm is influenced by both polymerization of o-benzoquinone and inhibition of tyrosinase by semi-*o*-benzoquinone. As a result, oxygen consumption curves are linear for longer reaction times than the curves of absorbance at 420 nm at equivalent tyrosinase activity.

The standard curves of catechol oxidase activity plotting the constant increasing rates of oxygen consumption and absorbance at 420 nm against the selected range of tyrosinase in Sigma activity units are well correlated for both the fluorescence oxygen probe method ($r^2 = 0.996$) (Figure 8-A) and the spectrophotometric method ($r^2 = 0.989$) (Figure 8-B). Consequently, there are no differences in sensitivity between the fluorescence oxygen probe method and the spectrophotometric method to determine catechol oxidase activity of tyrosinase within 40 Sigma units/mL.

Granny Smith apple PPO activities

Apple homogenates (0.073 g/mL) in a reaction mixture containing 0.5 mM *p*-cresol resulted in linear oxygen consumption over a reaction time of 30 min (Figure 9-A). Zone II, the second linear oxygen consumption zone detected in tyrosinase assays of 5 Sigma units/mL or more using 0.5 mM *p*-cresol substrate, was not observed in *p*-cresol oxidation catalyzed by apple PPO. Zone II was attributed to *p*-cresol oxidation by tyrosinase consuming oxygen ranging from 150 to 250 µM (Figure 2). Less than 100 µM of oxygen was consumed in apple PPO-catalyzed reaction of 30 min, indicating that the reaction did not yield sufficient 4-methyl catechol to act as a proton

donor in *p*-cresol oxidation. Absorbance at 400 nm of the reaction mixture containing clarified apple extract (0.073 g/mL) and 0.5 mM *p*-cresol decreased for the first 1.5 min after the reaction was initiated, followed by a linear increase (Figure 9-B). The lag period was also observed in an assay with the spectrophotometric method to determine monophenol oxidase activity of peach extracts (Espin and others 1998).

In the reaction mixture containing apple homogenates (0.0255 g/mL) and 50 mM catechol, oxygen was consumed at a constant rate for 1.9 min resulting in a correlation coefficient $r^2 = 0.998$ between oxygen consumption and reaction time (Figure 10-A). Appropriate dilution of the apple homogenate as PPO source was required to obtain the slope of oxygen consumption over reaction time corresponding to tyrosinase activity between 0 and 40 Sigma unit/mL for the assay. The increase in absorbance at 420 nm of the reaction mixture containing clarified apple extract (0.073 g/mL) and 50 mM catechol was linear for 1.9 min of reaction time exhibiting the correlation coefficient $r^2 = 0.994$ (Figure 10-B).

The slopes exhibiting constant increasing rates of oxygen consumption determined with the fluorescence oxygen probe method (Figures 9-A and 10-A) and absorbance determined with the spectrophotometric method (Figures 9-B and 10-B) were selected to translate *p*-cresol oxidase and catechol oxidase activities of Granny Smith apples to corresponding Sigma units of tyrosinase (Figure 11). The assay of *p*-cresol oxidase activity of Granny Smith apples with the fluorescence oxygen probe method resulted in smaller activity expressed as Sigma units and larger standard

deviations compared to the mean and standard deviations determined with the spectrophotometric method. The fluorescence oxygen probe method was less precise and reproducible determination of *p*-cresol oxidase activity than the spectrophotometric method. Catechol oxidase activity of Granny Smith apples was 81.9 and 19.0 Sigma units/g fw/mL determined with the fluorescence oxygen probe method and the spectrophotometric method, respectively. The catechol oxidase activity determined with the fluorescence oxygen probe method was 4.3 times greater and is attributed to the determination of oxygen consumption in reaction mixtures and the assay of apple homogenates.

The oxygen consumption rate over reaction time reflects catechol oxidase activity more accurately than the increasing rate of absorbance at 420 nm. The assay of catechol oxidase activity in the clarified apple extract with the fluorescence oxygen probe method resulted in 41.5 Sigma units/g fw/mL. The catechol oxidase activity obtained with the fluorescence oxygen probe method was 2.2 times greater compared to the activity obtained using clarified apple extract with the spectrophotometric method.

Another reason for the differences in catechol oxidase activity determined with two methods is that the fluorescence oxygen probe method uses apple homogenate as a PPO source. In fruits and vegetables, PPO is located in the chloroplasts bound to thylakoid membranes. Therefore, detergents are often added to extracting buffers to yield high activities of PPO in soluble fractions. The fluorescence

oxygen probe method used with apple homogenates includes both soluble and insoluble fractions enabling the determination of catechol oxidase activity in the entire tissue compared to the spectrophotometric method which assays catechol oxidase activity only in the soluble fractions. The differences of catechol oxidase activity in apple homogenate and clarified apple extract, both determined with the fluorescence oxygen probe method, may correspond to the PPO activity in the insoluble fraction of apples. The selection and assay of apple homogenates increases resulted in greater catechol oxidase activity determination by 2.0 times compared to the selection and assay of clarified apple extracts determined by the spectrophotometric method.

The ratio of catechol oxidase activity to *p*-cresol oxidase activity was 49.3 for the fluorescence oxygen probe method and 4.2 for the spectrophotometric method. Perez-Gilabert and Carmona (2000) reported the catechol oxidase/*p*-cresol oxidase ratio of 41.1 for eggplant PPO using the spectrophotometric method. Determination of monophenol oxidase activity of apple PPO with the fluorescence oxygen probe method is unlikely due to the small activity in apples and the small reproducibility of the method. However, the fluorescence oxygen probe method is desirable for the determination of diphenol oxidase activity in fruit and vegetable tissue homogenates with the fluorescence oxygen probe.

The preparation of an apple homogenate for the fluorescence oxygen probe method was carried out in 3 min, whereas the centrifugation and filtration procedures following homogenization to clarify the extract required 20 min per extract for the

spectrophotometric method. Although the assays with the fluorescence oxygen probe method to determine diphenol oxidase activity using catechol substrate were carried out for 3 to 5 min, the assay time can be reduced to 2 min to obtain the oxygen consumption rate and calculate PPO activity. In this study, the time necessary for determination of catechol oxidase activity with the fluorescence oxygen probe method was reduced by 70 to 80% compared to the conventional spectrophotometric method. In addition, the preparation of homogenates from plant tissues without centrifugation and filtration enables rapid determination of PPO activities in many fruits and vegetables.

CONCLUSIONS

The fluorescence oxygen probe method will contribute to the rapid determination of diphenol oxidase activity of polyphenol oxidase in fruits and vegetables. Assay of apple homogenates with the fluorescence oxygen probe method resulted in catechol oxidase activity more than four times greater than the activity determined with the conventional spectrophotometric method, attributed to the determination of oxygen consumption in reaction mixtures and the selection of homogenates as PPO sources. Assay time for the fluorescence oxygen probe method is reduced by 70 to 80% due to the assay of crude plant tissue homogenates when compared to the conventional spectrophotometric method that requires

homogenization, centrifugation, and filtration to prepare an optically clear extract of plant tissues.

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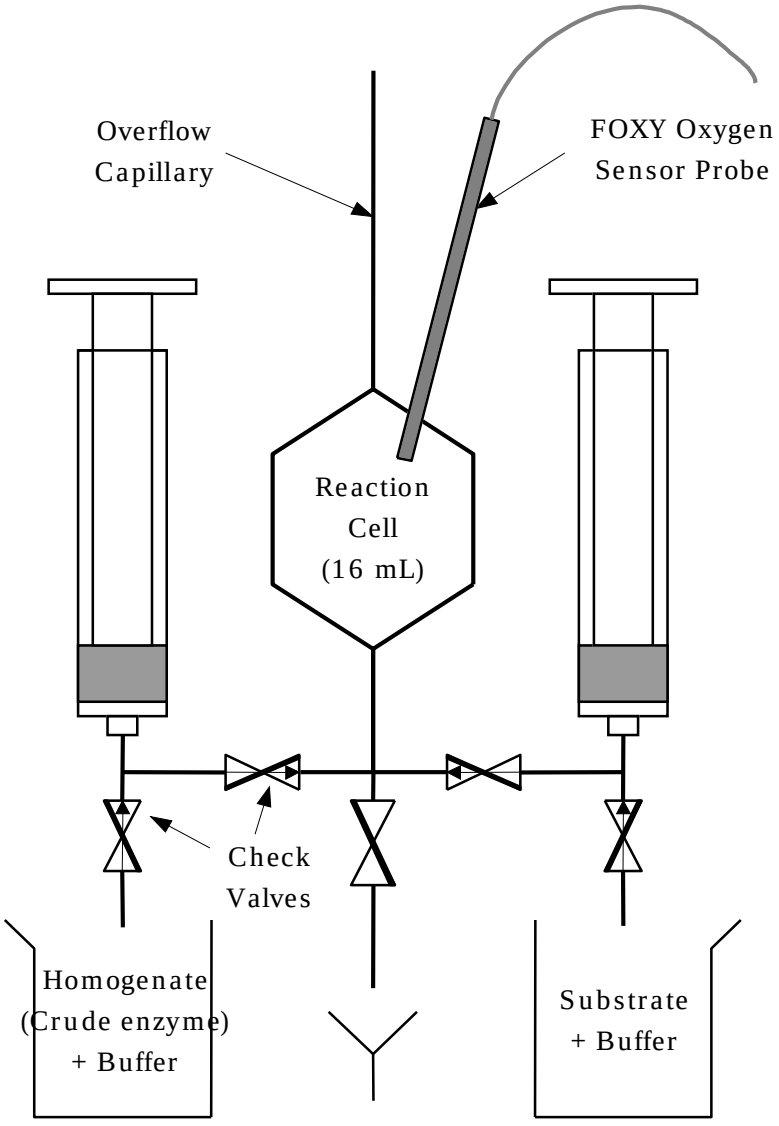


Figure 1: Schematic diagram of the fluorescence oxygen probe instrument.

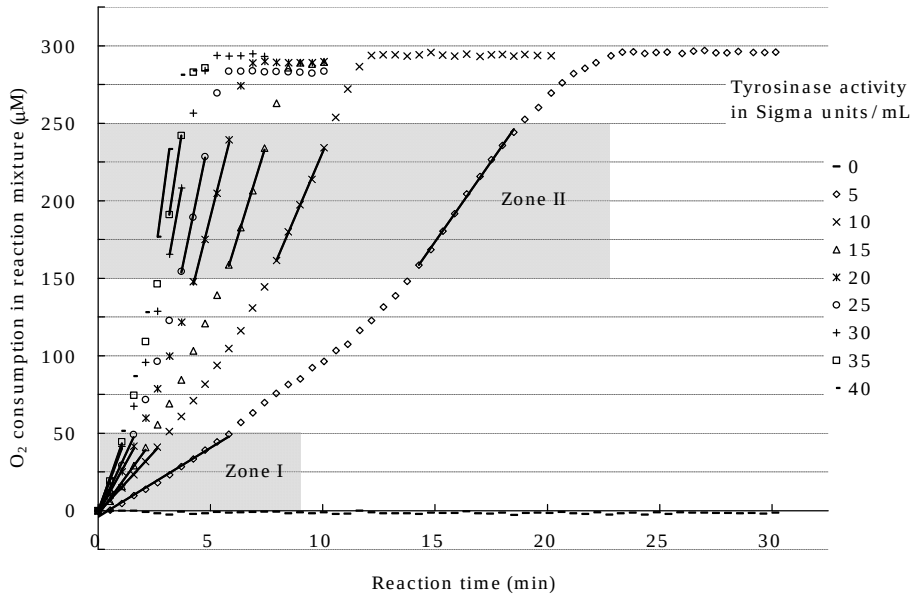


Figure 2: Oxygen consumption during *p*-cresol oxidation catalyzed by selected Sigma activity units of tyrosinase.

Reaction mixtures contained selected activity of tyrosinase, 0.5 mM *p*-cresol, and McIlvaine buffer at pH 6.5.

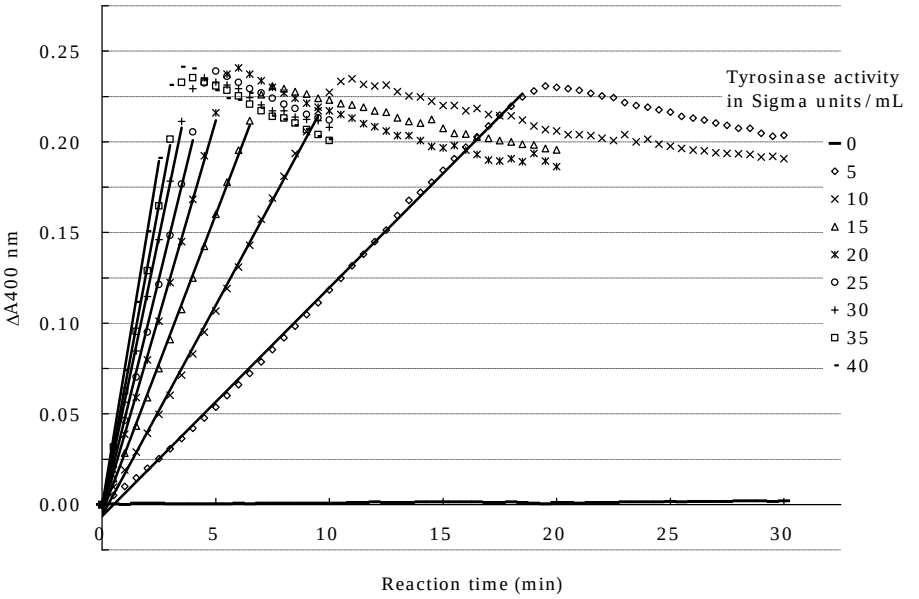


Figure 3: Absorbance at 400 nm during *p*-cresol oxidation catalyzed by selected Sigma activity units of tyrosinase.

Reaction mixtures consisted of selected activity of tyrosinase, 0.5 mM *p*-cresol, and McIlvaine buffer at pH 6.5.

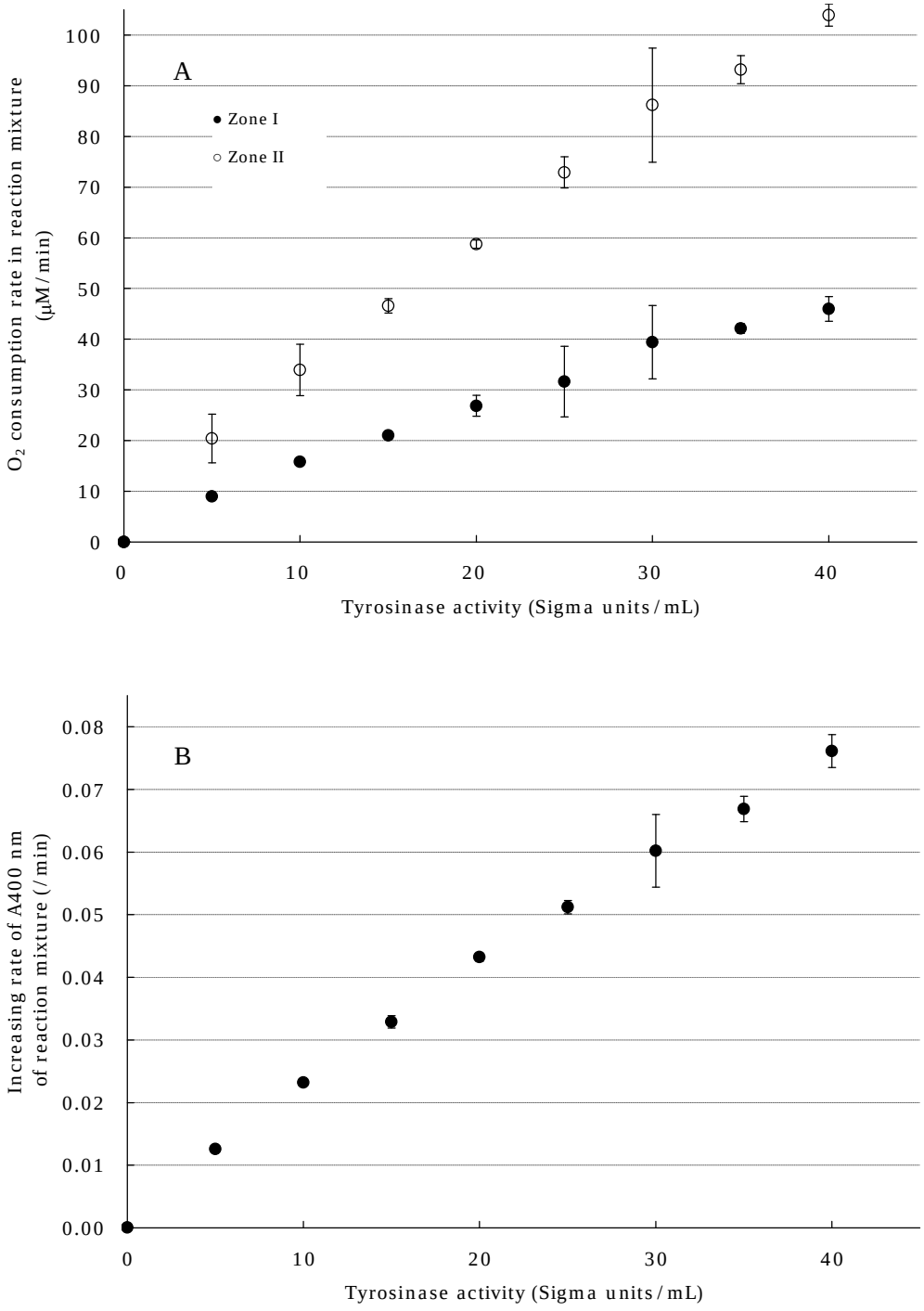
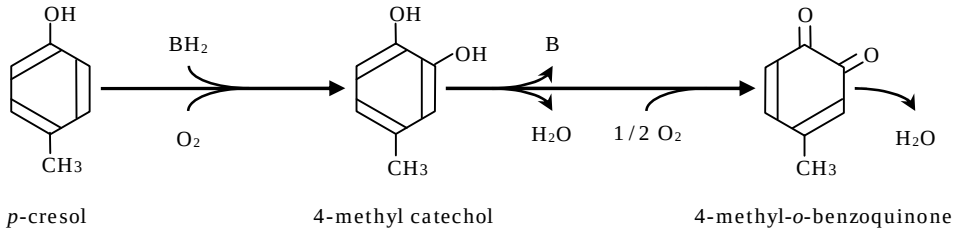


Figure 4: Oxygen consumption per min in zone I and II (A) and increase in absorbance at 400 nm per min (B) resulted from *p*-cresol oxidation by selected Sigma activity units of tyrosinase.

Zone I



Zone II

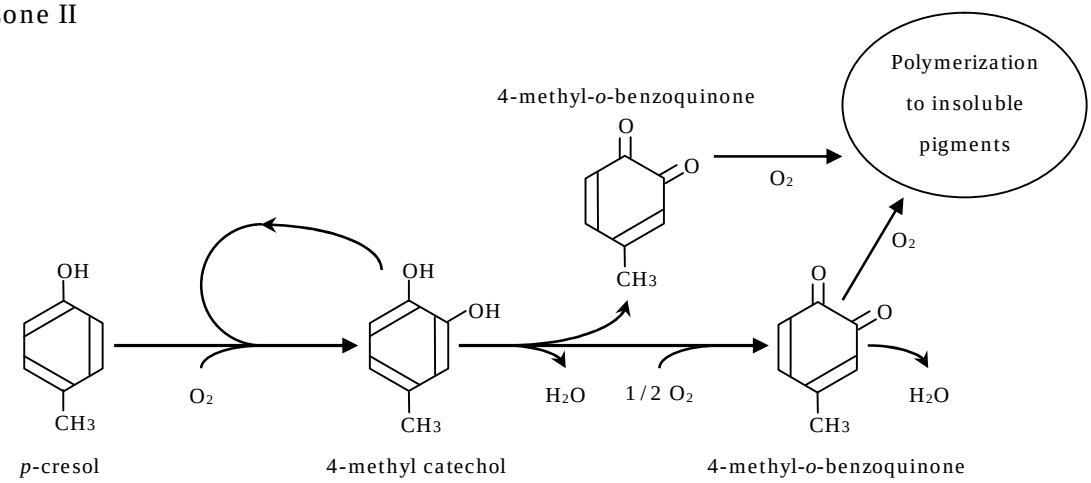


Figure 5: Proposed reactions predominant in zone I and zone II.

BH_2 presents any compound that acts as a proton donor.

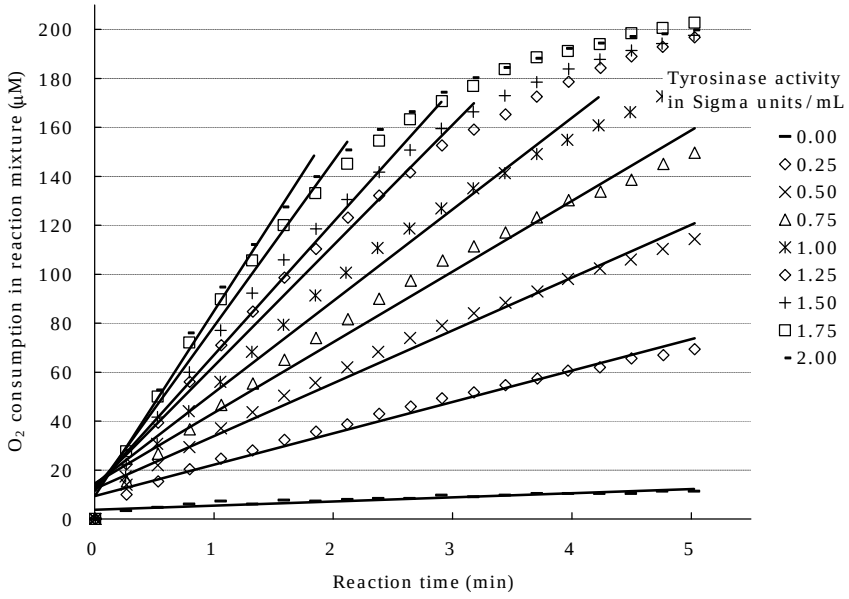


Figure 6: Oxygen consumption during catechol oxidation catalyzed by selected Sigma activity units of tyrosinase.

Reaction mixtures contained selected activity of tyrosinase, 50 mM catechol, and McIlvaine buffer at pH 6.5.

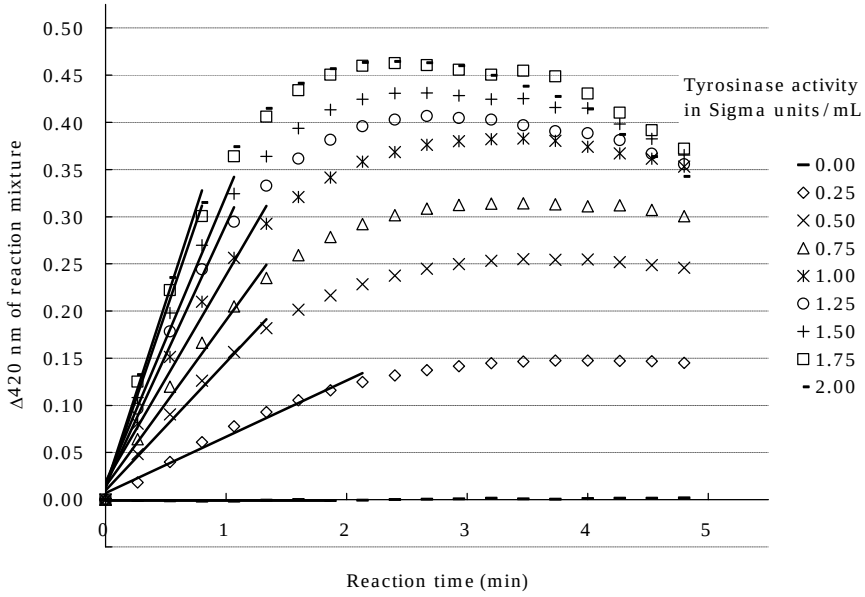


Figure 7: Absorbance at 420 nm during catechol oxidation catalyzed by selected Sigma activity units of tyrosinase.

Reaction mixtures consisted of selected activity of tyrosinase, 50 mM catechol, and McIlvaine buffer at pH 6.5.

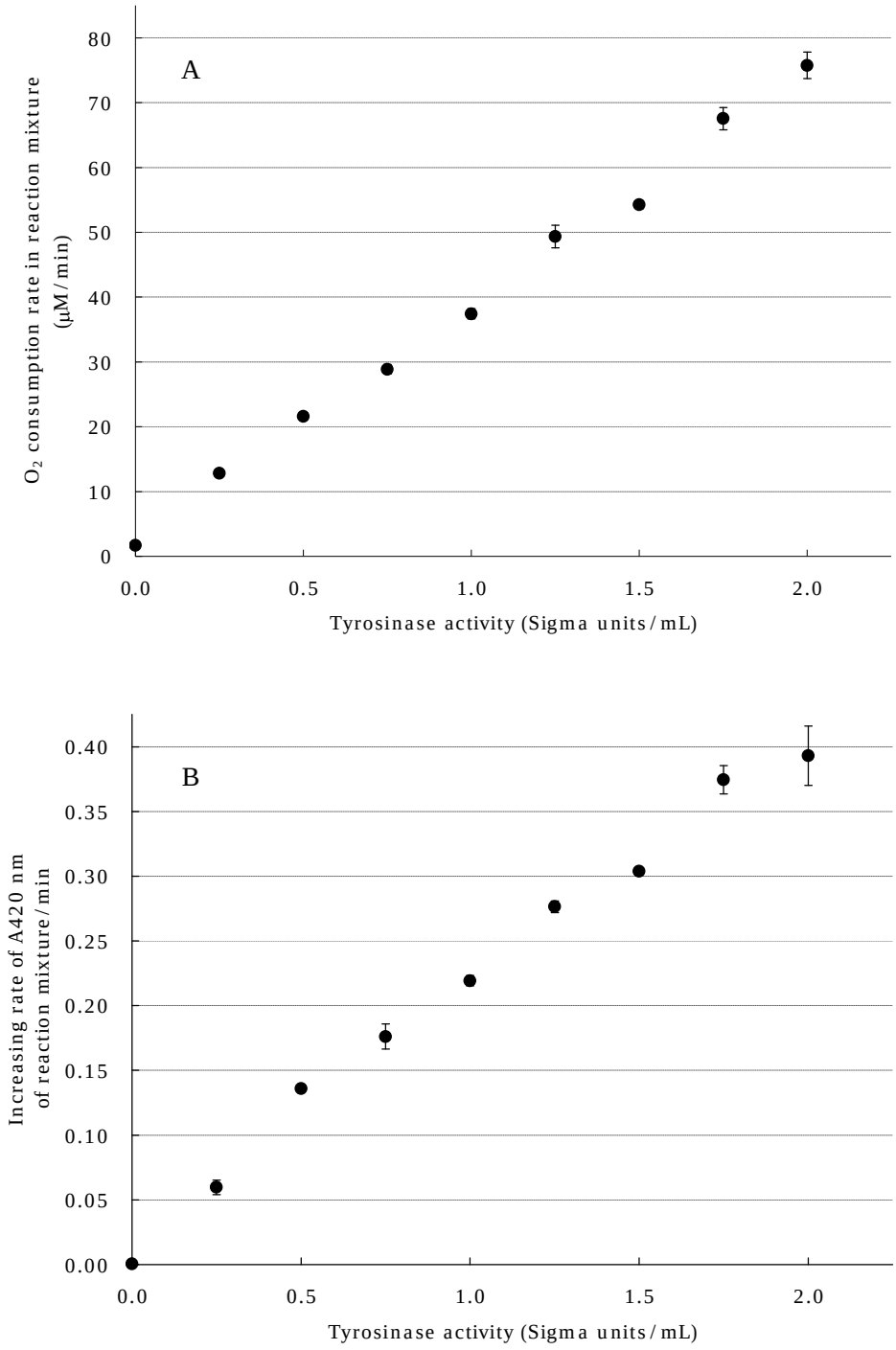


Figure 8: Oxygen consumption per min (A) and increase in absorbance at 420 nm per min resulted from catechol oxidation by selected Sigma activity units of tyrosinase.

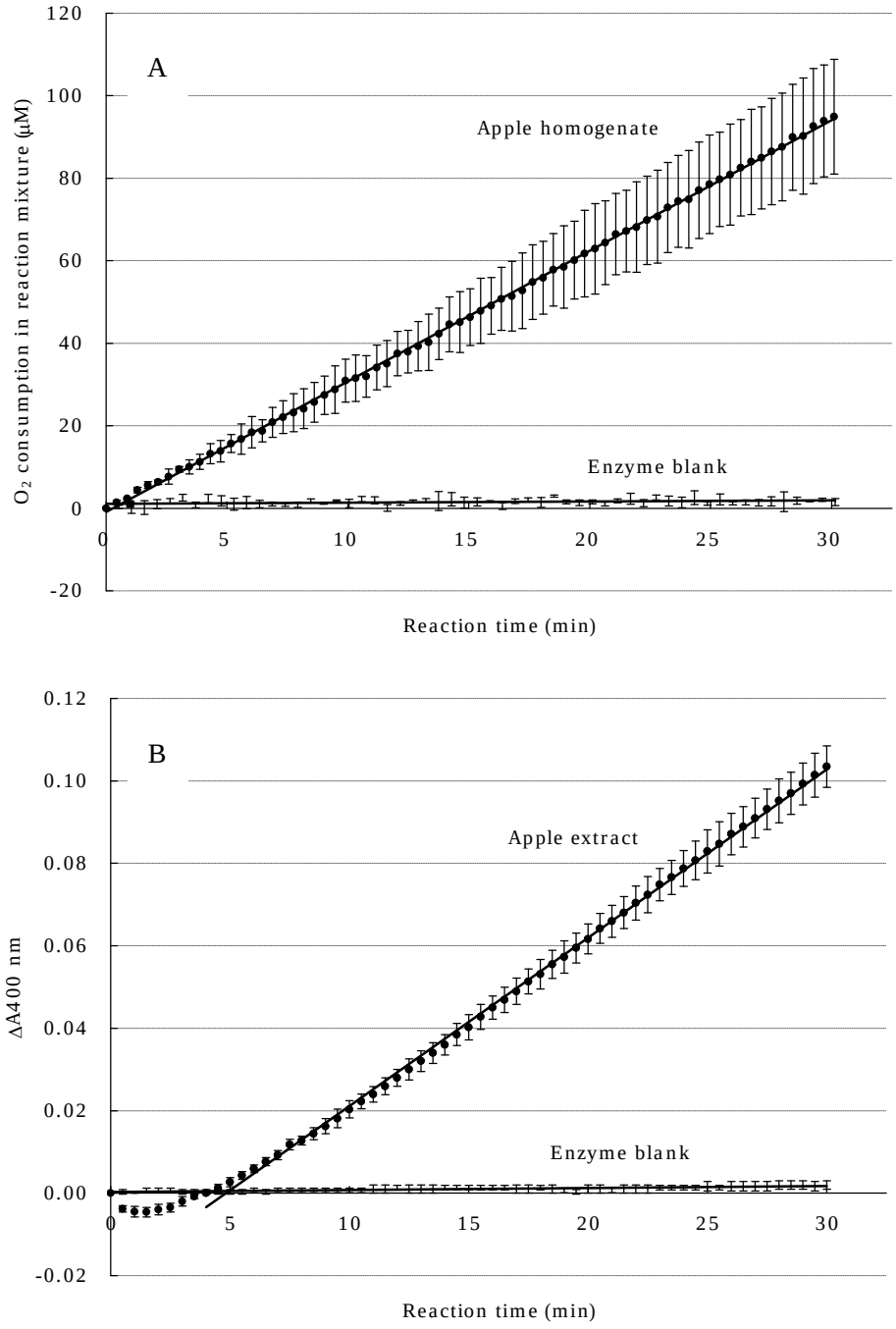


Figure 9: *p*-Cresol oxidase assays of Granny Smith apples with the fluorescence oxygen probe (A) and the spectrophotometric (B) methods.

Both reaction mixtures contained 0.073 g of apples/mL, 0.5 mM *p*-cresol, and McIlvaine buffer at pH 5.0. McIlvaine buffer was substituted for apple homogenate or extract for control assay. Vertical lines present ±1 × standard deviation in triplicates.

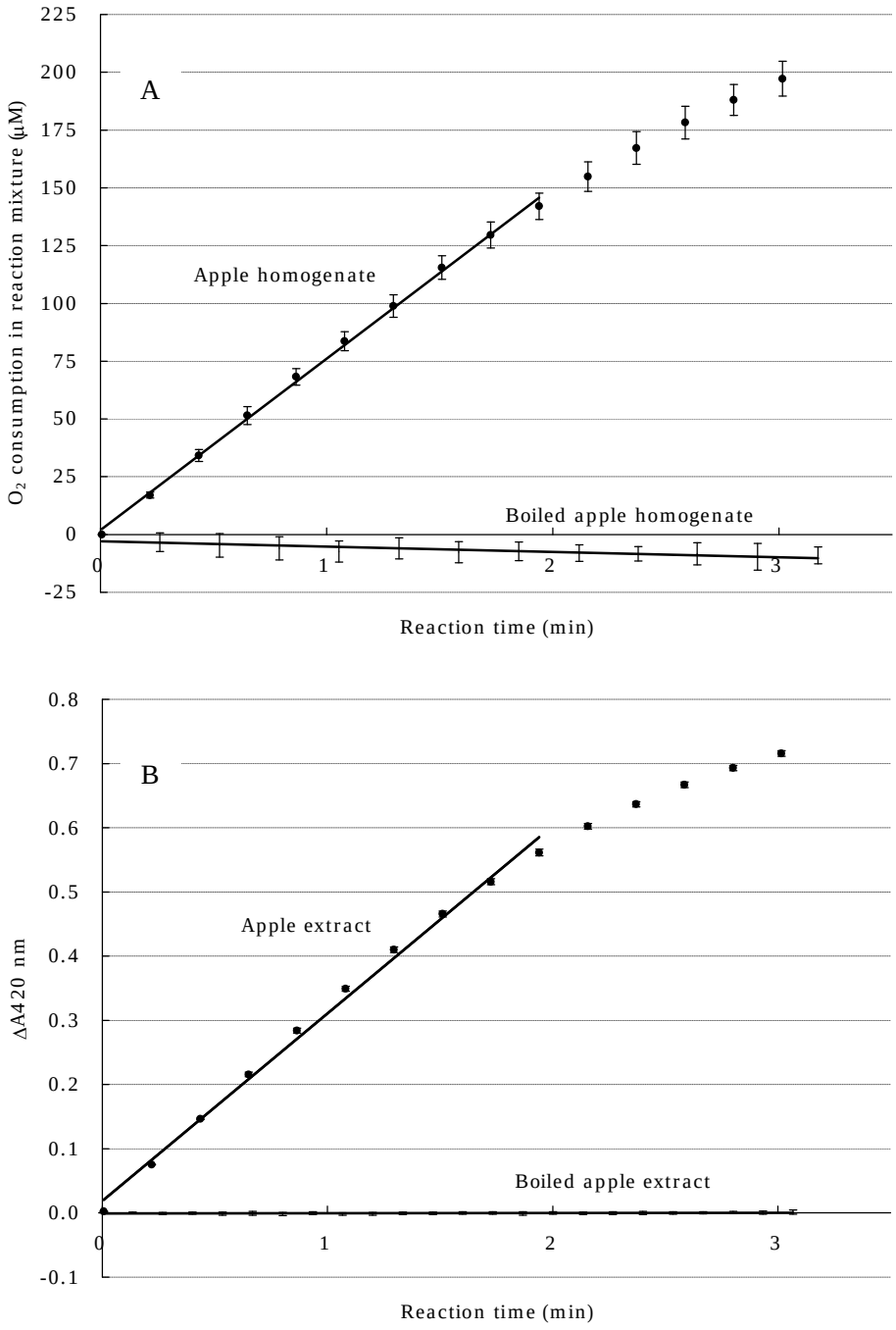


Figure 10: Catechol oxidase assays of Granny Smith apples with the fluorescence oxygen probe (A) and the spectrophotometric (B) methods.

Reaction mixtures contained 0.0255 g (A) or 0.0730 g (B) of apples / mL, 50 mM catechol, and McIlvaine buffer at pH 5.0. Vertical lines present ±1 × standard deviation in triplicates.

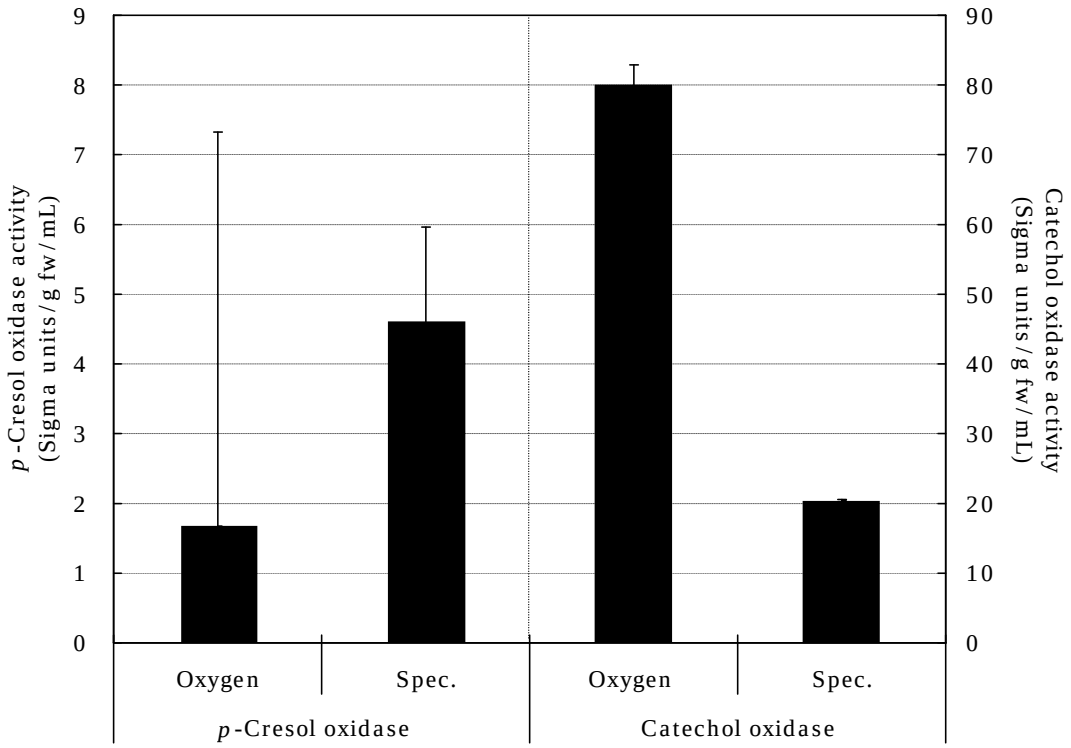


Figure 11: *p*-Cresol oxidase and catechol oxidase activities of Granny Smith apples determined with the fluorescence oxygen probe (Oxygen, n = 3) and the spectrophotometric (Spec., n = 3) methods.

Activities were calculated from the slopes of oxygen consumption and absorbance to equivalent Sigma tyrosinase activity units based on the standard curves.

CHAPTER 4

Ultra High Pressure Inactivation of Apple Polyphenol Oxidase by Papain and Combined Methods

ABSTRACT

A combined method of protease and ultra high pressure (UHP) treatment was examined to inactivate polyphenol oxidase (PPO) of Granny Smith apples and produce apple homogenates exhibiting acceptable color retention during storage. Among the cysteine proteases, bromelain, ficin, and papain, only papain significantly ($p \leq 0.05$) inhibited PPO activity in the presence of EDTA and cysteine in apple homogenates incubated at 4 °C for 30 min. PPO activity was effectively inhibited by adding 2.5 mg protein as papain powder/mL to apple homogenates containing 1 mM EDTA and 25 mM cysteine during incubation at 25 °C for 30 min. Eight apple homogenates containing selected concentrations of EDTA, cysteine, and papain were treated at 600 MPa and 25 °C, following incubation at 25 °C for 30 min. In apple homogenates containing cysteine and papain, more than 85% inhibition of PPO activity was achieved after UHP treatment at 600 MPa and 25 °C for 10 min. PPO activity was less effectively inhibited in apple homogenates containing only papain, or homogenates containing papain and EDTA during incubation and UHP treatment. Exogenous

papain in apple homogenates was stable during incubation at 25 °C for 30 min, followed by UHP treatment at 600 MPa and 25 °C for 10 min. After UHP treatment, apple homogenates containing cysteine exhibited light green to yellow color during storage at 4 or 25 °C for 28 d regardless of UHP dwelling time. Rapid discoloration of apple homogenates to dark brown without cysteine was observed immediately after homogenate preparation. The presence of cysteine was necessary to extend shelf life color of apple homogenates.

INTRODUCTION

Polyphenol oxidase (PPO), monophenol dihydroxy phenylalanine: oxygen oxidoreductase (E.C. 1.14.18.1), widely distributed in the plant kingdom, is an enzyme responsible for catalyzing the discoloration of fruits and vegetables (Mayer 1979). PPO catalyzes two specific oxidative reactions; monophenols to diphenols and diphenols to *o*-quinones. Subsequent polymerization of *o*-quinones with proteins and amino acids results in the development of dark colored pigments (Whitaker 1994). Plant PPO is located in the chloroplasts bound to thylakoid membranes (Tolbert 1973, Martinez and Whitaker 1995). Latent PPO becomes active after being released into the cytosol when plant tissues undergo physical damage such as insect or microbial invasion, bruising, or cutting during biological ripening and senescence.

Apple (*Pyrus malus*) PPO has been intensively studied because of its importance to the processing industry. PPO activity in apples varies with cultivar

(Janovitz-Klapp and others 1989), tissue (Janovitz-Klapp and others 1989), storage conditions (Lanciotti and others 1999; Soliva-Fortuny and others 2001; Rocha and Morais 2001), and fruit maturity (Murata and others 1995). The selection of antibrowning agents is one of the common practices in the food industry to control apple PPO activity and prevent discoloration of apple products. Antibrowning agents are classified into two groups; enzyme inhibitors and reducing agents.

Ethylenediaminetetraacetic acid (EDTA) chelates most metal ions possessing more than one ionic charge resulting in the formation of ethylenediaminetetra aceto complexes. EDTA as a PPO inhibitor chelates divalent copper ions located at the active site of PPO molecules. The presence of EDTA did not significantly reduce PPO activities extracted from mushrooms (Weemaes and others 1999) bananas (Yang and others 2000; Yang and others 2001), or artichokes (Aydemir 2004). However, inhibitory effects of EDTA were demonstrated with PPO from pineapple (Das and others 1997), avocados (Weemaes and others 1999; Soliva-Fortuny and others 2002), taro, potatoes (Duangmal and Owusu-Apenten 1999), or longan fruit (Jiang 1999).

Cysteine is an effective reducing agent to prevent browning of apple products (Molnar-Perl and Fiedman 1990a, Molnar-Perl and Fiedman 1990b, Ozoglu and Bayindirli 2002, Iyidogan and Bayindirli 2004). The mode of action of cysteine to inhibit brown color development is different from other reducing agents such as sulfites or L-ascorbic acid that reduce *o*-quinones to diphenols. Cysteine reacts with *o*-quinones derived from apple PPO-catalyzed oxidation of diphenols to form colorless

compounds called cysteine-quinone adducts (Richard and others 1991). In addition, the colorless cysteine-quinone adducts play a role as substrate analogs to inhibit PPO activity (Richard-Forget and others 1992).

Thermal processing is a conventional technique to inactivate pathogenic and spoilage microorganisms, as well as detrimental enzymes to extend shelf life to food products. With the increase in consumers' demand for high quality: good appearance, good flavor, and high nutritional value in addition to microbial safety, the introduction of nonthermal processing technologies are of great interest to the food industry (Hoover 1997, Butz 2002). Ultra high pressure (UHP) treatment is considered one of the potential nonthermal technologies as an alternative to thermal processing to extend shelf life and retain or modify sensory attributes (Pothakamury and others 1995; Tewari and others 1999; San Martin and others 2002). A number of studies were conducted to establish methods to inhibit PPO from selected sources by means of UHP. Apple PPO in cell free extracts were activated and inhibited by 1 min UHP treatments at 200 and 900 MPa, respectively (Anese and others 1995). Residual activity of purified apple (cv. Golden Delicious) PPO was determined after UHP treatment at 800 MPa for 30 min (Weemaes and others 1998). UHP inhibition of PPO in diced apples (cv. Golden Delicious and Granny Smith) was promoted by immersion in 4-hexylresorcinol for 15 min, whereas immersion in ascorbic acid or citric acid solutions for 15 min did not result in PPO sensitization to UHP treatment (Ibarz and others 2000).

Another approach to inhibition of PPO is the application of proteases. Papain (EC 3.4.22.2), ficin (EC 3.4.22.3), and bromelain (EC 3.4.22.4) are plant cysteine proteases commercially applied in the food industry to tenderize meat, clarify beers, and change the texture of baked goods (Cowan 1983). PPO molecules are potentially hydrolyzed by the action of cysteine proteases resulting in the loss of activity. Small numbers of studies were carried out to investigate the effects of plant cysteine proteases on PPO activity or discoloration of food products. The inhibitory effect of frozen pineapple juice on browning of fresh apple slices was smaller than canned pineapple juice, suggesting the contribution of components other than bromelain (Lozano-de-Gonzalez and others 1993). Ficin inhibited commercial mushroom tyrosinase activity and browning of shrimp and potatoes, whereas the addition of papain effectively inhibited the browning of apple slices (Labuza and others 1990).

The action of cysteine proteases is inhibited by oxidation of sulfhydryl groups located in active sites of the enzyme molecules (Wong 1995). The rate of the oxidative reaction and inhibition of protease activity increases in the presence of metal ions. Therefore, chelating agents and antioxidants are routinely added to extracts prior to assaying the activity of cysteine proteases. For example, a reaction mixture for the determination of papain activity includes 1 mM EDTA and 10 mM cysteine (Gomes and others 1997). In a reaction mixture including PPO, cysteine protease, cysteine, and EDTA, cysteine and EDTA are inhibitors of PPO activity and stabilizers for the cysteine protease; simultaneously PPO is potentially hydrolyzed by cysteine protease.

Studies showing the three-dimensional relationships involving PPO, plant proteases, and antibrowning agents are not well reported.

The objectives of this study are to examine effects of three plant proteases, papain, ficin, and bromelain on inhibiting of apple PPO in the presence of cysteine and EDTA, and establish a method to achieve extended shelf life of apple products by UHP treatment in combination with the application of selected proteases.

MATERIALS AND METHODS

Materials

Apples (*Malus* × *domestica*) cv. Granny Smith were purchased at a local market in Pullman, WA. Three plant origin proteases, bromelain (Catalog No. B-4882, 35% protein), ficin (F-6008, 90% protein), and papain (P-4762, 91% protein) were purchased from Sigma Chemical Co. (St. Louis, MO). Catechol (C-9510), L-cysteine (C-7352), and Triton® X-100 (T-9284) were purchased from Sigma Chemical Co. as well. EDTA disodium salt (8993-1), disodium phosphate dibasic (Na_2HPO_4, 3928-05), potassium phosphate monobasic (KH_2PO4, 3246) and citric acid monohydrate (0110-01) were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Deionized water was used for the preparation of solutions.

Preparation of Granny Smith apple homogenates

After washing, the apples were peeled, cut into four wedges, cored, and cut into 1 cm cubes. Diced apple flesh was blended with an equal weight of water or

selected solutions in a commercial Waring blender for 1 min. Selected proteases were added as powders and dispersed in the apple homogenates immediately after blending.

PPO assay

PPO activity in the apple homogenates was assayed with a fluorescence oxygen probe (Ocean Optics Catalog # FOXY-R, Dunedin, FL) to determine oxygen consumption in the reaction cell containing selected reaction mixtures. The fluorescence oxygen probe was calibrated using oxygen-saturated water (280 μM O_2) and oxygen-free water (0 μM O_2) obtained from the continuous flow of atmospheric air or nitrogen gas through deionized water for 20 min, respectively. An apple homogenate dilution was prepared by mixing 1 mL of apple homogenate with 11 mL of McIlvaine buffer pH 5.0 in a 15 mL plastic test tube. Using two syringes, 8.5 mL of the apple homogenate dilution and 8.5 mL of 100 mM catechol solution in McIlvaine buffer pH at 5.0 were injected simultaneously into the reaction cell with 16 mL volume, allowing overflow of the mixture. Fluorescence of the reaction mixture at 610 nm was determined at 23 °C at 16 s intervals for 3 min. Fluorescence data obtained from assays were collected by LabVIEW (National Instruments, Austin, TX) software and converted into PPO activity in micromole oxygen per minute (μM/min) based on the linear oxygen consumption rate computed with Microsoft Excel software. PPO activity was expressed as percent residual activity using PPO activity in homogenates assayed immediately after blending or addition of papain as 100%. The assay was conducted in triplicate for each apple homogenate.

Papain assay

Papain activity was assayed spectrophotometrically using the casein digestion method of Kunitz (1947) with the addition of cysteine and EDTA to reaction mixtures. A stock solution of 1% casein was prepared by dispersing casein powder in 0.06 M Sorensen's phosphate buffer at pH 6.2 including 20 mM cysteine and 2 mM EDTA. The stock solution in a glass bottle was heated in boiling water for 15 min to dissolve casein. A test tube containing 1 mL of papain solution and 1 mL of 1% casein stock solution was incubated for precisely 20 min in a 25 °C water bath. Three milliliters of 5% TCA was added to the test tube to stop the reaction. One milliliter of 1% casein stock solution and 1 mL of the papain solution were subsequently mixed with 3 mL of TCA solution in a test tube to obtain the corresponding blank. The resulting supernatant after centrifugation 1,755 × g for 15 min at room temperature was used to determine absorbance at 280 nm. Papain activity was expressed as percent residual activity using papain activity obtained by assaying equivalent concentrations of papain dispersed in 0.06 M Sorensen's phosphate buffer at pH 6.2 as 100%. The assay was carried out in triplicate for each apple homogenate containing papain.

Experimental protocols to determine incubation conditions

In a series of experiments, potencies of the three plant cysteine proteases bromelain, ficin, and papain, L-cysteine, and EDTA to inhibit apple PPO were evaluated. Effects of incubation temperature and the addition of Triton X-100 to homogenates were also investigated with an expectation that the reactivity between

proteases and apple PPO would increase. Apple homogenates containing selected concentrations of protease, cysteine, and /or EDTA in 15 mL graduated plastic test tubes were incubated at 4, 15, or 25 °C for 30 min. PPO activity in apple homogenates was determined immediately after the dispersion of proteases and at selected times during incubation for 30 min.

Single use of protease. Thirty milligrams of protein as bromelain, ficin, or papain powder was dispersed in 8 mL of apple homogenate and incubated at 4 °C. PPO activity in apple homogenates without protease was used as a control.

Cysteine protease activators. The concentrations of EDTA and cysteine as activators of cysteine proteases were determined following the method of Gomes and others (1997). Apple homogenates including 1 mM EDTA, 10 mM cysteine, or 1 mM EDTA and 10 mM cysteine were incubated at 4 °C. PPO activity in apple homogenates without cysteine protease activators was used as a control.

Combination of proteases and activators. Thirty milligrams of protein as bromelain, ficin, or papain powder was dispersed in 8 mL of apple homogenate including 1 mM EDTA and 10 mM cysteine, and incubated at 4 °C. PPO activity in apple homogenates including 1 mM EDTA and 10 mM cysteine was used as a control.

Combination of papain, EDTA, and cysteine. To determine if papain required both activators, 30 mg of protein as papain powder was dispersed in 8 mL of apple homogenates including 1 mM EDTA, 10 mM cysteine, or 1 mM EDTA and 10 mM

cysteine, and incubated at 4 °C. PPO activity in apple homogenates without papain, EDTA, and cysteine was used as a control.

Papain concentration. Selected quantities (15 to 30 mg) of protein as papain powder were dispersed in 8 mL of apple homogenates including 1 mM EDTA and 10 mM cysteine and incubated at 4°C. PPO activity in apple homogenates including 1 mM EDTA and 10 mM cysteine was used as a control.

Preincubation of papain with activators. To examine the effect of activators of papain to promote inhibition of apple PPO, preincubation of papain with cysteine and EDTA prior to adding the mixture to apple homogenates was compared to the direct dispersion method. Preincubation was achieved by dispersing 20 mg of protein as papain powder in 2 mL of water including 1 mM EDTA and 10 mM cysteine, followed by incubation at 25 °C for 30 min. The papain mixture after preincubation was mixed with 6 mL of apple homogenate prepared by blending 100 g of diced apples and 50 g of water with and without 3 mM EDTA and 30 mM cysteine immediately. The apple homogenates containing preincubated papain were incubated at 4 °C for 30 min. PPO activity in apple homogenates containing 1 mM EDTA, 10 mM cysteine, and directly dispersed papain (2.5 mg protein as papain powder/mL) were used as a control.

EDTA and cysteine concentrations. Apple homogenates containing 2.5 mg protein as papain powder/mL and selected concentrations of EDTA (0 to 2 mM) and cysteine (0 to 50 mM) were incubated at 4 °C for 30 min

Incubation temperature. Apple PPO activity was determined during the incubation of apple homogenates containing 2.5 mg protein as papain powder/mL, 1 mM EDTA and 25 mM cysteine at 4, 15, or 25°C.

Detergent. Diced apples were blended in an equivalent weight of water containing 2 mM EDTA, 50 mM cysteine, or containing 2 mM EDTA 50 mM cysteine, and Triton X-100 at concentrations of 0.02, 0.2, or 2%. Apple homogenates mixed with 2.5 mg protein as papain powder/mL were incubated at 4 or 25 °C.

Incubation-UHP treatments of apple homogenates

Defined as incubation-UHP treatments, apple homogenates containing selected constituents were incubated at 25 °C for 30 min, then treated under UHP conditions of 600 MPa at 25 °C for selected dwelling times after the come-up-time.

Apple homogenate preparations. To clarify the roles of papain, EDTA, and cysteine during and after incubation-UHP treatments, eight apple homogenates were prepared with selected combinations of EDTA, cysteine, and papain (Table 1). Each apple homogenate was filled into six sealed polyethylene bags; three 2 × 5 cm bags for enzyme assays and three 8 × 8 cm bags for color determination, respectively. The apple homogenates were incubated at 25 °C for precisely 30 min prior to UHP treatment. On the other hand, assays for PPO and papain were carried out immediately after homogenate preparation and incubation at 25 °C for 30 min.

Table 1: Constituents of apple homogenates subjected to incubation-UHP treatments.

Group	A	A	B	B	C	C	D	D
Code	N	P	E	EP	C	CP	EC	ECP
Apple (% w/w)	50	50	50	50	50	50	50	50
Cysteine (mM)	0	0	0	0	25	25	25	25
EDTA (mM)	0	0	1	1	0	0	1	1
Papain (mg/mL)	0	2.5	0	2.5	0	2.5	0	2.5

UHP treatment. Apple homogenates in sealed polyethylene bags were exposed to UHP treatment in an isostatic pressing system (Engineered Pressure Systems, Inc., Andover, MA) at 600 MPa at an initial temperature of 25 °C for selected dwelling times of 0, 2, 5, or 10 min. Pressurization was initiated precisely 30 min after starting the incubation of apple homogenates. The come-up time to reach 600 MPa was 230 ± 3 s and "0 min" homogenates were prepared by decompressing the pressure vessel immediately after the come-up-time. PPO assay of eight apple homogenates and papain assay of four apple homogenates containing papain were conducted after UHP treatment.

Determination of apple homogenate discoloration during storage. UHP treated apple homogenates were stored at 4 or 25 °C for 28 d. Homogenate color (L*, a*, and b*) was determined at 7 d intervals with a Minolta CM-2002 spectrophotometer (Minolta Camera Co., Osaka, Japan). A standard ceramic white plate, L* = 96.44, a* = -0.10, and b* = 3.56, was used to standardize the instrument. Hue angle (= $\tan^{-1}$ (b*/a*)) and chroma (= $(a^{*2} + b^{*2})^{1/2}$) were calculated based on the obtained a* and b* values.

Statistical analysis

Calculations of means and standard deviations as well as analysis of variance (ANOVA) of the effect of experimental conditions on residual PPO activity, residual papain activity, and apple homogenates color parameters were performed using Minitab Release 14 software (Minitab Inc., State College, PA). Significant differences are defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

EDTA, Cysteine, and apple PPO activity

PPO activity in apple homogenates as a control naturally decreased by 11.0% after incubation at 4 °C for 30 min, while the addition of 1 mM EDTA or 10 mM cysteine to apple homogenates did not exhibit an inhibitory effect on PPO activity during incubation at 4 °C for 30 min (Figure 1).

In the presence of 1 mM EDTA, residual apple PPO activity after incubation at 4 °C for 10 or 15 min was significantly larger ($p \leq 0.05$) than the control PPO activity after equivalent incubation. The addition of 1 mM EDTA to apple homogenates may result in a stable apple PPO activity, whereas equivalent concentration of EDTA inhibited pineapple PPO activity by 45% (Das and others 1997) or longan fruit PPO activity by 25% (Jiang 1999).

No significant differences ($p \leq 0.05$) in apple PPO activities were observed during incubation of apple homogenates containing 10 mM cysteine compared to the

control activity in homogenates. Several studies demonstrated the effectiveness of cysteine as an antibrowning agent with colorimetric methods. For example, conventional spectrophotometric assays of extracted apple PPO to determine the formation of *o*-quinones reported a decrease in PPO activity in the presence of cysteine (Molnar-Perl and Friedman 1990b). In addition, sliced apples dipped in cysteine solutions exhibited smaller color changes expressed as Hunter L*, a*, and b* values compared to sliced apples that were not treated with cysteine solutions (Molnar-Perl and Friedman 1990a). The results demonstrating the efficacy of cysteine to inhibit apple PPO is attributed to the action of cysteine as an antibrowning agent and the selected PPO assay method.

In the fluorescence oxygen probe method that determines oxygen consumption in reaction mixtures, oxygen concentrations decrease as PPO-catalyzed oxidation of catechol substrates to *o*-benzoquinone reactants progress. Therefore, oxygen molecules in PPO assay reaction mixtures are consumed, being involved in the oxidation of catechol to *o*-benzoquinone regardless of the presence of cysteine that reacts with *o*-benzoquinone. In addition, incubation of apple homogenates containing 10 mM cysteine at 4 °C for 30 min may result in insufficient formation of cysteine-quinone adducts as PPO inhibitors.

The antibrowning effect of the addition of cysteine to apple homogenates was observed visually. The observed color of apple homogenates containing cysteine or both cysteine and EDTA in our experiments did not change during blending of apples

and incubation of apple homogenates at 4 °C for 30 min, whereas apple homogenates that did not contain cysteine exhibited discoloration immediately after blending.

A significant decrease of apple PPO activity was achieved with the addition of both 1 mM EDTA and 10 mM cysteine after 30 min incubation at 4 °C compared to the control apple PPO activity after equivalent incubation. However, statistical comparisons of PPO activity in apple homogenates containing cysteine and both EDTA and cysteine resulted in no significant change in PPO activity attributed to combination of cysteine with EDTA.

Cysteine proteases, EDTA, cysteine, and apple PPO activity

Preliminary experiments to calculate mean PPO activity in Granny Smith apple homogenates resulted in 187.5 ± 23.6 Sigma units/mL. Experimental concentrations of 3.75 mg protein as cysteine protease powders/mL were determined by doubling the concentration of ficin expected to inactivate equivalent Sigma units of mushroom tyrosinase (Labuza and others 1990).

Bromelain did not inhibit PPO activity in apple homogenates, whereas apple PPO was stable in the presence of ficin or papain (Figure 2). When compared to the control PPO activity after equivalent incubation, PPO activity in apple homogenates was greater ($p \leq 0.05$) after 5 min incubation with ficin, or after 10, 15, 25, or 30 min incubation with papain.

Bromelain or ficin did not significantly inhibit PPO activity in apple homogenates including 1 mM EDTA and 10 mM cysteine during incubation at 4 °C for

30 min (Figure 3). While bromelain did not alter PPO activity in apple homogenates during incubation for 30 min, stability of PPO in apple homogenates was increased ($p \leq 0.05$) by ficin after incubation at 4 °C for 30 min.

On the other hand, PPO activity in apple homogenates containing 1 mM EDTA and 10 mM cysteine were significantly inhibited by papain ($p \leq 0.05$) after incubation at 4 °C for 10, 15, or 20 min (Figure 3). Residual PPO activities in apple homogenates containing EDTA, cysteine, and papain were 64.2 and 56.5% after incubation at 4 °C for 15 and 30 min, respectively. Two different PPO stabilities of PPO in apple homogenates were observed in the presence of papain: a stable apple PPO when incubated with papain alone and a inhibition of PPO activity when incubated in the presence of EDTA and cysteine.

Papain, EDTA, cysteine, and apple PPO activity

An experiment using selected apple homogenates was conducted to determine whether the presence of EDTA and cysteine are required for the inhibition of apple PPO by papain. As a result, PPO activity in apple homogenates was significantly decreased by papain when incubated with EDTA and cysteine at 4 °C for 15 min (Figure 4). When incubated with either EDTA or cysteine, papain did not alter observed apple PPO activity during incubation at 4 °C for 30 min. We observed that only papain, among selected plant cysteine proteases, inhibited apple PPO activity in the presence of both EDTA and cysteine.

Residual PPO activity in apple homogenates after incubation at 4 °C for 15 or 30 min was dependent on papain concentrations from 1.875 to 3.75 mg protein as powder/mL (Figure 5). Papain at a concentration of 1.875 mg protein as powder/mL did not inhibit PPO activity during incubation at 4 °C for 30 min compared to apple PPO activity in the absence of papain. Apple PPO was significantly ($p \leq 0.05$) inhibited after incubation of apple homogenates containing papain at a concentration of 3.125 mg protein as powder/mL at 4 °C for 15 min. Significant inhibition of apple PPO activity was also achieved with 2.5 mg protein as papain powder/mL or larger concentrations after incubation at 4 °C for 30 min. Therefore, papain concentration of 2.5 mg/mL and incubation at 4 °C for 30 min were employed in the following experiments to determine incubation conditions for apple PPO inhibition by papain.

Papain dispersed in a solution containing 1 mM EDTA and 10 mM cysteine and placed at 25 °C for 30 min prior to the addition to apple homogenates designated as preincubation did not exhibit an increased inhibition of PPO activity in apple homogenates during the following incubation at 4 °C for 30 min (Figure 6). When papain was added directly to apple homogenates containing 1 mM EDTA and 10 mM cysteine, apple PPO exhibited equivalent inhibition during incubation at 4 °C for 15 or 30 min regardless of the preincubation of papain with EDTA and cysteine. Preincubated papain did not inhibit PPO activity in apple homogenates containing 0.1 mM EDTA and 1 mM cysteine.

Commercial papain was dissolved in a buffer containing 1 mM EDTA and 10 mM cysteine to prepare the papain solution selected for UHP experiments (Gomes and others 1997). Cysteine enhances papain activity by reducing disulfide bonds to sulfhydryl groups (Wong 1995). Our results indicate that papain was neither enhanced nor activated during preincubation with 1 mM EDTA and 10 mM cysteine. The requirement of 1 mM EDTA and 10 mM cysteine in apple homogenates to inhibit apple PPO activity during incubation at 4 °C regardless of the preincubation with EDTA and cysteine. The absence of increased papain activation during preincubation was attributed to the protective effects of EDTA and cysteine on papain in apple homogenates potentially containing active compounds resulting in oxidation of sulfhydryl groups of papain molecules.

The inhibitory effect of papain at a concentration of 2.5 mg protein as powder/mL on PPO activity in apple homogenates during incubation at 4 °C was dependent on incubation times and concentrations of EDTA and cysteine (Figure 7). Significant decreases ($p \leq 0.05$) in apple PPO activity were achieved with papain in the presence of 25 mM cysteine after incubation at 4 °C for 30 min. Papain at a concentration of 3.75 mg protein as powder/mL in the presence of 10 mM cysteine did not inhibit apple PPO activity under equivalent incubation conditions (Figure 4). Therefore, the increase in cysteine concentrations from 10 to 25 mM in apple homogenates resulted in inhibition of apple PPO activity either by papain activation at

large cysteine concentrations or independently by large cysteine concentrations acting as a reducing agent.

The addition of 1 mM EDTA to apple homogenates effectively promoted apple PPO inhibition by papain in the presence of cysteine at concentrations from 2.5 to 25 mM. Among selected concentrations of EDTA from 0.5 to 2.0 and cysteine from 2.5 to 50 mM, the largest inhibitory effect of 2.5 mg papain / mL on apple PPO activity was achieved at concentrations of 2 mM EDTA and 25 mM cysteine after incubation at 4 °C for 15 min, and at concentrations of 1 mM EDTA and 25 mM cysteine after incubation at 4 °C for 30 min. The addition of 1 mM EDTA or 25 mM cysteine to apple homogenates containing 1 mM EDTA and 25 mM cysteine did not result in increased inhibition of apple PPO activity by papain after incubation at 4 °C for 15 or 30 min. Consequently, the presence of 1 mM EDTA and 25 mM cysteine in apple homogenates may fully protect papain molecules at concentrations of 2.5 mg protein as powder / mL to provide a significant inhibitory effect on apple PPO during incubation at 4 °C for 30 min.

Incubation temperature and apple PPO activity

PPO activities in apple homogenates containing 1 mM EDTA and 25 mM cysteine were more rapidly inhibited in the presence or the absence of papain as incubation temperature and incubation time increased (Figure 8). In control apple homogenates, significant inhibition ($p \leq 0.05$) of PPO activity in apple homogenates containing 1 mM EDTA and 25 mM cysteine was observed after incubation at 15 or

25 °C for 30 min, while PPO activity remained constant during incubation at 4 °C for 30 min. The inhibitory effect resulting from the addition of 1 mM EDTA and 25 mM cysteine was observed by elevating incubation temperatures to 15 or 25 °C and may be attributed to the increased EDTA chelation of copper atoms from the active site of PPO molecules, or formation of cysteine-quinone adducts that act as substrate analogs to inhibit PPO reactions with catechol.

PPO inhibition by papain in apple homogenates containing 1 mM EDTA and 25 mM cysteine was also promoted as incubation temperatures increased (Figure 8). Based on the initial apple PPO activity determined immediately after starting incubation, residual apple PPO activities after 30 min incubation with papain, EDTA, and cysteine at 4, 15, or 25 °C were 49.4, 40.4, and 34.4%, respectively. Residual apple PPO activity was significantly inhibited ($p \leq 0.05$) during incubation for 30 min as incubation temperatures increased from 4 to 25 °C. Incubation at 25 °C for 30 min is the most desirable condition for inhibition of residual apple PPO activity by papain, EDTA, and cysteine.

Triton X-100 and apple PPO activity

Triton X-100 was examined to assess solubilization of membrane-bound apple PPO and potential increases in apple PPO reactivity in the presence of water soluble papain, EDTA, and cysteine-quinone adducts during incubation. However, the addition of Triton X-100 to apple homogenates containing 2.5 mg papain/mL, 1

mM EDTA, and 25 mM cysteine did not alter apple PPO activity during incubation at 4 or 25 °C for 30 min (Figure 9).

In a preliminary assay for catechol oxidase activity with the fluorescence oxygen probe method, Granny Smith apple PPO activities in homogenates in the presence of 0.1 and 1% Triton X-100 were 170 and 79%, respectively, of PPO activity in the absence of Triton X-100. The increased apple PPO activity observed in the presence of 0.1% Triton X-100 may be lost by preferential reactivity of papain, cysteine-quinone adducts, and/or EDTA in the soluble fraction of apple homogenates. The probability of increased inhibition of apple PPO activity in the soluble fraction of apple homogenates is supported by the absence of any apparent inhibition of PPO activity resulting from inclusion of Triton X-100 during incubation at 4 or 25 °C because the efficacy of detergents generally increases as temperature increases.

PPO activity in apple homogenates after incubation-UHP treatments

Little change in the pH of apple homogenates was observed by adding 1 mM EDTA, 25 mM cysteine, or 1mM EDTA and 25 mM cysteine, while the addition of papain at a concentration of 2.5 mg protein as powder/mL increased pH of the homogenates (Table 2).

Table 2: pH of apple homogenates subjected to incubation-UHP treatments.

Group	A	A	B	B	C	C	D	D
Code	N	P	E	EP	C	CP	EC	ECP
pH	3.57	3.68	3.58	3.68	3.58	3.78	3.58	3.75

Constituents of apple homogenates are presented in Table 1 (p. 117).

In three individual experiments, residual PPO activities in apple homogenates containing 2.5 mg protein as papain powder/mL, 1 mM EDTA, and 25 mM cysteine and incubated at 25 °C for 30 min were 45.7 ± 3.1% (Figure 7), 34.4 ± 0.9% (Figure 8), or 45.8 ± 0.8% (Figure 9), respectively, based on the initial PPO activity determined immediately after starting incubation. Additional inhibitory effects on apple PPO were examined by combining incubation with UHP treatment. Inhibition of apple PPO followed two distinctive trends after incubation-UHP treatment (Figure 10).

As a control, PPO activity in apple homogenates was inhibited during UHP treatment, whereas the addition of papain to apple homogenates reduced the inhibitory effects on apple PPO activity during incubation and UHP treatments (Figure 10-A). Similar reduction of the inhibition of apple PPO activity in the presence of papain during incubation-UHP treatment were observed in apple homogenates containing 1 mM EDTA (Figure 10-B). No significant differences ($p \leq 0.05$) were observed in residual apple PPO activity during incubation or UHP treatment between control homogenates and homogenates containing EDTA. The addition of 1 mM EDTA to apple homogenates did not significantly affect apple PPO activity or papain action on apple PPO during the selected incubation-UHP treatments.

PPO activity increases in the presence of proteolytic enzymes other than plant cysteine proteases. Treatment of PPO with trypsin, for example, increased the activity of PPO extracted from grapes (Sanchez-Ferrer and others 1989), broad beans (King and Flurkey 1987), and peaches (Laveda and others 2001). Trypsin hydrolyzes

inactive broad bean PPO to form a slightly smaller molecular weight enzyme that retained PPO activity (King and Flurkey 1987). Thus, proteolytic enzymes may potentially hydrolyze and reduce the molecular weight of PPO resulting in changes in configuration and solubility of PPO molecules. In this study, the addition of papain to apple homogenates resulted in an increase in pressure tolerance of apple PPO regardless of the presence of EDTA.

PPO activity in apple homogenates decreased continually after the addition of 25 mM cysteine (Figure 10-C), or 1 mM EDTA and 25 mM cysteine (Figure 10-D) in the absence and the presence of papain during the incubation-UHP treatments. Increased inhibition of apple PPO was achieved by adding papain at a concentration of 2.5 mg/mL to the apple homogenates containing only cysteine or both EDTA and cysteine. Apple PPO activity in apple homogenates containing only cysteine treated with UHP for 0 or 10 min, or in apple homogenates containing EDTA and cysteine treated with UHP for 0, 2, or 10 min was significantly inhibited ($p \leq 0.05$) by the addition of papain to the apple homogenates.

To assess the efficacies of incubation and UHP treatments to inhibit apple PPO, the first-order kinetic constant ($k \times 10^{-3}$•min) of apple PPO inhibition in eight homogenates during 30 min incubation and 10 min UHP treatment, and the percentage inhibition of apple PPO activity after UHP treatment for 10 min are summarized (Table 3).

Table 3: First-order kinetic constants k ($\times 10^{-3} \bullet min^{-1}$) of apple PPO inhibition by incubation and UHP treatments and percentage inhibition of apple PPO activity after UHP treatment for 10 min.

Group Code	A N	A P	B E	B EP	C C	C CP	D EC	D ECP
k Incubation 25 °C/30 min	5.5 ± 3.7	0.9 ± 0.6	9.2 ± 1.9	1.7 ± 0.5	14.6 ± 1.7	22.2 ± 1.6	12.7 ± 7.9	24.7 ± 2.2
k UHP 25 °C/10 min	103.8 ± 12.9	42.0 ± 7.9	102.5 ± 12.9	38.0 ± 6.2	86.7 ± 15.5	89.4 ± 25.4	65.1 ± 10.4	96.1 ± 24.2
% inhibition after UHP for 10 min	85.3 ± 1.0	43.0 ± 6.8	86.5 ± 1.7	38.2 ± 3.1	81.5 ± 2.8	87.1 ± 3.1	75.0 ± 1.5	87.1 ± 2.9

Constituents of apple homogenates are presented in Table 1.

More than 85% inhibition of apple PPO activity was achieved after a 10 min UHP treatment of 1) control apple homogenates and apple homogenates containing 2) EDTA, 3) cysteine and papain, or 4) EDTA, cysteine, and papain. Apple PPO activity in the first two homogenates (homogenates 1) and 2)) was less effectively inhibited during incubation, yet rapidly inhibited during the come-up-time of UHP treatment. On the other hand, apple PPO activity in the latter two homogenates (homogenates 3) and 4)) was significantly inhibited ($p \leq 0.05$) during incubation. No significant differences ($p \leq 0.05$) in the kinetic constants (k) for the UHP treatments were observed with four apple homogenates (1) ~ 4)) exhibiting greater than 85% inhibition of apple PPO after UHP treatment at 600 MPa for 10 min.

Therefore, the presence of two apple PPO fractions is hypothesized in relation to the mode of inhibition during incubation or UHP treatments. The first hypothesized PPO fraction is inhibited by pressurization during the come-up-time of UHP treatment in the absence of papain and cysteine (homogenates 1) and 2)), or inhibited by papain-catalyzed PPO hydrolysis and reactions with cysteine-quinone adducts during incubation in the presence of papain and cysteine (homogenates 3) and 4)). A second hypothesized PPO fraction is then structurally altered leading to inhibition during UHP treatment at 600 MPa. More than 85% inhibition of apple PPO activity results after incubation and UHP treatment at 600 MPa by the addition of selected inhibitory additives such as EDTA, cysteine, and papain to apple homogenates.

Papain activity after incubation-UHP treatments

Exogenous papain activity in apple homogenates is more stable to UHP treatment at 600 MPa than Granny Smith apple PPO in apple homogenates (Figure 11). Papain activity was not inhibited in control apple homogenates during incubation-UHP treatments. Significant inhibition of papain activity ($p \leq 0.05$) was observed in apple homogenates containing EDTA, cysteine, or EDTA and cysteine after the come-up-time of UHP treatment. UHP treatment for 2 min was required to significantly inhibit papain activity ($p \leq 0.05$) in apple homogenates containing EDTA and cysteine. Residual papain activity after UHP treatments of four apple homogenates containing papain for 10 min ranged from 74.4 to 88.9% depending on constituents of apple homogenates compared to the activity determined immediately

after dispersion of papain powders in apple homogenates prior to incubation (Figure 11). Gomes and others (1997) also demonstrated the barostability of papain under pressures of 600 MPa at initial ambient temperatures for 10 min. Papain was inhibited by UHP treatment of 600 MPa by increasing the initial and UHP treatment temperatures to 60 °C.

Papain activity determined immediately after addition to apple homogenates was dependent on homogenate constituents. Relatively small papain activity was observed in control apple homogenates or apple homogenates containing only EDTA when compared to papain activity in apple homogenates containing only cysteine or EDTA and cysteine. Therefore, the absence of cysteine in apple homogenates results in an inhibition of papain activity. Apple PPO was stable to pressure in control apple homogenates containing papain (Figure 10-A) or apple homogenates containing papain and EDTA (Figure 10-B). Increased inhibition of PPO activity by the addition of papain was observed in apple homogenates containing cysteine (Figure 10-C), or EDTA and cysteine (Figure 10-C). Further studies such as electrophoretic or spectral analyses to investigate structural changes of apple PPO molecules in the presence of papain are required related to the addition of cysteine.

Discoloration of incubation-UHP treated apple homogenates

The Hunter L*, a*, and b* values of eight apple homogenates during storage at 4 or 25 °C are presented in Table 4. The calculated hue angles and chromas are presented in Table 5. The color data was analyzed based on the constituents in apple

homogenates and storage temperatures. UHP dwell times did not affect discoloration of apple homogenates during storage.

Control apple homogenates or apple homogenates containing EDTA exhibited rapid brown discoloration immediately after blending in the absence or the presence of papain. The resulting brown color of the four apple homogenates immediately after UHP treatment was not acceptable as described by mean L* of 35.4, hue angle of 65.5 °, and chroma of 13.8. The increase in hue angles of the UHP treated apple homogenates during storage at 4 or 25 °C was attributed to a decrease in a* values. The reduction of redness of apple homogenate color described as decrease in a* during storage resulted in observed fading of brown.

Apple homogenates containing cysteine or apple homogenates containing EDTA and cysteine retained the original flesh color of apples during blending and incubation-UHP treatments. The addition of papain to the apple homogenates did not alter the color of homogenates during incubation-UHP treatments. The four apple homogenates containing at least cysteine immediately after UHP treatment exhibited an acceptable chartreuse color with a mean L* of 43.9, hue angle of 100.9 °, and chroma of 10.2. The acceptable chartreuse color was retained during storage at 4 °C for 28 d.

The addition of papain in apple homogenates containing only cysteine or EDTA and cysteine resulted in relatively rapid brown discoloration involving significant decreases ($p \leq 0.05$) in L* values and hue angles after storage at 25 °C for

28 d compared to equivalent apple homogenates without papain at equivalent storage conditions. The decreases in hue angles of apple homogenates containing papain and cysteine, or papain, EDTA, and cysteine were attributed to change from chartreuse to yellow, degradation of chlorophylls, and the resulting increase in a* values during storage at 25 °C. The color of the four UHP treated apple homogenates containing at least cysteine was acceptable after storage at 4 or 25 °C for 28 d.

Small residual PPO activity and storage at refrigerated temperatures inhibits rate of brown discoloration of UHP treated avocado puree (Lopez-Malo and others 1999), banana puree (Palou and others 1999), and peach puree (Guerrero-Beltran and others 2004) during storage. The color stability of UHP treated guacamole is dependent on the number of pressurization cycles and storage temperatures (Palou and others 2000). The color of Granny Smith apple homogenates are retained by the addition of cysteine and concomitant treatment with UHP of 600 MPa at an initial temperature of 25 °C.

CONCLUSIONS

Among the bromelain, ficin, and papain proteases, only papain significantly inhibits PPO activity in Granny Smith apple homogenates. The presence of both EDTA and cysteine as activators in the apple homogenates is necessary to promote significant papain activity. The minimum papain concentration demonstrating inhibition of PPO activity was 2.5 mg/mL of apple homogenate for 30 min incubation

at 4 °C. The largest inhibitory effect of papain on PPO activity was observed in apple homogenates containing 1 mM EDTA and 25 mM cysteine. Preincubation of papain with EDTA and cysteine prior to addition to apple homogenates, or addition of Triton X-100 to apple homogenates did not promote papain-catalyzed-inhibition of PPO activity. The inhibition of apple PPO activity by papain increased as incubation temperatures to 25 °C.

Incubation of apple homogenates containing selected concentrations of papain, EDTA, and cysteine at 25 °C for 30 min followed by UHP treatment at 600 MPa and 25 °C for 10 min resulted in two characteristic inhibition plots of apple PPO activity depending on the constituents of apple homogenates. Incubation of apple homogenates with added cysteine and papain effectively inhibited apple PPO activity. PPO activity in control apple homogenates or apple homogenates containing 1 mM EDTA was significantly inhibited during the come-up-time of UHP treatment. The consequent dwell times of the apple homogenates under UHP at 600 MPa for 10 min resulted in more than 85% inhibition of apple PPO activity. Papain was baroresistant to experimental incubation-UHP treatments.

Apple homogenates containing at least cysteine exhibited chartreuse to light yellow color after incubation-UHP treatments, while rapid brown discoloration characterized apple homogenates immediately after apple homogenate preparation in the absence of cysteine. Apple homogenate color was retained during incubation-UHP treatments, as well as during storage at 4 or 25 °C for 28 d. The combined methods of

adding papain and cysteine to the apple homogenates followed by UHP treatment is a potential application for quality preservation of apple products such as sauce, juice and puree exhibiting low residual PPO activity and original apple color during storage at refrigerated or ambient temperatures.

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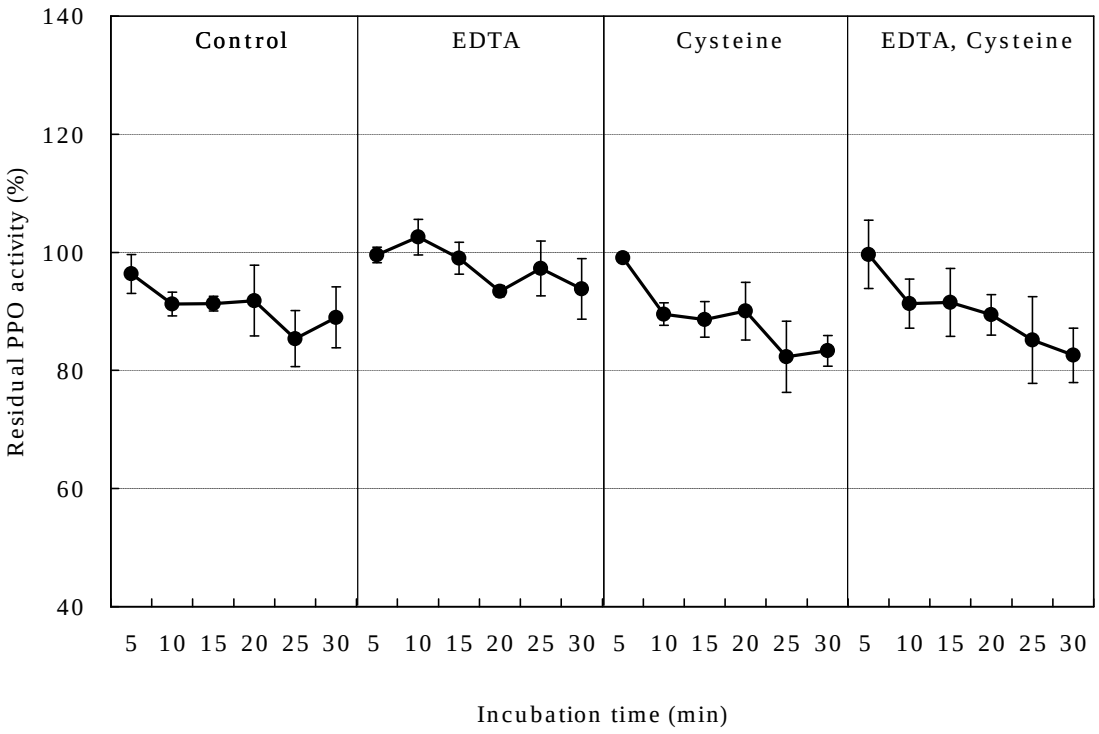


Figure 1: PPO activity in Granny Smith apple homogenates containing 1 mM EDTA, 10 mM cysteine, or 1 mM EDTA and 10 mM cysteine during incubation at 4 °C (n = 3).

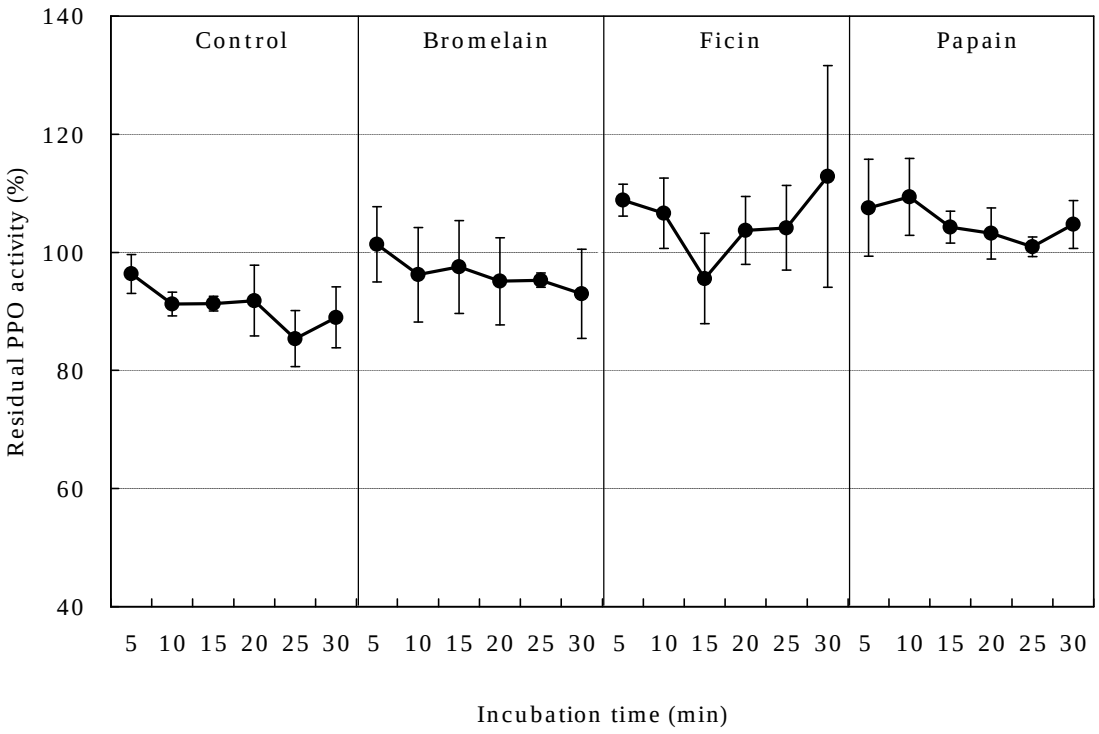


Figure 2: PPO activity in Granny Smith apple homogenates during incubation with 3.75 mg protein as bromelain, ficin, or papain powder/mL at 4 °C (n = 3).

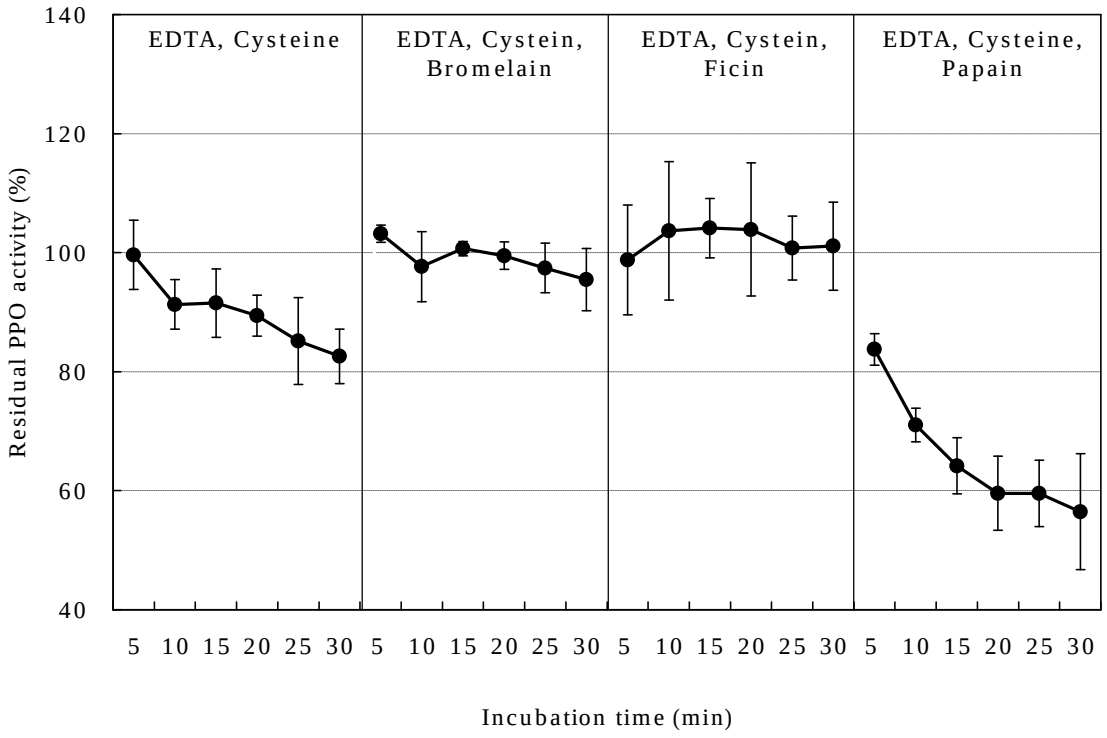


Figure 3: PPO activity in Granny Smith apple homogenates containing 1 mM EDTA and 10 mM cysteine during incubation with 3.75 mg protein / mL of bromelain, ficin, or papain at 4 °C (n = 3).

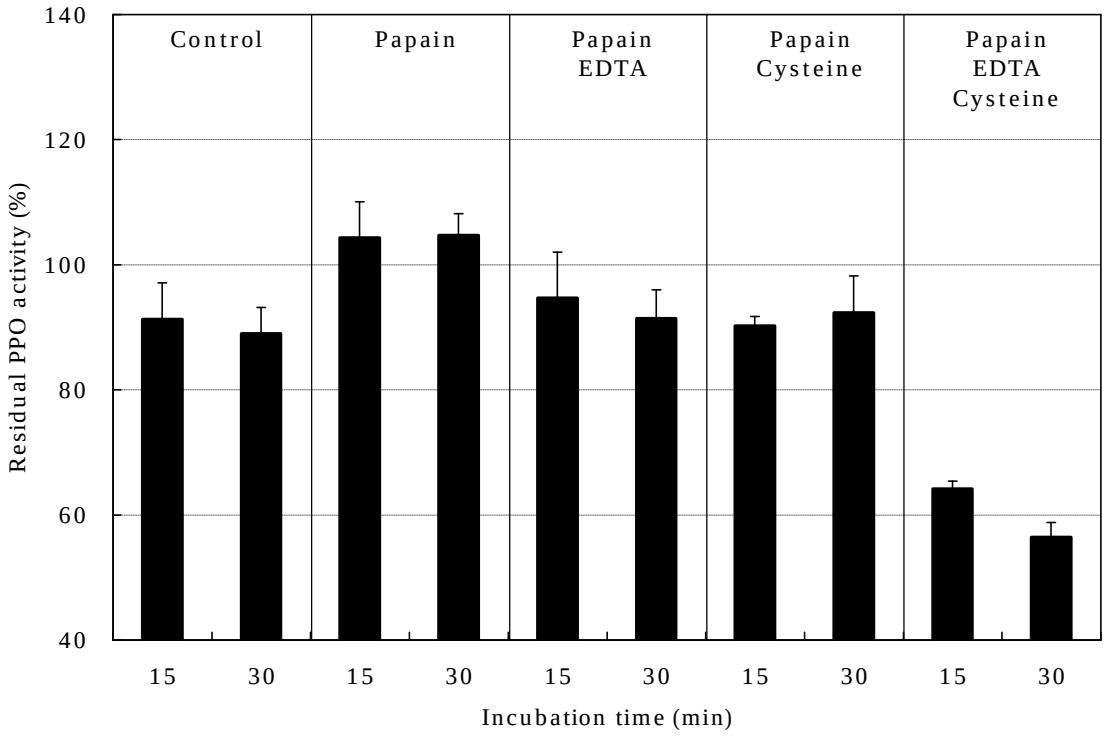


Figure 4: PPO activity in Granny Smith apple homogenates containing selected combinations of 1 mM EDTA and 10 mM cysteine during incubation with 3.75 mg protein as papain powder/mL at 4°C (n = 3).

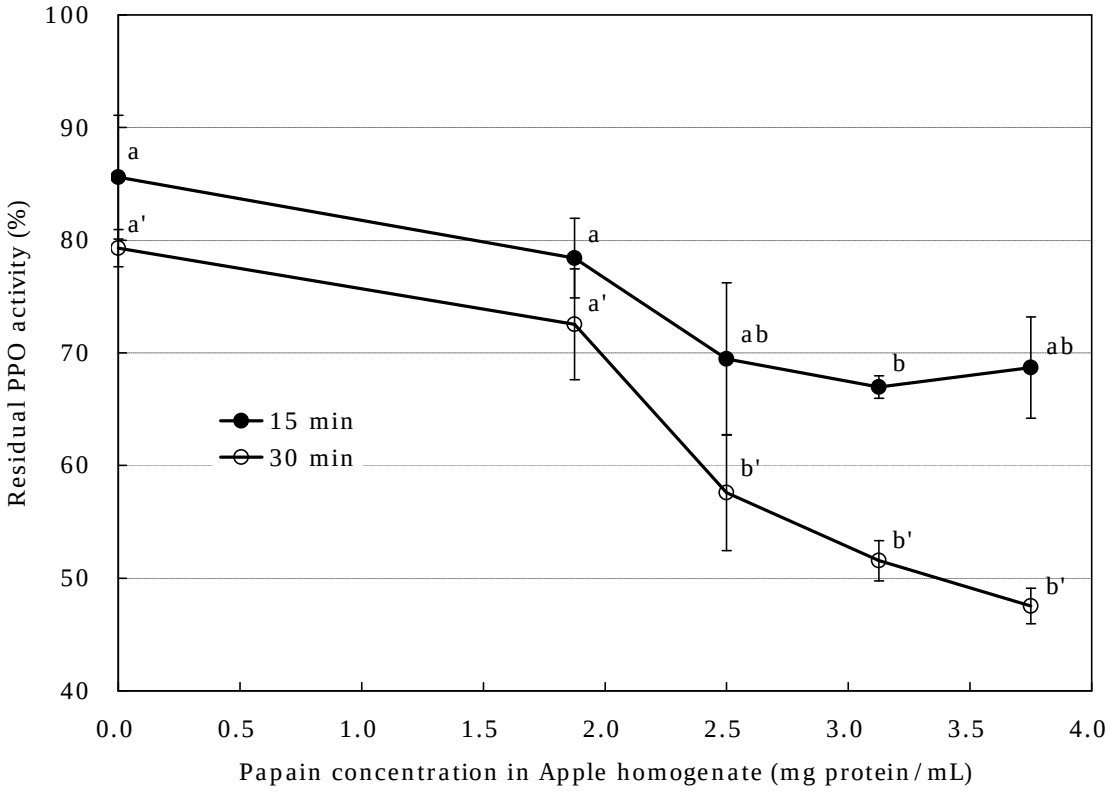


Figure 5: PPO activity in Granny Smith apple homogenates containing 1 mM EDTA and 10 mM cysteine during incubation with selected concentrations of protein as papain powder / mL at 4 °C for 15 or 30 min (n = 3).

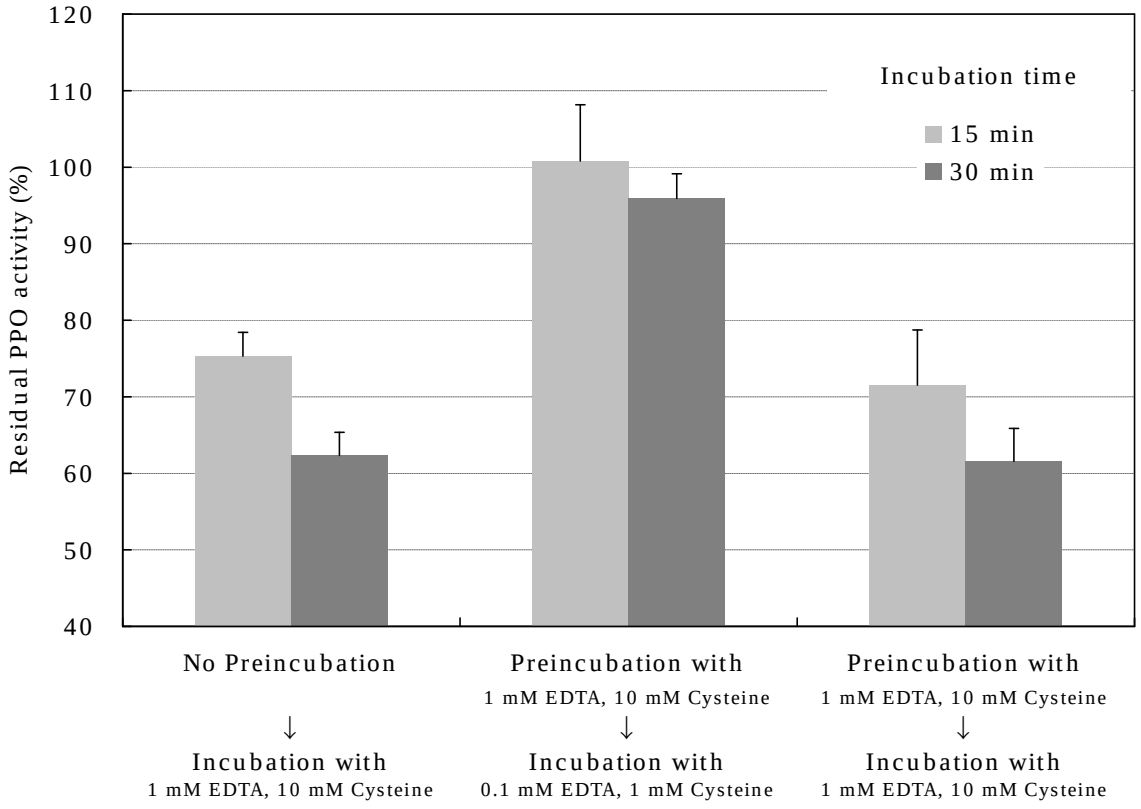


Figure 6: PPO activity in Granny Smith apple homogenates containing directly added or preincubated papain during incubation at 4°C for 15 or 30 min (n = 3).

Preincubation was assessed by incubating 20 mg protein as papain with EDTA and cysteine at 25 °C for 30 min. Preincubated or non-preincubated papain was added and incubated in 8 mL of apple homogenate containing EDTA and cysteine.

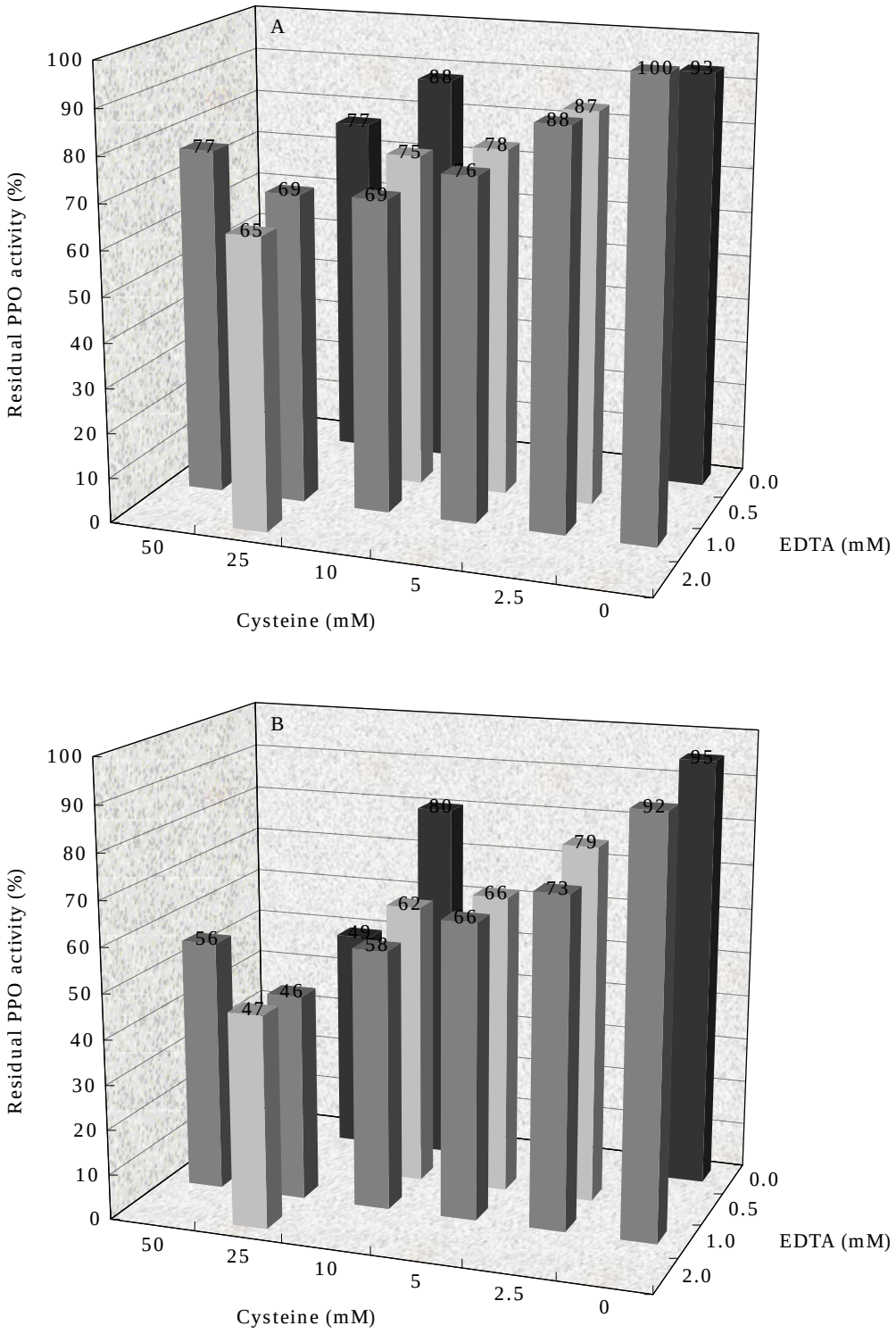


Figure 7: PPO activity in Granny Smith apple homogenates containing selected concentrations of EDTA and cysteine during incubation with 2.5 mg protein as papain powder/mL at 4°C for15 (A) or 30 min (B).

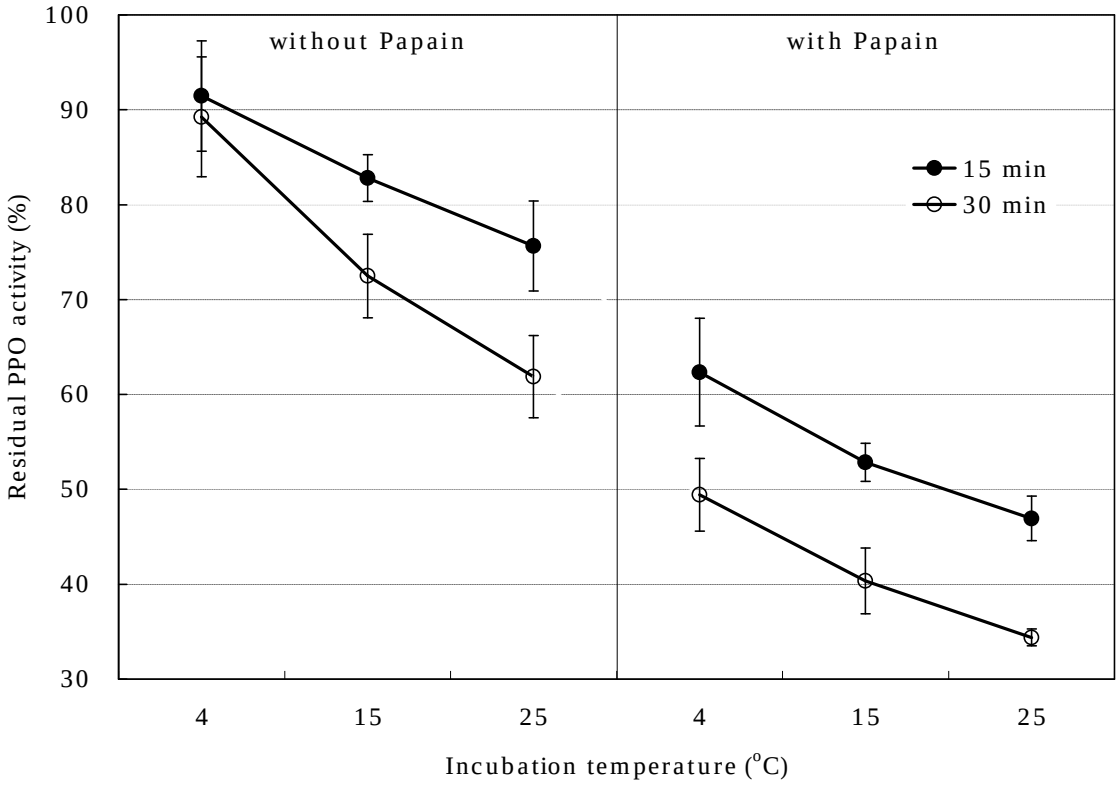


Figure 8: PPO activity in Granny Smith apple homogenates containing 1 mM EDTA and 25 mM cysteine during incubation with or without 2.5 mg protein as papain powder/mL at 4, 15, or 25 °C for 15 or 30 min (n = 3).

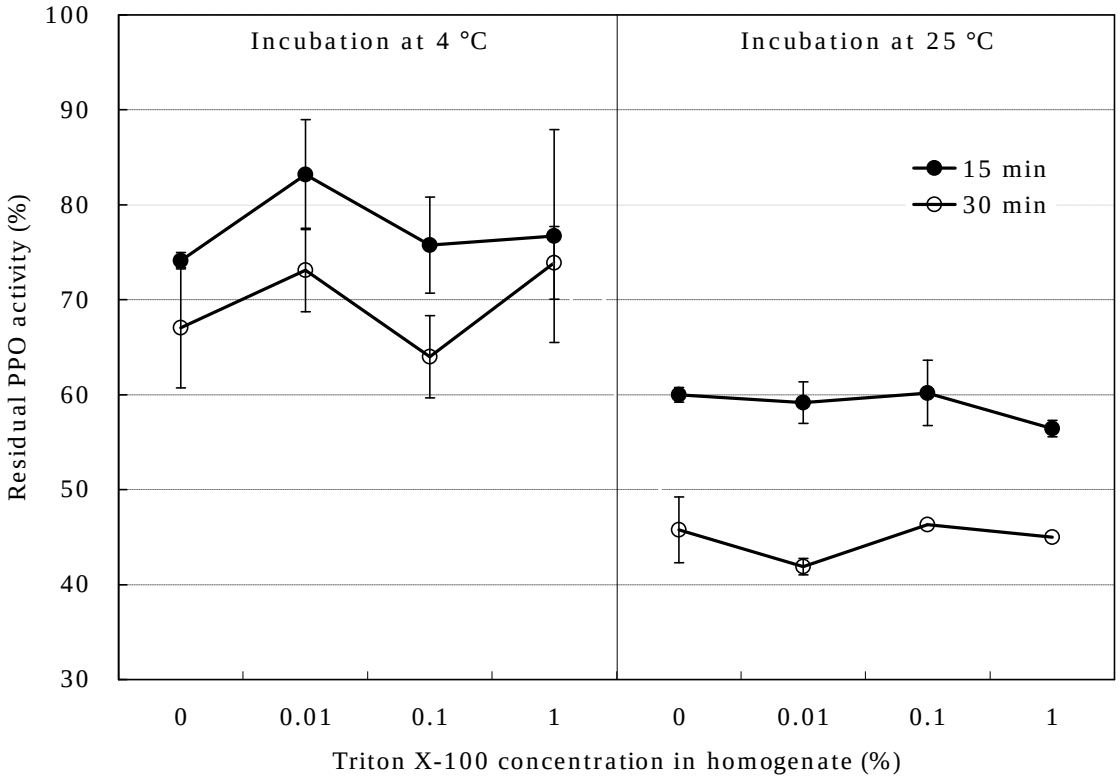


Figure 9: PPO activity in Granny Smith apple homogenates containing 1 mM EDTA, 25 mM cysteine, 2.5 mg protein as papain powder/mL in the presence of selected concentrations of Triton X-100 during incubation at 4 or 25 °C.

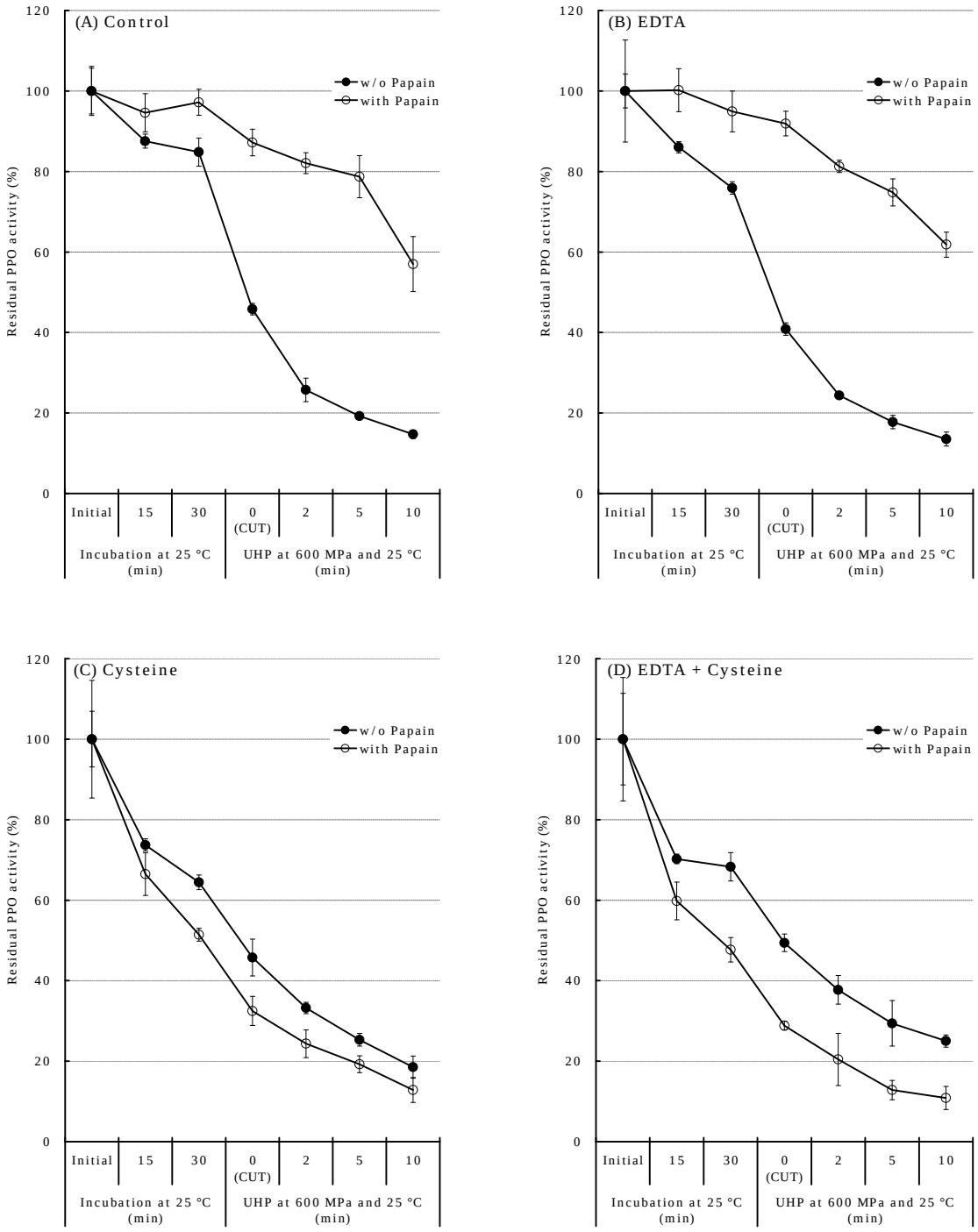


Figure 10: Apple PPO activity during incubation at 25 °C for 30 min followed by UHP treatment at 600 MPa and 25 °C for 10 min (n = 3).

Apple homogenates (A) and apple homogenates containing 1 mM EDTA (B), 25 mM cysteine (C), and 1 mM EDTA and 25 mM cysteine (D) with and without 2.5 mg protein as papain powder/mL. ('CUT' = come-up-time)

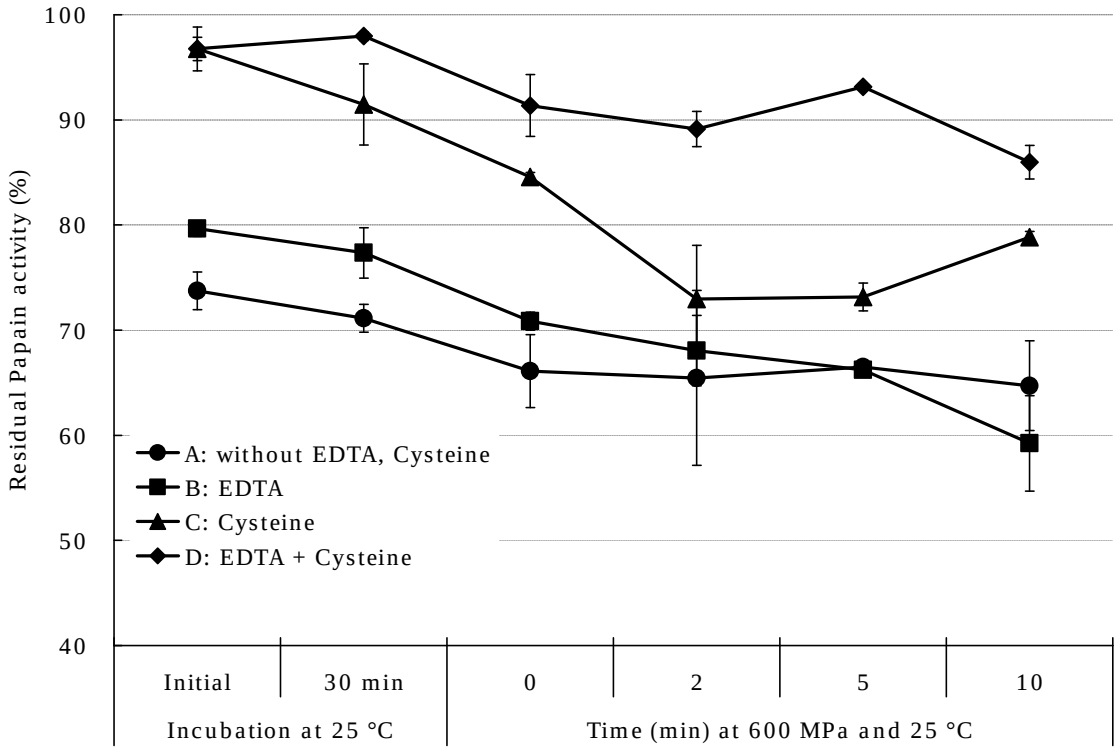


Figure 11: Exogenous papain activity in Granny Smith apple homogenates during incubation at 25 °C for 30 min followed by UHP treatment at 600 MPa and 25 °C for 10 min (n = 3).

Apple homogenates (A), and homogenates containing 1 mM EDTA (B), 25 mM cysteine (C), and 1 mM EDTA and 25 mM cysteine (D) during incubation at 25 °C for 30 min followed by UHP treatment at 600 MPa and 25 °C for 10 min.

HHP	Storage	L					a					b				
(min)	°C | days	0	7	14	21	28	0	7	14	21	28	0	7	14	21	28
Control																
0	4	36.0	32.9	33.6	31.3	32.4	6.5	5.6	6.1	4.6	4.4	12.8	11.2	13.2	12.2	11.0
2	4	35.7	34.9	33.6	31.6	32.9	6.0	7.0	6.5	5.1	4.6	13.1	14.2	14.6	12.5	10.8
5	4	34.5	38.9	32.2	31.8	33.1	6.0	4.2	5.2	4.8	3.9	13.4	9.6	11.8	11.7	9.5
10	4	32.9	37.0	33.2	32.8	34.1	4.8	6.9	4.6	4.5	4.0	11.1	13.7	11.4	11.4	9.3
0	25	36.0	34.4	37.9	36.9	34.5	6.5	4.0	4.4	4.8	4.1	12.8	11.9	11.6	11.0	10.9
2	25	35.7	33.4	35.2	35.2	33.9	6.0	4.5	5.5	4.1	4.6	13.1	12.5	14.2	9.7	12.5
5	25	34.5	32.8	35.4	35.8	33.7	6.0	4.5	6.3	4.0	4.5	13.4	12.7	15.5	9.3	12.7
10	25	32.9	34.0	37.4	35.7	34.9	4.8	3.8	5.0	3.9	4.8	11.1	10.9	13.3	9.8	13.3
2.5 mg Papain/mL																
0	4	34.7	36.3	36.0	34.6	37.8	6.3	5.7	5.5	4.7	4.2	13.3	13.3	14.0	13.8	12.1
2	4	37.5	38.3	34.7	34.2	37.5	6.6	6.6	5.2	5.2	4.4	13.7	14.8	13.5	14.2	11.7
5	4	37.3	36.9	36.1	35.1	37.8	6.2	5.2	4.9	4.7	5.0	13.3	12.7	12.3	12.9	13.2
10	4	37.0	36.9	37.1	34.6	37.4	6.1	5.8	5.3	4.8	4.7	13.4	13.6	13.6	13.4	13.3
0	25	34.7	35.2	35.1	36.9	35.3	6.3	5.0	5.7	4.5	4.3	13.3	14.3	16.6	13.4	13.7
2	25	37.5	35.8	35.8	35.7	35.3	6.6	5.0	4.8	3.8	4.7	13.7	14.2	14.9	10.3	14.5
5	25	37.3	35.7	35.5	37.7	36.5	6.2	4.6	4.6	3.6	4.4	13.3	13.2	14.8	10.0	14.2
10	25	37.0	34.7	35.9	37.3	35.9	6.1	5.5	4.9	3.7	4.8	13.4	15.0	14.8	9.5	14.8
1 mM EDTA																
0	4	33.4	34.9	35.0	31.8	33.0	5.0	5.7	5.0	4.6	5.1	11.1	12.0	11.3	10.2	12.3
2	4	33.2	34.5	33.0	32.2	33.0	5.1	5.0	5.0	4.6	5.3	10.9	11.0	12.3	11.5	13.1
5	4	34.0	32.2	34.9	33.4	32.7	5.2	4.7	5.1	4.5	5.1	10.6	10.8	12.6	10.6	13.7
10	4	34.1	33.2	33.9	32.7	33.4	5.6	3.9	5.3	4.5	4.9	12.3	9.6	12.9	10.1	13.4
0	25	33.4	34.4	35.1	35.4	33.9	5.0	4.5	5.2	4.1	4.8	11.1	10.9	13.8	9.9	12.0
2	25	33.2	34.0	35.0	35.7	33.7	5.1	4.2	4.6	4.3	4.8	10.9	10.8	13.9	10.4	14.3
5	25	34.0	33.8	33.4	35.8	34.2	5.2	5.4	4.4	4.1	4.9	10.6	13.8	13.4	11.3	14.3
10	25	34.1	34.7	34.7	35.9	33.6	5.6	4.9	4.5	4.1	5.0	12.3	12.5	13.7	10.7	14.4
1 mM EDTA + 2.5 mg Papain/mL																
0	4	36.1	35.8	35.8	38.2	36.8	5.9	5.8	4.7	4.1	5.1	13.3	13.5	13.4	11.3	15.1
2	4	36.3	35.4	37.5	36.9	36.9	5.0	5.0	4.7	4.7	5.3	12.1	12.8	13.0	12.6	15.2
5	4	35.7	34.6	37.1	38.1	37.1	5.4	3.8	5.2	4.3	5.4	12.9	12.3	14.2	11.3	15.3
10	4	37.3	37.2	38.9	37.1	37.3	5.3	4.0	4.6	4.5	5.1	12.8	12.5	12.7	12.2	15.0
0	25	36.1	37.6	38.8	38.3	36.7	5.9	4.9	4.5	3.9	5.2	13.3	14.2	15.3	11.6	16.1
2	25	36.3	37.8	38.8	38.8	38.0	5.0	4.9	4.6	3.9	5.0	12.1	15.0	15.2	10.8	15.3
5	25	35.7	38.5	38.6	40.2	37.8	5.4	4.7	4.8	4.1	4.8	12.9	13.7	15.2	11.3	14.7
10	25	37.3	37.4	39.8	39.5	38.0	5.3	5.2	5.3	4.1	4.5	12.8	15.4	16.2	11.3	13.7
25 mM Cysteine																
0	4	43.2	43.5	43.1	40.4	42.5	-2.1	-1.8	-2.3	-1.8	-1.4	11.1	10.5	9.5	8.8	7.6
2	4	42.9	44.1	42.3	41.6	41.4	-2.2	-2.0	-2.0	-1.8	-1.6	10.8	9.5	9.6	8.3	9.0
5	4	42.7	45.6	43.4	40.2	41.7	-2.2	-1.9	-2.1	-1.7	-1.7	9.6	10.0	8.8	8.2	9.0
10	4	43.2	46.9	45.0	41.2	41.1	-2.1	-1.9	-1.6	-1.6	-1.7	10.5	11.9	9.0	7.4	8.5
0	25	43.2	40.5	41.5	41.0	39.1	-2.1	-1.4	-1.3	-0.8	-0.7	11.1	8.6	8.1	7.3	6.7
2	25	42.9	40.6	42.0	42.6	38.9	-2.2	-1.0	-1.2	-0.5	-0.6	10.8	9.5	7.2	7.0	7.5
5	25	42.7	40.3	40.8	41.9	39.5	-2.2	-1.2	-1.4	-0.5	-0.7	9.6	9.7	6.9	6.5	6.2
10	25	43.2	42.6	41.4	41.7	40.2	-2.1	-1.1	-1.2	-0.5	-0.6	10.5	10.4	8.6	6.6	6.5
25 mM Cysteine + 2.5 mg Papain/mL																
0	4	44.6	42.0	42.5	41.2	42.2	-2.1	-2.0	-1.7	-1.7	-1.7	9.9	9.1	10.5	8.2	9.0
2	4	43.4	42.2	42.5	42.3	42.3	-2.2	-1.9	-1.6	-1.4	-1.5	9.8	10.3	9.7	8.7	10.2
5	4	43.2	44.1	42.1	42.4	42.4	-2.1	-1.7	-1.6	-1.5	-1.6	9.0	10.4	10.3	9.0	10.7
10	4	44.9	43.3	44.1	42.1	42.2	-1.9	-1.6	-1.6	-1.4	-1.5	11.2	11.5	9.7	9.1	10.4
0	25	44.6	41.2	39.2	36.2	37.3	-2.1	-0.8	-0.6	-0.2	0.4	9.9	10.5	9.2	5.3	8.4
2	25	43.4	40.8	40.4	38.6	37.5	-2.2	-0.7	-0.7	0.0	0.4	9.8	10.9	8.2	6.1	8.7
5	25	43.2	40.4	40.3	38.7	37.3	-2.1	-0.7	-0.4	0.1	0.5	9.0	10.4	9.7	5.6	9.0
10	25	44.9	41.5	40.0	38.7	38.2	-1.9	-0.7	-0.3	0.1	0.4	11.2	10.5	10.2	6.1	8.9
1 mM EDTA + 25 mM Cysteine																
0	4	42.9	43.5	42.1	41.4	41.6	-1.9	-1.7	-1.8	-1.4	-1.5	9.7	10.0	9.3	7.6	9.5
2	4	42.9	42.7	43.9	41.1	42.0	-2.0	-1.5	-1.7	-1.5	-1.6	9.1	10.3	9.5	7.1	9.9
5	4	42.8	42.4	45.6	43.4	42.2	-1.9	-1.6	-1.7	-1.3	-1.4	9.0	9.1	9.7	8.1	10.0
10	4	42.5	42.8	44.6	42.8	41.5	-2.0	-1.7	-1.5	-1.5	-1.7	8.9	9.3	8.5	7.7	9.5
0	25	42.9	42.7	43.7	41.9	39.3	-1.9	-1.0	-0.9	-0.7	-0.4	9.7	9.8	9.4	4.6	7.5
2	25	42.9	42.8	44.7	41.4	39.5	-2.0	-0.9	-0.8	-0.5	-0.2	9.1	10.3	9.3	5.3	7.3
5	25	42.8	41.6	44.6	41.2	39.8	-1.9	-1.2	-0.9	-0.6	-0.5	9.0	8.7	7.8	4.3	6.6
10	25	42.5	44.3	45.2	40.8	39.4	-2.0	-0.8	-0.5	-0.4	-0.1	8.9	10.1	8.6	5.2	7.1
1 mM EDTA + 25 mM Cysteine + 2.5 mg Papain/mL																
0	4	43.9	42.1	43.5	43.2	41.9	-1.8	-1.5	-1.3	-1.2	-1.3	10.2	10.4	11.1	9.2	9.9
2	4	45.1	43.3	43.2	42.8	43.3	-1.7	-1.3	-1.4	-1.1	-1.2	11.4	11.5	10.6	8.0	9.6
5	4	44.8	45.0	44.4	43.7	42.3	-1.7	-1.3	-1.3	-1.2	-1.2	11.0	11.0	10.3	8.9	9.7
10	4	45.4	44.3	44.3	42.5	41.1	-1.7	-1.3	-1.2	-1.2	-1.2	11.1	10.8	11.9	8.6	9.4
0	25	43.9	40.4	43.5	40.9	39.1	-1.8	-0.6	-0.2	-0.2	0.0	10.2	9.8	10.1	6.5	8.2
2	25	45.1	41.4	43.8	41.6	40.5	-1.7	-0.6	-0.2	-0.1	0.4	11.4	10.2	10.4	6.6	9.1
5	25	44.8	40.8	43.5	41.1	40.3	-1.7	-0.6	0.0	0.1	0.2	11.0	9.7	11.2	6.4	8.5
10	25	45.4	40.6	42.2	41.0	40.1	-1.7	-0.6	-0.1	0.0	0.0	11.1	10.5	10.0	5.7	8.3

Table 4: Hunter L*, a*, and b* values of incubation-UHP treated Granny Smith apple homogenates during storage at 4 or 25 °C for 28 d.

HHP (min)	Storage °C | days	Hue 0	7	14	21	28	Chroma 0	7	14	21	28
Control											
0	4	63.0	63.4	65.3	69.3	68.3	14.4	12.5	14.5	13.0	11.9
2	4	65.2	63.6	65.8	67.7	67.2	14.4	15.8	16.0	13.5	11.8
5	4	65.9	66.3	66.2	68.0	67.6	14.7	10.5	12.9	12.7	10.3
10	4	66.6	63.1	68.0	68.6	66.6	12.1	15.3	12.3	12.2	10.1
0	25	63.0	71.3	69.3	66.4	69.6	14.4	12.6	12.4	12.0	11.6
2	25	65.2	70.0	68.9	66.8	69.6	14.4	13.3	15.3	10.5	13.3
5	25	65.9	70.7	68.0	67.0	70.5	14.7	13.5	16.7	10.1	13.5
10	25	66.6	70.7	69.4	68.4	70.0	12.1	11.6	14.2	10.6	14.1
2.5 mg Papain/mL											
0	4	64.7	66.6	68.4	71.1	70.6	14.7	14.4	15.0	14.6	12.8
2	4	64.4	65.8	68.9	69.8	69.4	15.2	16.2	14.4	15.1	12.5
5	4	64.9	67.6	68.2	70.0	69.4	14.7	13.7	13.3	13.7	14.1
10	4	65.5	66.9	68.8	70.1	70.4	14.8	14.8	14.6	14.2	14.1
0	25	64.7	70.8	71.0	71.2	72.7	14.7	15.2	17.5	14.1	14.4
2	25	64.4	70.7	72.2	69.7	72.1	15.2	15.1	15.6	11.0	15.2
5	25	64.9	70.8	72.7	70.2	72.7	14.7	13.9	15.5	10.6	14.9
10	25	65.5	69.9	71.7	68.8	72.1	14.8	15.9	15.6	10.2	15.6
1 mM EDTA											
0	4	65.8	64.5	66.3	65.8	67.5	12.2	13.3	12.4	11.2	13.3
2	4	65.1	65.3	67.7	68.0	67.9	12.0	12.1	13.3	12.4	14.1
5	4	64.0	66.3	68.0	67.0	69.6	11.8	11.8	13.6	11.5	14.6
10	4	65.5	67.8	67.8	65.9	69.7	13.5	10.4	13.9	11.1	14.2
0	25	65.8	67.6	69.4	67.7	68.1	12.2	11.8	14.7	10.7	12.9
2	25	65.1	68.7	71.8	67.6	71.5	12.0	11.6	14.7	11.2	15.0
5	25	64.0	68.5	71.9	70.1	70.9	11.8	14.9	14.1	12.0	15.1
10	25	65.5	68.8	71.7	69.0	70.7	13.5	13.4	14.4	11.4	15.3
1 mM EDTA + 2.5 mg Papain/mL											
0	4	65.9	67.0	70.5	70.2	71.5	14.5	14.7	14.2	12.1	16.0
2	4	67.6	68.5	70.1	69.4	70.7	13.1	13.7	13.8	13.5	16.1
5	4	67.2	72.9	69.7	69.3	70.5	14.0	12.9	15.1	12.1	16.3
10	4	67.6	72.3	70.1	69.9	71.3	13.9	13.1	13.5	13.0	15.8
0	25	65.9	71.1	73.5	71.5	72.2	14.5	15.0	15.9	12.3	16.9
2	25	67.6	72.0	73.1	70.0	72.0	13.1	15.8	15.9	11.5	16.1
5	25	67.2	70.9	72.6	70.2	71.8	14.0	14.5	16.0	12.0	15.5
10	25	67.6	71.3	71.8	69.9	71.9	13.9	16.2	17.0	12.0	14.5
25 mM Cysteine											
0	4	100.7	99.6	103.4	101.6	100.5	11.2	10.6	9.7	9.0	7.7
2	4	101.3	101.6	101.5	102.2	100.4	11.0	9.7	9.8	8.5	9.1
5	4	103.1	100.9	103.3	101.8	100.8	9.9	10.2	9.1	8.4	9.1
10	4	101.3	99.0	100.3	102.1	101.5	10.7	12.0	9.2	7.6	8.6
0	25	100.7	99.4	99.4	96.1	95.7	11.2	8.7	8.2	7.4	6.7
2	25	101.3	96.2	99.1	94.4	94.3	11.0	9.6	7.3	7.0	7.5
5	25	103.1	96.9	101.6	94.1	96.1	9.9	9.7	7.0	6.5	6.2
10	25	101.3	96.2	98.0	94.6	95.6	10.7	10.5	8.7	6.7	6.5
25 mM Cysteine + 2.5 mg Papain/mL											
0	4	101.9	102.0	99.2	101.4	100.5	10.2	9.3	10.7	8.4	9.1
2	4	102.5	100.5	99.5	99.1	98.3	10.0	10.4	9.8	8.8	10.3
5	4	103.0	99.4	98.8	99.2	98.4	9.3	10.5	10.5	9.2	10.8
10	4	99.5	98.0	99.3	98.8	98.1	11.3	11.6	9.8	9.2	10.5
0	25	101.9	94.4	93.8	92.0	87.4	10.2	10.5	9.3	5.3	8.4
2	25	102.5	93.6	94.5	89.7	87.6	10.0	10.9	8.3	6.1	8.7
5	25	103.0	94.1	92.2	89.2	86.6	9.3	10.4	9.7	5.6	9.0
10	25	99.5	93.8	91.7	89.5	87.4	11.3	10.5	10.2	6.1	8.9
1 mM EDTA + 25 mM Cysteine											
0	4	100.9	99.4	100.6	100.5	98.7	9.9	10.1	9.5	7.7	9.7
2	4	102.3	98.2	100.0	102.0	99.0	9.3	10.4	9.6	7.2	10.0
5	4	101.9	99.8	99.8	99.4	98.2	9.2	9.2	9.9	8.2	10.1
10	4	102.6	100.4	100.0	101.2	100.0	9.1	9.4	8.7	7.9	9.7
0	25	100.9	96.1	95.7	98.2	93.2	9.9	9.8	9.5	4.7	7.5
2	25	102.3	95.2	95.0	95.4	91.8	9.3	10.4	9.4	5.3	7.3
5	25	101.9	98.1	96.9	97.9	94.3	9.2	8.7	7.8	4.3	6.7
10	25	102.6	94.7	93.2	94.0	90.6	9.1	10.2	8.6	5.2	7.1
1 mM EDTA + 25 mM Cysteine + 2.5 mg Papain/mL											
0	4	100.2	98.4	96.6	97.2	97.4	10.4	10.5	11.2	9.3	9.9
2	4	98.4	96.4	97.3	97.6	96.9	11.5	11.5	10.7	8.1	9.7
5	4	99.0	96.9	97.4	97.8	97.1	11.2	11.1	10.4	9.0	9.8
10	4	98.6	96.8	95.8	98.2	97.5	11.2	10.8	12.0	8.7	9.5
0	25	100.2	93.6	91.0	92.1	89.9	10.4	9.8	10.1	6.5	8.2
2	25	98.4	93.5	91.3	90.8	87.8	11.5	10.2	10.4	6.6	9.1
5	25	99.0	93.4	90.2	89.0	88.4	11.2	9.8	11.2	6.4	8.5
10	25	98.6	93.4	90.8	89.6	89.9	11.2	10.5	10.0	5.7	8.3

Table 5: Hue angle (°) and Chroma of incubation-UHP treated Granny Smith apple homogenates during storage at 4 or 25 °C for 28 d.

CHAPTER 5

Firmness of Strawberry Fruit: Combined Mild Heat and Ultra High Pressure Treatments

ABSTRACT

Pectin methylesterase (PME) exhibits greater stability under elevated pressure and temperature conditions compared to equivalent pressures or temperatures alone. Combined mild heat and ultra high pressure (UHP) treatments of strawberries were examined to improve firmness of strawberry fruit attributed to endogenous strawberry PME activity and exogenous $CaCl_2$. A mild heat treatment was conducted prior to UHP treatment by covering strawberries with water or selected concentrations of $CaCl_2$ solution at 60 °C in polyethylene bags. Strawberries were immersed in blanching water at 60 °C for 6 min, filled into polyethylene bags, and covered with water or selected concentrations of $CaCl_2$ solution at 60 °C were also exposed to UHP treatment. Strawberry PME activity was stable during UHP treatments at 600 MPa and an initial pressure media temperature of 23 °C for 10 min regardless of mild heat treatments. Firmness of strawberries covered with heated or non-heated syrups prior to pressurization decreased after the UHP treatments and was attributed to physical damage of tissues by UHP and insufficient endogenous PME activity to form calcium

bridges. However the firmness loss of strawberry tissues after UHP treatments was inhibited as concentrations of $CaCl_2$ in syrups increased to 0.5, 1.0, or 1.5%. Strawberries preheated in blanching water and covered with heated syrups prior to UHP treatments exhibited significant decreases in firmness after UHP treatments regardless of selected concentrations of $CaCl_2$ in syrups. Strawberry fruit firmness was susceptible to the combined mild heat treatments including blanching of strawberries followed by UHP treatments resulting in cell wall disintegrations by heat and pressure. UHP treatment of 600 MPa was a potential processing method to enhance UHP treated strawberries at ambient temperature by the addition of $CaCl_2$ to syrups.

INTRODUCTION

Pectic substances, the main cell wall components of fruits and vegetables together with cellulose and hemicellulose, are located in the middle lamella playing a role of “glue” to adhere one cell wall to another. Pectic substances include two groups, pectic acid and pectin. Pectic acid is homogeneous composed of galacturonic acid linked by α-1-4 glycoside bonds. On the other hand, pectins are heterogeneous polysaccharide complexes naturally present in fruits and vegetables. Pectin is composed of galacturonic acid linked by α-1-4 glycoside bonds as well; however some of carboxyl groups at the carbon-6 position of galacturonic acid are methyl esterified. Pectins are also characterized by the presence of other natural sugars such as

galactose, glucose, rhamnose, arabinose, and xylose in both main and side chains of the molecules. The degree of methyl esterification in pectin molecules varies depending on plant source and maturity.

Pectin methylesterase (PME) is an endogenous enzyme in plants to catalyze deesterification of carboxyl groups in pectin molecules yielding galacturonic acid and methanol. The resulting galacturonic acid in pectin molecules are potential substrates for polygalacturonase (PG). PG is also an endogenous enzyme that catalyzes hydrolytic cleavage of α-1-4 glycoside bonds between galacturonic acid residues, resulting in shorter pectin chains and tissue softening of fruits and vegetables. Deesterifying action of PME on pectin molecules yields not only substrates for PG, but also for subsequent reactions in the presence of divalent cations. Carboxyl groups located at the carbon-6 position of galacturonic acid residues between adjacent pectin chains are linked by way of divalent cations such as calcium to form calcium bridges, increasing tissue firmness (Whitaker 1996).

Strawberries (*Fragaria ananassa*) are highly perishable fruits exhibiting rapid softening during maturation and postharvest handling and storage. Although it is important to elucidate the mechanism of strawberry fruit softening, the contributions of pectin degradation involving PME and PG to strawberry tissue softening are not well documented.

Pectic substances of strawberries account for approximately 50% of cell wall materials by dry weight (Legentil and others 1995). Both PME and PG activities are

present in strawberry fruit. After the investigations of pectic enzyme activity, pectin composition, and firmness during the ripening stage of twelve strawberry cultivars, Lefever and others (2004) concluded that the increase in PME activity exhibited potential as an indicator to screen strawberry maturity relative to the decrease in fruit firmness and the increase in water-soluble pectin concentration. Nogata and others (1993) reported that the predominant strawberry exo-PG activity decreased gradually as the ripening stage proceeded from small green to overripe. Moreover, Redondo-Nevado (2001) pointed out that a transcript level expression of endo-PG genes was detected in strawberry fruit only at very beginning of the ripening stage. Therefore, strawberry PG may play insignificant roles in fruit softening, while strawberry PME is potentially a contributor to maintenance of cell wall integrity by yielding galacturonic acid residues resulting in the reaction with intrinsic divalent cations and the formation of calcium bridges (Castillejo and others 2004).

Instead of the role of PG, the presence of pectate lyase was suggested as an important contributor to cell wall pectin degradation of strawberries (Medina-Escobar and others 1997; Jimenez-Bermudez and others 2002). In addition, involvements of other enzymes, such as β-galactosidase (Trainotti and others 2001), endo-β-(1, 4)-glucanase (Woolley and others 2001; Palomer and others 2004), and β-xylosidase (Martinez and others 2004) in the degradation of cell walls in strawberry fruit were studied in relation to gene expression during the ripening stages.

Endogenous or exogenous PME activity together with divalent cations is utilized to for the purpose of texture improvement of strawberry fruit. Immersion of strawberries in 1% $CaCl_2$ solution at 45 °C for 15 min prior to storage at 18 °C reduced postharvest decay due to the retention of firmness and soluble solid concentrations (Garcia and others 1996). Immersion of strawberries with 1% $CaCl_2$ and fungal PME under the vacuum conditions resulted in the production of frozen strawberries suitable for jam manufacturing, exhibiting a firm texture after thawing (Suutarinen and others 2002a; Suutarinen and others 2002b). Compared to passive osmosis and pressure-assisted infusion methods, vacuum method effectively promoted the penetration of exogenous PME and $CaCl_2$ into strawberry tissues resulting in the production of strawberries possessing 70% of initial raw strawberry firmness after subsequent ultra high pressure (UHP) treatment at 400 to 550 MPa (Duvetter and others 2005).

UHP processing is one of the nonthermal technologies proposed as an alternative to conventional thermal processing in the food industry. The aim of UHP is the shelf life extension of foods by inactivating pathogenic and spoilage microorganisms, and detrimental enzymes. UHP processing is also regarded as a potential technology for preserving high quality food and reducing undesirable changes in flavors, nutrients or color during processing, storage and distribution that occur during thermal preservation of food.

UHP treatment of strawberries results in preservation of naturally occurring vitamin C (Sancho and others 1999) and anthocyanins (Zabetakis and others 2000; Gimenes and others 2001) during processing and storage. UHP treatment at 600 MPa or more for 15 min inactivated 99% of polyphenol oxidase (PPO) in strawberry homogenates (Garcia-Palazon and others 2004). Strawberry PME was pressure resistant as demonstrated by the smaller inactivation kinetic constant *k* at 600 MPa and 10 °C than the constant *k* at atmospheric pressure and 54 °C (Ly-Nguyen and others 2002).

The effects of UHP treatment on activities of tomato PG and PME are intensively studied. At the treatment temperature of 45 °C, PG in diced tomatoes was effectively inactivated by UHP treatment at 600 MPa, while PME was activated during pressure treatment at 400 MPa and was scarcely inactivated at 600 or 800 MPa (Shook and others 2001). UHP treatment at 100 to 600 MPa under elevated temperature at 60 to 75 °C exhibited antagonistic effects on inactivation of tomato PME compared to the UHP treatment at ambient temperature (Crelier and others 2001). The presence of a heat stable fraction of tomato PG to thermal treatment at 55 to 70 °C and the absence of a pressure stable fraction of tomato PG to UHP treatment at 350 to 500 MPa were demonstrated (Fachin and others 2003). UHP treatment at 500 MPa and 25 °C for 15 min resulted in significant inactivation of both heat stable PG I and heat labile PG II exhibiting identical behaviors, potentially attributed to the

rapid dissociation of a β-subunit from PG I to form PG II under pressurized conditions (Peeters and others 2004).

Both heat labile and stable PME fractions were identified in orange juice from kinetic studies (Chen and Wu 1998). In addition, a pressure stable fraction of PME present in orange juice was scarcely inactivated by UHP treatment at 900 MPa and ambient temperature (Nienaber and Shellhammer 2001). The heat stable fraction of orange PME was stabilized by UHP treatment at 600 MPa combined with elevated temperature at 70 or 80 °C, while the stable fraction of orange PME was inactivated at atmospheric pressure by thermal treatment at 73 °C or more (Van den Broeck and others 2000).

The objective of this study is to combine the application of mild heat and UHP treatments to improve the firmness of preserved strawberries utilizing endogenous PME activity and promoting exogenous $CaCl_2$ penetration into strawberry tissues.

MATERIALS AND METHODS

Materials

Strawberries were purchased at a local market in Pullman, WA. Strawberries cut into four wedges were used in all experiments. Pectin (P-9135, methoxy content 9.4%) and calcium chloride dihydrate (C-5080) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chloride (S271-3) was purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water was used for the preparation of solutions.

Strawberry PME assay conditions

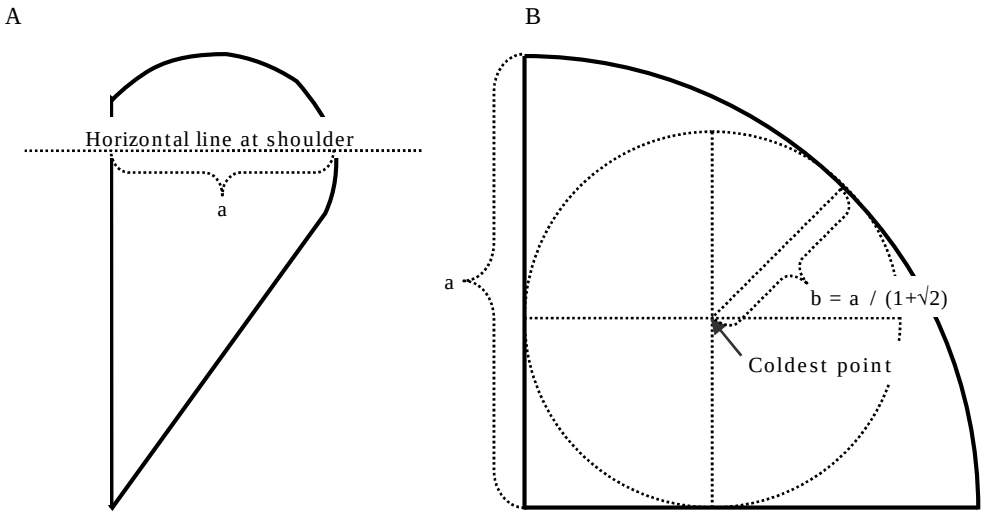
Approximately 100 g of strawberry wedges were blended with an equivalent weight of water at 4 °C in a commercial Waring blender for 1 min. The resulting homogenate was poured into a glass bottle and kept at 4 °C until analyses. PME activity was determined titrimetrically based on the method of Anthon and others (2002) modifying the amount of crude enzyme solutions added to pectin substrate solutions. Substrate solutions containing 1% pectin and selected concentrations of NaCl and $CaCl_2$ were heated to temperature of 30 °C and adjusted to pH 7.0 by adding 0.01 N NaOH prior to assays of strawberry homogenates. After the addition of 2.00 g of the strawberry homogenate to 30 mL of the pectin substrate solution, 0.005 N NaOH was manually titrated to maintain the pH of the reaction mixture at 7.00. The titrated volume of 0.005 N NaOH was recorded periodically during the reaction or at 10 min of reaction time. A blank assay was carried out by assaying strawberry homogenates filled into capped test tubes and heated in boiling water at temperature above 95 °C for 20 min. One PME activity unit was defined as the activity releasing one microequivalent of H^+ during reaction for 10 min.

Strawberry tissue blanching condition

The coldest point of strawberry wedges during blanching was geometrically determined (Figure 1). One thermocouple was placed at the cold point considering the radii of the wedge arc (b) from outer surface of the strawberry in Figure 1, and another was immersed in water bath maintained at 60 °C. Following the immersion of

the strawberry wedge in water, temperatures were determined at 0.1 min intervals for 6.7 min. The temperature determinations were repeated in triplicate. The mean weight of strawberry wedges was 6.31 ± 0.29 g and the mean radius of strawberries at shoulder (the value of 'a') was 18.27 ± 0.64 mm.

Figure 1: Cut surfaces of a strawberry wedge (A; vertical) and at shoulder (B; horizontal).



Strawberry preparation for UHP treatments

Strawberries were tempered at 23 °C for 3 h to equilibrate the fruits to ambient temperature. Approximately 55 g (8 ~ 9 strawberry wedges) were selected for each analysis. Polyethylene bags (12 × 8 cm) were used as containers for UHP treatment.

There were three experimental groups depending on heat treatments of strawberry wedges before UHP treatment. Group A strawberry wedges were placed in

polyethylene bags, a two-fold weight of water or selected concentrations of 0.5, 1.0, or 1.5% $CaCl_2$ solutions at 23 °C was added, and the bags were heat sealed. A two-fold weight of water or selected $CaCl_2$ solutions heated to 60 °C was poured into the bags identified as group B. Strawberry wedges in group C were immersed in water at 60 °C for 6 min prior to placing in the bags, and a two-fold weight of water or selected $CaCl_2$ solutions heated to 60 °C were poured into the bags.

UHP treatments of strawberry wedges

Strawberry wedges in groups A, B, and C in sealed polyethylene bags were exposed to UHP treatment in an isostatic pressing system (Engineered Pressure Systems, Inc., Andover, MA) at 600 MPa and initial temperature of 24 °C for selected dwelling times of 0, 2, 5, and 10 min. The come-up time to reach 600 MPa was 230 ± 3 s and designated as "0 min" dwelling time were prepared by decompressing the pressure vessel immediately after the come-up-time.

Temperatures of dipping solutions and strawberry wedges at the cold points were determined in triplicate before and after UHP treatment of strawberry wedges in groups A, B, and C.

Weight of strawberries after UHP treatments

Weights of raw and processed strawberry wedges were determined to examine weight gain or loss resulting from UHP treatment. After UHP treatment, the strawberry wedges were drained in a sieve, and excess dipping solutions removed by rolling the strawberry wedges on triple-layered paper towels for 1 min. The difference

of weights of strawberry wedges before and after UHP processing was expressed in percentage based on the weights recorded before processing.

Color determination of UHP treated syrups

After drainage through a sieve, syrups were filtered through Whatman No. 1 paper filter to remove fine particles. Absorbance at 500 nm of each solution was determined with a 8452A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) using water as a blank.

PME assay

Strawberry homogenates were prepared by blending UHP treated strawberry wedges and cold water in a Waring blender for 1 min. Regardless of weight gain or loss of strawberry wedges after UHP treatments, the weight of cold water added to the blender was adjusted so that total homogenate weight was twice (2×) the weight of raw strawberry wedges. Pectin substrate solutions for strawberry PME assay contained 1% pectin, 0.2M NaCl, and 0.5% $CaCl_2$. The determinations of PME activity in raw strawberries and UHP treated strawberries were conducted in four replicates following the protocol of titration described previously. Strawberry PME activity after UHP treatments was expressed as percent residual activity using PME activity in raw strawberries as 100%.

Firmness

Firmness of raw strawberry wedges and UHP treated strawberry wedges were determined with a TA.TX2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) at room temperature following the method of Anthon and others (2005). A cork borer with 9 mm inside diameter was horizontally inserted into strawberry wedges from the arc center of outer surface of the strawberry shoulder to the line formed by two cut surfaces of the wedge. The resulting cylinders of strawberry tissues were 10 mm in length and placed on a plate of the texture analyzer with the outer side of the tissue upward. The cylinders were compressed with a 25 mm radius probe (TA-25) to 75% strain at a rate of 1 mm/s. The maximum force in Newtons during compression was expressed as tissue firmness. Texture determinations were carried out in six replicates for each process.

Statistical analysis

Analysis of variance (ANOVA) of the effect of experimental conditions on residual strawberry PME activity and firmness were performed using Minitab Release 14 software (Minitab Inc., State College, PA). Significant differences are defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Determination of strawberry PME assay conditions

After the addition of strawberry homogenates to 1% pectin substrate solutions, the volume of 0.005 N NaOH titrated to maintain the pH of reaction mixtures to 7.00 is proportional to the amount of hydrogen ions produced by strawberry PME-catalyzed pectin degradation. No alkali was needed to maintain a pH of 7.00 after 3 min of reaction time in the absence of NaCl, whereas the plots of 0.005 N NaOH volumes titrated in the presence of NaCl at concentrations of 0.1 to 0.5 M exhibited linear increases for 30 min (Figure 2). Larger volumes of titrant were required during 30 min of strawberry PME catalyzed pectin degradation as concentrations of NaCl in the pectin substrate solutions increased. The slopes exhibiting the increasing rates of titrant volume over reaction times from initiation of the reaction to 10 min were correlated to NaCl concentrations from 0.1 to 0.5 M with correlation coefficient $r^2 = 0.977$. On the other hand, the titrant volumes titrated for 10 min of reaction time were correlated to NaCl concentrations from 0.1 to 0.5 M with correlation coefficient $r^2 = 0.993$. Consequently, strawberry PME activity increases as NaCl concentrations increase in the range from 0.1 to 0.5 M in pectin substrate solutions.

Acceptable tomato PME activity is routinely determined using 1% pectin solutions containing 0.2 M NaCl (Anthon and others 2002). Papaya and acerola PME activities are determined using 1% pectin substrate solutions containing 0.15 M NaCl

(Fayyaz and others 1995; Assis and others 2000). Although the substrate pectin solutions contained 0.15 M NaCl, the largest activity yield of soursop PME was achieved with extraction buffer containing 1.92 M NaCl (Arbaisah and others 1997). The presence of salt increases the ionic strengths of pectin substrate solutions, potentially promoting the solubilization of membrane-bound PME molecules and increasing reactivity of PME with water soluble pectins. The large ionic strength in pectin substrate solutions may result in the activation of soluble PME attributed to open configuration of the PME molecule. In addition to NaCl, the presence of $CaCl_2$ in both strawberry homogenates and pectin substrate solutions was hypothesized to increase PME activity determined by the titrimetric method.

PME activity in strawberry homogenates determined in pectin substrate solutions containing 0.2 M NaCl and 0.5% $CaCl_2$ was equivalent to PME activity determined in pectin substrate solutions containing only 0.2 M NaCl (control) (Figure 3). The addition of 0.5% $CaCl_2$ to pectin substrate solutions containing 0.2 M NaCl did not increase strawberry PME activity.

While fixing the NaCl concentration at 0.2 M in pectin substrate solutions, PME activity in strawberry homogenates containing 1.0% $CaCl_2$ determined in pectin substrate solutions without $CaCl_2$ was significantly smaller ($p \leq 0.05$) than PME activities in the same homogenates determined in pectin substrate solutions containing 0.5% $CaCl_2$, PME activity of control, or PME activity in strawberry

homogenates determined in pectin substrate solutions containing 0.5% $CaCl_2$ (Figure 3).

In a preliminary experiment, strawberry PME exhibited the greatest activity in pectin substrate solutions containing 0.5% $CaCl_2$ together with 0.2 M NaCl. A gradual decrease in PME activity was observed in pectin solutions containing concentrations of 1.0% and 1.5% $CaCl_2$. The decrease in strawberry PME activity in the presence of large concentrations of $CaCl_2$ is attributed to a general property of proteins including enzymes that are insolubilized and potentially denatured in solutions containing large concentrations of salts (Damodaran 1996). The activity of insolubilized or denatured strawberry PME in homogenates containing 1% $CaCl_2$ was smaller in pectin substrate solutions containing only 0.2 M NaCl compared to the activity determined in pectin substrate solutions containing 0.2 M NaCl and 0.5% $CaCl_2$ (Figure 3). The reduction in strawberry PME activity observed upon the addition of 1.0% $CaCl_2$ to homogenates was reversible by readjusting $CaCl_2$ concentration at 0.5% in pectin substrate solutions.

Our study involved the UHP treatments of strawberries surrounded in two-fold weights of water or solutions containing $CaCl_2$ at concentrations of 0.5, 1.0, or 1.5% in polyethylene bags. The application of UHP to strawberries covered with $CaCl_2$ solutions is one of the potential methods to promote diffusion and absorption of calcium into strawberry tissues (Duvetter and others 2005). Assuming the concentration of $CaCl_2$ approaches equilibrium between strawberry tissues and 1.5%

$CaCl_2$ solution after UHP treatments, PME activity in strawberry homogenates containing 1% $CaCl_2$ is determined with the assay using pectin substrate solutions containing 0.2 M NaCl and 0.5% $CaCl_2$.

Blanching conditions and strawberry PME activity

Starting at room temperatures of 18 °C, the coldest point of strawberry wedges (Figure 1) reached mean temperatures of 57.8 ± 0.4 °C and 58.8 ± 0.3 °C after blanching treatment conducted by immersion of strawberry wedges in water at 60 °C for 5 and 6 min, respectively (Figure 4). The blanching time of 6 min was selected to distribute strawberry tissue temperature uniformly to approximately 60 °C for UHP experiments.

There were no significant differences in strawberry PME activities determined before or after the blanching treatments, indicating adequate stability of PME in strawberry wedges immersed in water at 60 °C for 6 min (Figure 5). Ly-Nguyen and others (2002) reported heat treatments at temperature ranging from 54 to 63 °C for prolonged times up to 500 min inactivated heat-labile fractions of purified strawberry PME. The blanching treatment to resulting in nearly uniform temperature distribution of 60 °C to strawberry wedges (Figure 4) did not inhibit strawberry PME activity attributed to the short heating time for 6 min. Balogh and others (2004) observed greater heat stability of PME in diced carrots compared to purified carrot PME or PME in carrot juice. PME in strawberry wedges may also be more stable to heat than purified strawberry PME.

The blanching treatments of strawberry wedges in presence of $CaCl_2$ inhibited PME activity in strawberry wedges very little (Figure 5). The mean weight gain of strawberry wedges heated in blanching solutions was 3.36 ± 1.30% for the five treatments based on the initial weights of strawberry wedges. The absorption of $CaCl_2$ occurring during the blanching treatment of strawberry wedges was reflected in the weight gains. Van den Broeck and others (1999) observed increased thermal inactivation of orange PME at temperatures ranging from 45 to 67 °C upon addition of 0.5 to 1.5 M $CaCl_2$ to the PME solutions. Anthon and others (2005) reported larger PME activity in tomato slices during incubation in 0.5% $CaCl_2$ at 65 °C for 10 to 50 min compared to PME activity in tomato slices incubated in water at equivalent temperature. The trend observed in our results exhibiting smaller strawberry PME activities after the blanching treatment as $CaCl_2$ concentrations in blanching solutions increased indicated little stability of strawberry PME in the presence of $CaCl_2$ under elevated temperature conditions.

Temperature of strawberry wedges and syrups after UHP treatments

A temperature profile in the pressure vessel filled with pressure medium (5% Mobil Hydrasol 78) during pressurization at 675 MPa for 10 min was characterized by adiabatic heating of 12.6 °C during the come-up-time for 4.5 min, gradual decrease in temperature of 9.7 °C at constant pressure of 675 MPa for 10 min, and rapid decrease in temperature to 8.2 °C associated with decompression of the vessel (Figure 6).

Temperatures of the coldest point of strawberry wedges as well as water surrounding the strawberry wedges in the polyethylene bags determined immediately after UHP treatment were affected by the changes in pressure medium temperature during UHP treatment, UHP dwelling times, and heat transfer between strawberry wedges and water in bags (Figure 7). When strawberries in group A surrounded in water at 21 °C were treated with UHP, the temperatures of strawberry wedges after UHP treatment decreased gradually as UHP dwelling time became longer. Immersed in water at 60 °C before UHP treatment, the temperatures of strawberry wedges in group B increased slightly after UHP treatment for 0 or 2 min, and decreased after UHP treatment for 5 or 10 min. Treated in blanching water at 60 °C for 6 min reaching the temperature at coldest point of strawberry wedges at approximately 60 °C prior to UHP treatment, temperatures of strawberry wedges in group C rapidly decreased to 27.3 °C after decompression of the pressure vessel following the come-up-time, and decreased to 24.9, 21.3, and 19.1 °C after UHP dwelling times of 2, 5, and 10 min, respectively.

Temperatures of water surrounding strawberry wedges for group A and C were nearly identical to the temperatures at the coldest point of strawberry wedges after corresponding UHP treatments of groups A and C, respectively. Little differences in temperatures between strawberry wedges and water were observed after UHP treatment of strawberry wedges in group B for 2 min or longer.

The rapid decreases in temperature of water (group B and C) and strawberries (group C) after UHP treatment for 0 min or longer may result from heat transfer from the large volume of pressure media during decompressing of the pressure vessel.

Strawberry wedges and syrups after UHP treatments

The weights of strawberry wedges in groups A and B increased while the weights of strawberry wedges in group C decreased during UHP treatments (Figure 8). Weight gains of strawberry wedges in groups A and B were attributed to absorption of dipping solutions and $CaCl_2$ during UHP treatments. However, no specific trends in weight gain were observed relative to $CaCl_2$ concentration in the syrups, UHP dwelling times, or initial temperature of dipping solutions. The mean weight gains of strawberry wedges in groups A and B were 9.2 ± 4.6% and 6.1 ± 3.1%, respectively. Strawberry wedges in group C exhibited a mean weight loss of -9.0 ± 3.7%. Since little insoluble particles were visually observed in UHP treated syrups surrounding strawberries in group C, the weight loss of strawberries resulted from elusion of soluble solids from the blanched strawberries to syrups during UHP treatment. Water absorption as well as heat transfer of strawberries during the blanching treatment at 60 °C followed by UHP treatment at 600 MPa possibly promoted cell decompartmentalization of strawberry tissues.

The larger concentration of $CaCl_2$ in syrups resulted in the lower pH of both strawberry homogenates (Table 1) and UHP treated syrups (Table 2) prepared after UHP treatments. The UHP dwelling times did not significantly change the pH of

strawberry homogenates or syrups, indicating nearly complete infusion of UHP treated syrups occurred within the come-up-time of UHP treatments.

UHP treatments at 600 MPa of strawberries in groups A, B, or C covered with syrups in polyethylene bags resulted in orange colorization of UHP treated syrups detected with determinations of absorbance at 500 nm (Table 3). UHP dwelling times did not affect absorbance at 500 nm of UHP treated syrups in groups A, B, or C suggesting the colorization of syrups occurred during the come-up-time of UHP treatment. The large values of absorbance at 500 nm of UHP treated syrups in groups A, B, or C resulting from increased concentrations of $CaCl_2$ in the syrups was attributed to the effect of syrup pH (Table 2) on anthocyanin color (Von Elbe JH and Schwartz SJ 1996). Two predominant anthocyanins in strawberries, pelargonidin 3-glucoside and pelargonidin 3-rutinoside, were retained in the fruit during UHP treatments at 200 to 800 MPa of strawberries vacuum sealed in polyethylene bags (Zabetakis and others 2000). When strawberries dipped in syrups were subjected to UHP treatments, color loss of strawberry fruit is inevitable due to elution of anthocyanins from strawberries to syrups by pressurization. Absorbance at 500 nm of UHP treated syrups in groups B or C containing initial $CaCl_2$ concentrations of 0.5, 1.0, and 1.5% were larger than absorbance at 500 nm of UHP treated syrups in group A containing equivalent concentrations of $CaCl_2$, indicating mild heat treatments designated as preparations of strawberries in groups B and C prior to UHP treatment promoted UHP-induced elution of anthocyanins during UHP treatments.

Strawberry PME activity after UHP treatments

UHP treatment at 600 MPa with initial temperature of 24 °C for 2, 5, or 10 min did not significantly reduce PME activity of strawberries in A, B, or C groups when compared to PME activity after UHP treatments at 600 MPa for the come-up-time (Figure 9).

As a control, PME activities in strawberry wedges dipped in water at 21 °C were not inhibited by UHP treatments at 600 MPa at 23 °C for 10 min. PME activity in orange juice was inhibited by more than 88% after UHP treatment at 600 MPa and 25 °C for 5 min (Nienaber and Shellhammer 2001). Significant inhibition of orange PME after UHP treatment at 600 MPa and ambient temperatures for 5 to 5.8 min is also reported (Truong and others 2002; Boff and others 2003). Tomato PME activities were stable under UHP conditions at 600 MPa and 25 °C for 5 to 15 min (Shook and others 2001; Fachin and others 2002). Our results demonstrating stable PME activity in control strawberries under UHP conditions of 600 MPa are supported the description of Ly-Nguyen and others (2002) that strawberry PME is more pressure resistant than carrot or orange PME at 10 °C.

PME activity in strawberry wedges was also stable during UHP treatment at 600 MPa for 10 min following dipping of the strawberry wedges in syrups at 60 °C (group B) or heated in water at 60 °C for 6 min followed by dipping of strawberry wedges in syrups at 60 °C (group C) prior to UHP treatment. Strawberry PME stability is similar to PME in diced tomatoes that exhibits little decrease in activity during UHP

treatment at 600 MPa and 45 °C for 5 min (Shook and others 2001). Increased inhibition of PME activity during UHP treatment at 600 MPa combined with mild heat up to 65 °C was demonstrated after prolonged treatment of crude and purified PME from oranges (Van den Broeck and others 2000), carrots (Ly-Nguyen and others 2003), and white grapefruit (Guiavarc'h and others 2005).

The selected concentrations of $CaCl_2$ in the dipping solutions did not alter the stability of strawberry PME during 10 min UHP treatments (group A). However, mean residual PME activities in strawberries (group A) during 10 min UHP treatments were decreased ($p \leq 0.05$) as concentration of $CaCl_2$ in dipping solutions increased from 0.5 to 1.5%. Similar trends of smaller residual PME activities in strawberry wedges in groups B and C after UHP treatment for 0 to 10 min were observed when dipped in $CaCl_2$ solutions. Since neither the presence of 1% $CaCl_2$ in strawberry homogenates nor the heat treatment of strawberry wedges at 60 °C for 6 min or did not affect strawberry PME activity determined with pectin substrate solutions containing 0.2 M NaCl and 0.5% $CaCl_2$ (Figures 3 and 5), the inhibition of strawberry PME activity is attributed to inhibitory effect of $CaCl_2$ achieved with infusion of $CaCl_2$ during the come-up-time of the UHP treatment.

Residual PME activities in strawberry wedges in group C after UHP treatment at 600 MPa for 0 to 10 min were smaller than PME activities in strawberry wedges in group A or B dipped in equivalent concentrations of $CaCl_2$ and treated with equivalent UHP conditions. The loss of PME activity in strawberry wedges in group C is

attributed to the blanching treatment at 60 °C for 6 min prior to UHP treatment resulting in elution of PME from strawberry tissue to syrups as well as elution of soluble solids from strawberry wedges during the come-up-time of the UHP treatment (Figure 8).

Firmness of strawberry fruit after UHP treatments

The loss of firmness of strawberry tissues in group A or B resulting from physical damage by UHP treatment was inhibited as the concentrations of $CaCl_2$ in syrups increased (Figure 10). Incubation of diced tomatoes under atmospheric pressure at 23 or 70 °C for 5 min resulted in significant increase in firmness as concentrations of $CaCl_2$ in dipping solutions increased up to 1% (Anthon and others 2005). Our results also demonstrated the significance of $CaCl_2$ concentrations in syrups affecting the formation of calcium bridges between pectin molecules promoted by endogenous strawberry PME activity during UHP treatment of strawberry wedges. However, mild heat treatments conducted by adding syrups heated to 60 °C (group B) did not alter the firmness of strawberry wedges compared to the firmness of controls (group A). In addition, UHP dwelling times did not affect the firmness of strawberry wedges (groups A and B) regardless of $CaCl_2$ concentrations in syrups. Therefore, firmness of strawberry tissues after the come-up-time was retained during the ensuing 10 min UHP treatment at 600 MPa.

Increases in firmness of strawberry tissues were observed when strawberries dipped in solutions containing exogenous PME activity together with $CaCl_2$ were

treated under vacuum pressures (Suutarinen and others 2001a; Duvetter and others 2005). The method to select UHP treatments of 327 MPa at 23 to 47 °C for 10 min for infusion of exogenous PME and $CaCl_2$ to strawberry tissues resulted in firmness of strawberries equivalent to the firmness of raw strawberries after UHP treatments (Duvetter and others 2005). Even though the addition of $CaCl_2$ to syrups contributed to enhancement of strawberry firmness during UHP treatments, our results exhibiting significant decreases in firmness of strawberry wedges were attributed to physical damage of strawberry tissues during UHP treatment at 600 MPa and insufficient endogenous PME activity in strawberries to form calcium bridges involving $CaCl_2$ added to syrups.

The significant losses of tissue firmness of strawberry wedges (group C) heated at 60 °C for 6 min, dipped in syrups heated to 60 °C, followed by UHP treatment at 600 MPa was observed regardless of the concentrations of $CaCl_2$ in syrups (Figure 10). The combined heat and UHP treatment of strawberry wedges may result in considerable cell wall disintegration in strawberry tissues accompanied by elution of soluble solids (Figure 8) and PME activity (Figure 9) from strawberry wedges. The addition of $CaCl_2$ to syrups did not contribute to inhibition of strawberry firmness loss although PME activity was stable during UHP treatments (Figure 9).

CONCLUSIONS

PME activity in strawberry wedges was stable during UHP treatment at 600 MPa and 23 °C for 10 min. The addition of $CaCl_2$ to syrups reduced residual PME activity compared to PME activity in raw strawberries. The addition of $CaCl_2$ to syrups enhanced the firmness of strawberry wedges by UHP treatments as concentrations of $CaCl_2$ increased. The selection of water or $CaCl_2$ solutions heated to 60 °C as syrups did not alter weight gain of strawberry wedges, elution of color pigments, PME stability, or firmness of strawberry wedges after UHP treatments at 600 MPa after an initial temperature of 23 °C for 10 min. Strawberry wedges heated in water at 60 °C for 6 min followed by immersing and packaging in syrups heated to 60 °C exhibited weight loss and significant decrease in tissue firmness after UHP treatments of 600 MPa at an initial temperature of 23 °C for 10 min regardless of concentration of $CaCl_2$ in syrups. Although strawberry PME was stable during UHP treatment at 600 MPa, the combined methods utilizing UHP treatment of strawberry wedges preheated to 60 °C is unlikely to improve firmness of strawberry fruit because of susceptibility of strawberry tissues to combined heat and UHP treatments.

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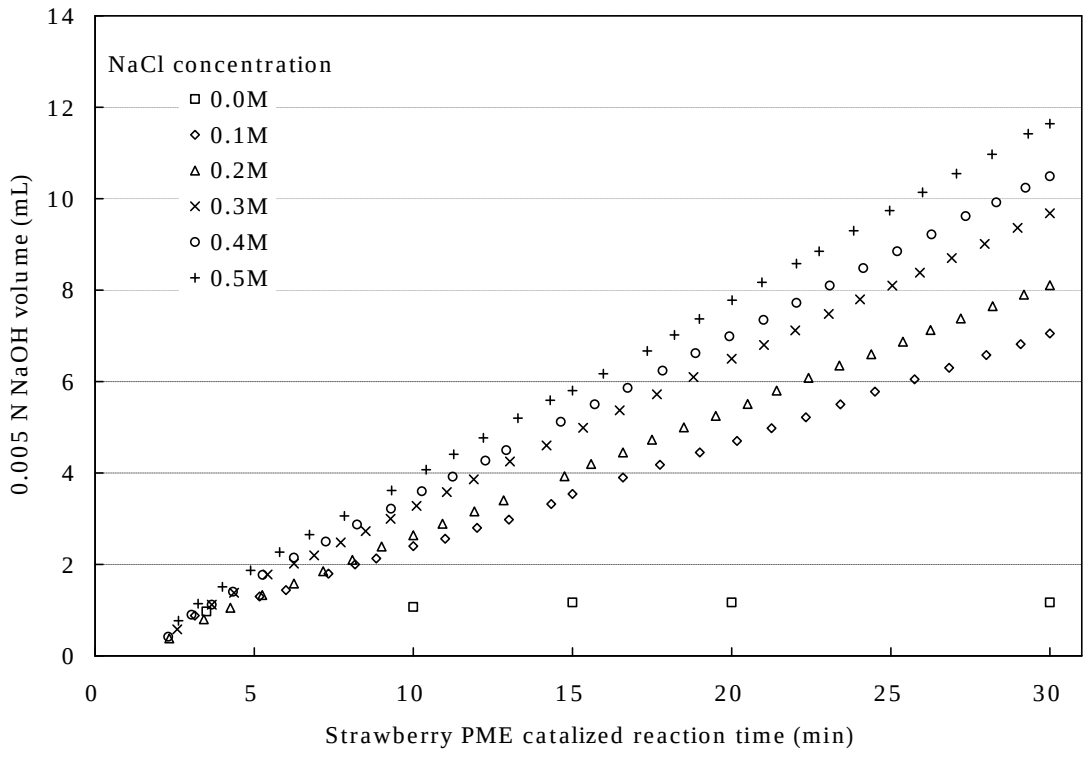


Figure 2: 0.005 N NaOH titration of strawberry homogenates.

Substrate solutions consisted of 1% pectin and selected concentrations of NaCl.

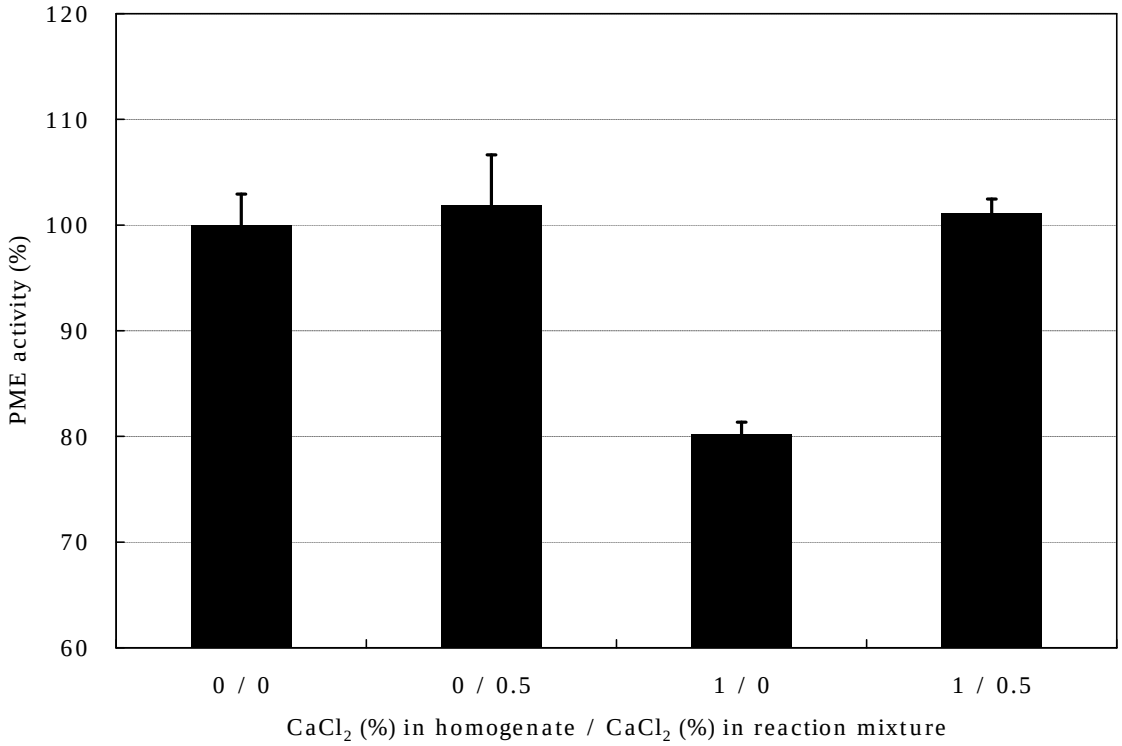


Figure 3: PME activity in strawberry homogenates.

Strawberry homogenates contained 0 or 1% $CaCl_2$. Substrate solutions consisted of 1% pectin, 0.2 M NaCl, and selected concentrations of $CaCl_2$. Each bar presents the + standard deviation range in triplicates.

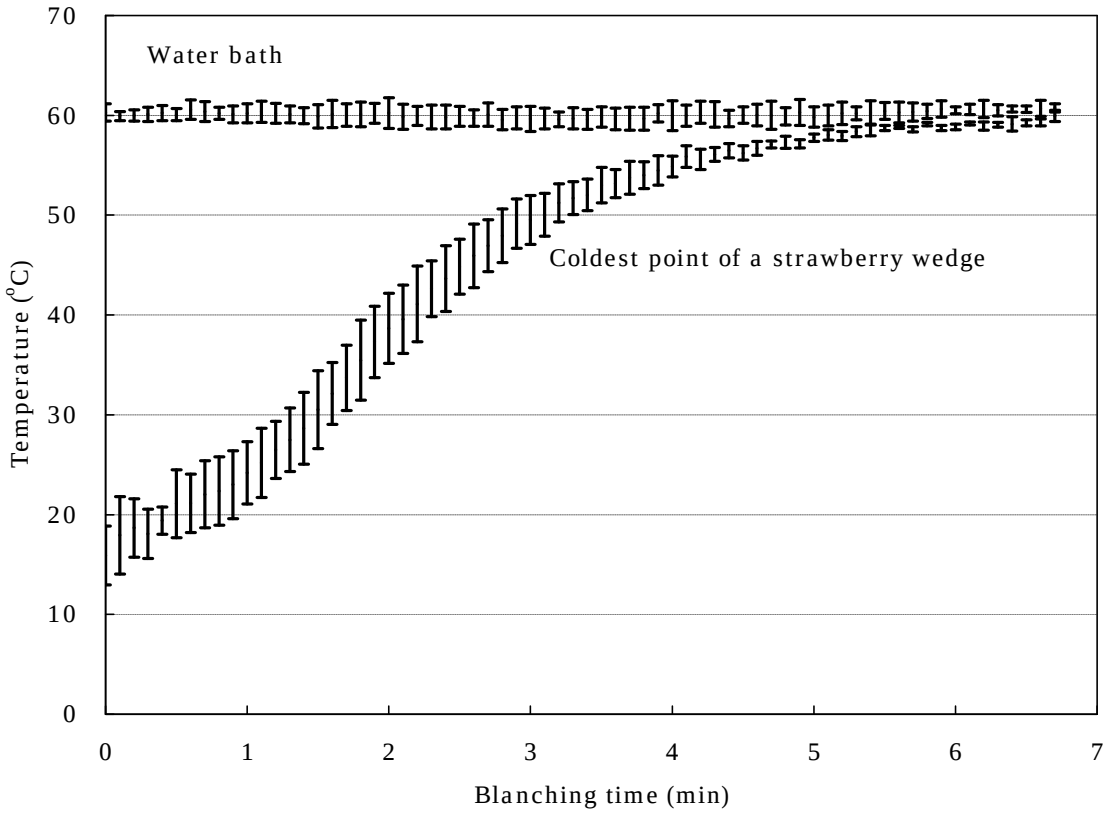


Figure 4: Temperature at coldest point of strawberry wedges during blanching treatment at 60 °C.

Each bar presents the ± standard deviation range in triplicates.

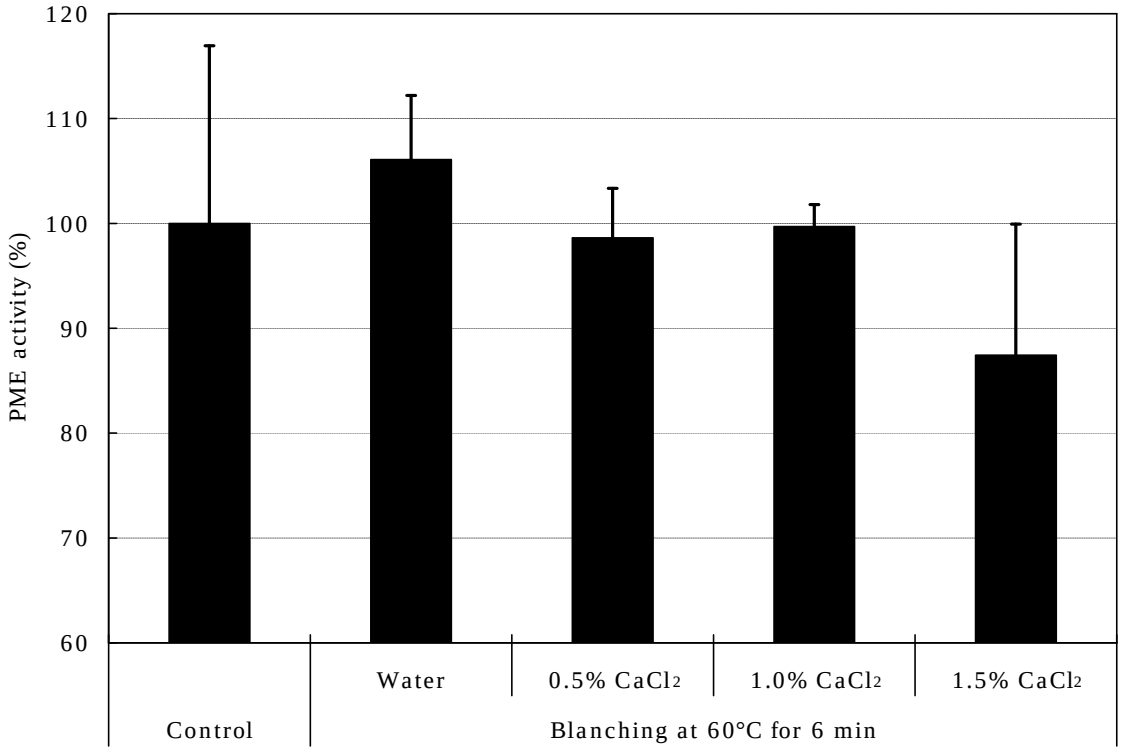


Figure 5: Strawberry PME activity before (control) and after blanching.

Each bar presents the range of + standard deviation in triplicates.

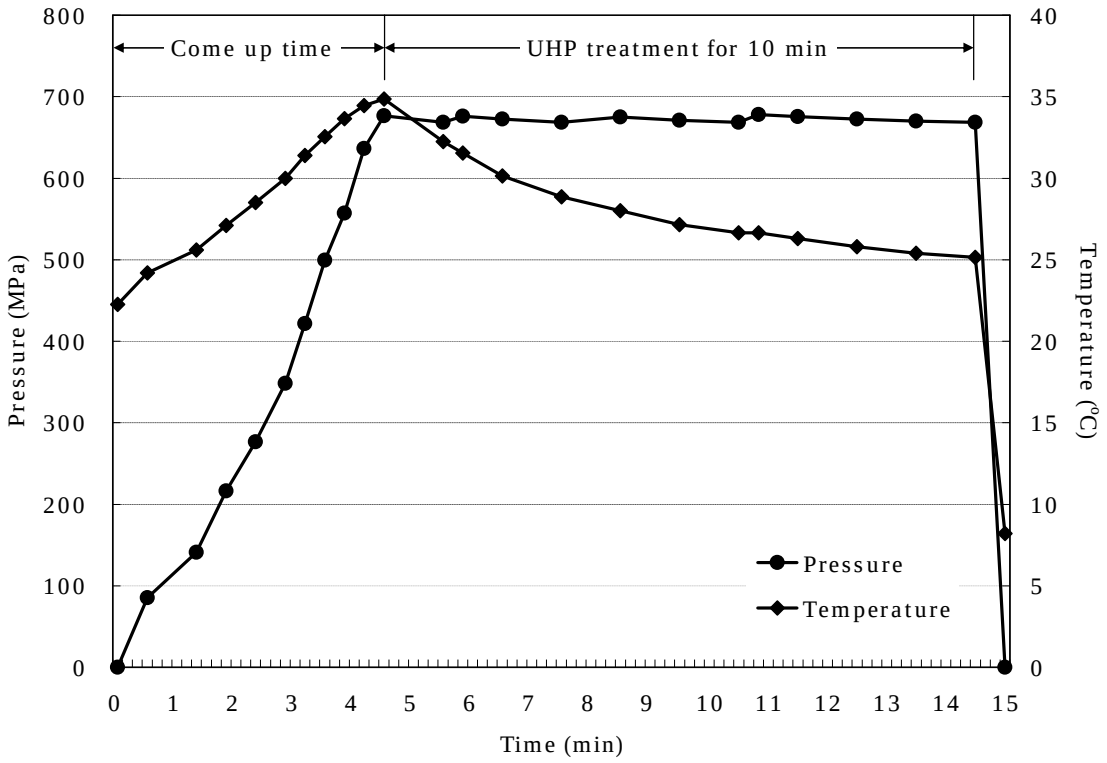
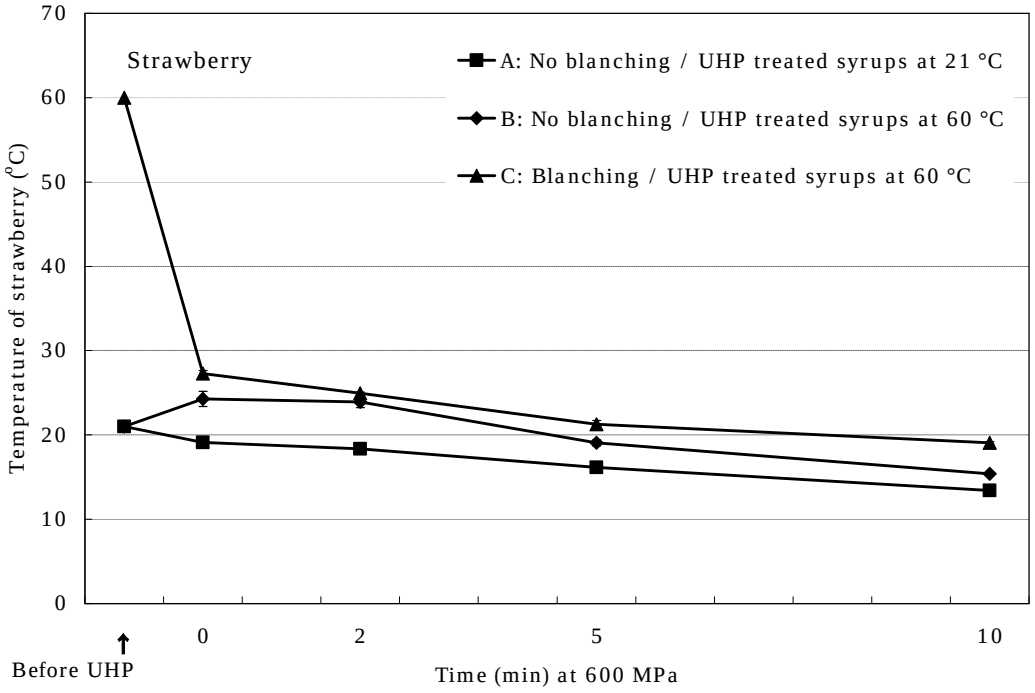


Figure 6: Pressure and temperature profiles of pressure medium (5% Mobil Hydrasol 78) during 10 min-UHP processing at 675 MPa and ambient temperature.



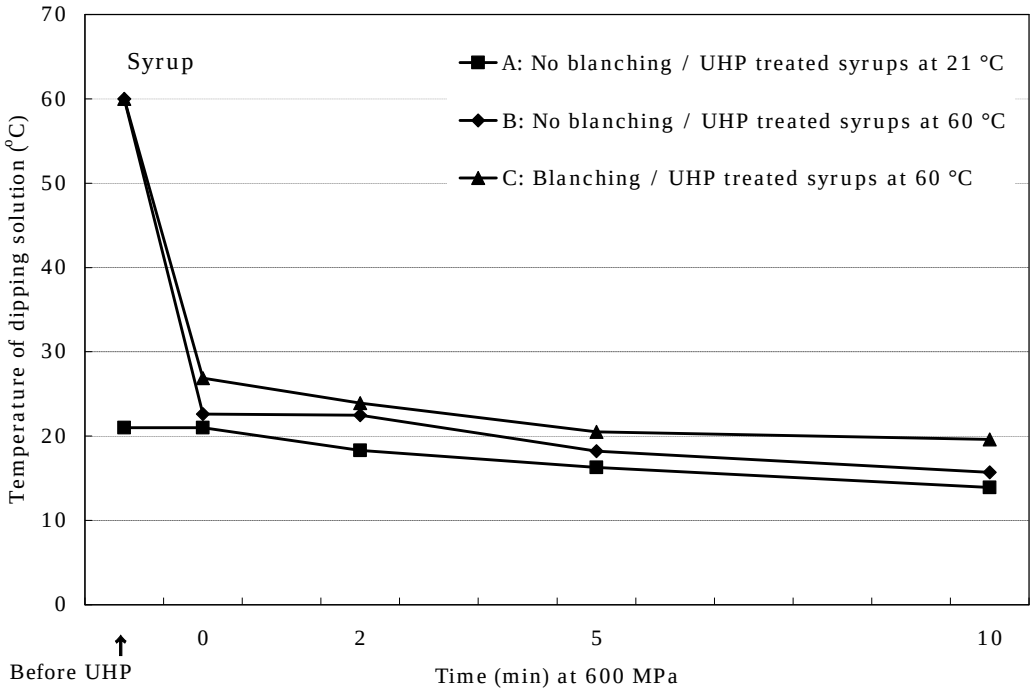


Figure 7: Temperatures at the coldest point of strawberry wedges and syrups before and after UHP treatments.

Each bar presents the range of ± standard deviation in triplicates.

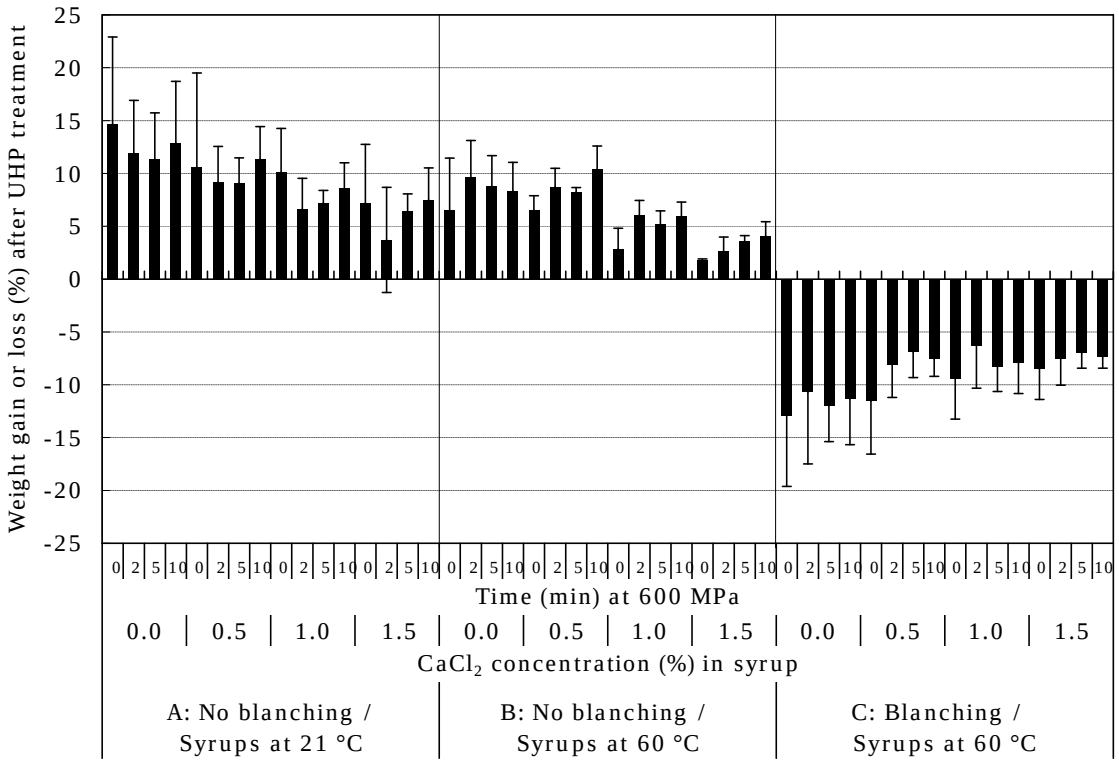


Figure 8: Weight gain or loss (%) of strawberry wedges after UHP treatments.

Each bar presents the range of ± standard deviation in triplicates.

$CaCl_2$ (%)	0.0				0.5				1.0				1.5			
UHP (min)	0	2	5	10	0	2	5	10	0	2	5	10	0	2	5	10
Group																
A	3.68 ± 0.02	3.64 ± 0.06	3.71 ± 0.01	3.64 ± 0.05	3.42 ± 0.00	3.44 ± 0.02	3.42 ± 0.04	3.42 ± 0.05	3.36 ± 0.01	3.32 ± 0.05	3.28 ± 0.04	3.35 ± 0.03	3.24 ± 0.03	3.23 ± 0.04	3.22 ± 0.08	3.23 ± 0.02
B	3.81 ± 0.02	3.80 ± 0.11	3.84 ± 0.03	3.91 ± 0.05	3.46 ± 0.05	3.52 ± 0.06	3.53 ± 0.08	3.50 ± 0.04	3.38 ± 0.07	3.37 ± 0.08	3.40 ± 0.13	3.40 ± 0.14	3.29 ± 0.12	3.28 ± 0.12	3.26 ± 0.15	3.26 ± 0.08
C	3.80 ± 0.19	3.71 ± 0.00	3.68 ± 0.04	3.68 ± 0.06	3.43 ± 0.11	3.47 ± 0.01	3.52 ± 0.01	3.53 ± 0.01	3.42 ± 0.01	3.44 ± 0.04	3.41 ± 0.04	3.41 ± 0.10	3.31 ± 0.05	3.33 ± 0.01	3.28 ± 0.14	3.36 ± 0.10

Table 1: pH of strawberry homogenates prepared after UHP treatments.

$CaCl_2$ (%)	0.0				0.5				1.0				1.5			
UHP (min)	0	2	5	10	0	2	5	10	0	2	5	10	0	2	5	10
Group																
A	3.69 ± 0.13	3.71 ± 0.15	3.72 ± 0.07	3.69 ± 0.10	3.43 ± 0.06	3.40 ± 0.10	3.38 ± 0.07	3.38 ± 0.06	3.31 ± 0.13	3.33 ± 0.11	3.29 ± 0.08	3.29 ± 0.09	3.21 ± 0.12	3.23 ± 0.07	3.22 ± 0.05	3.23 ± 0.10
B	3.63 ± 0.09	3.65 ± 0.13	3.66 ± 0.08	3.69 ± 0.09	3.35 ± 0.09	3.36 ± 0.09	3.39 ± 0.06	3.38 ± 0.09	3.27 ± 0.09	3.26 ± 0.09	3.27 ± 0.07	3.26 ± 0.08	3.18 ± 0.08	3.14 ± 0.07	3.17 ± 0.07	3.17 ± 0.08
C	3.67 ± 0.16	3.65 ± 0.12	3.72 ± 0.10	3.64 ± 0.07	3.36 ± 0.06	3.34 ± 0.07	3.36 ± 0.05	3.38 ± 0.07	3.27 ± 0.07	3.27 ± 0.06	3.28 ± 0.05	3.26 ± 0.06	3.19 ± 0.09	3.20 ± 0.08	3.19 ± 0.03	3.21 ± 0.08

Table 2: pH of syrups after UHP treatments.

$CaCl_2$ (%)	0.0				0.5				1.0				1.5			
UHP (min)	0	2	5	10	0	2	5	10	0	2	5	10	0	2	5	10
Group																
A	0.31 ± 0.04	0.35 ± 0.08	0.38 ± 0.07	0.36 ± 0.07	0.40 ± 0.13	0.38 ± 0.07	0.45 ± 0.13	0.42 ± 0.06	0.43 ± 0.08	0.40 ± 0.09	0.40 ± 0.10	0.44 ± 0.10	0.39 ± 0.13	0.42 ± 0.12	0.43 ± 0.12	0.44 ± 0.11
B	0.43 ± 0.05	0.43 ± 0.10	0.45 ± 0.07	0.40 ± 0.18	0.47 ± 0.06	0.51 ± 0.09	0.58 ± 0.11	0.48 ± 0.09	0.63 ± 0.07	0.54 ± 0.05	0.63 ± 0.12	0.53 ± 0.07	0.66 ± 0.12	0.63 ± 0.12	0.58 ± 0.14	0.57 ± 0.04
C	0.44 ± 0.09	0.37 ± 0.02	0.33 ± 0.07	0.31 ± 0.00	0.53 ± 0.06	0.46 ± 0.04	0.58 ± 0.05	0.45 ± 0.02	0.58 ± 0.09	0.53 ± 0.06	0.51 ± 0.02	0.57 ± 0.02	0.60 ± 0.05	0.55 ± 0.00	0.60 ± 0.04	0.59 ± 0.01

Table 3: Absorbance at 500 nm of syrups after UHP treatments.

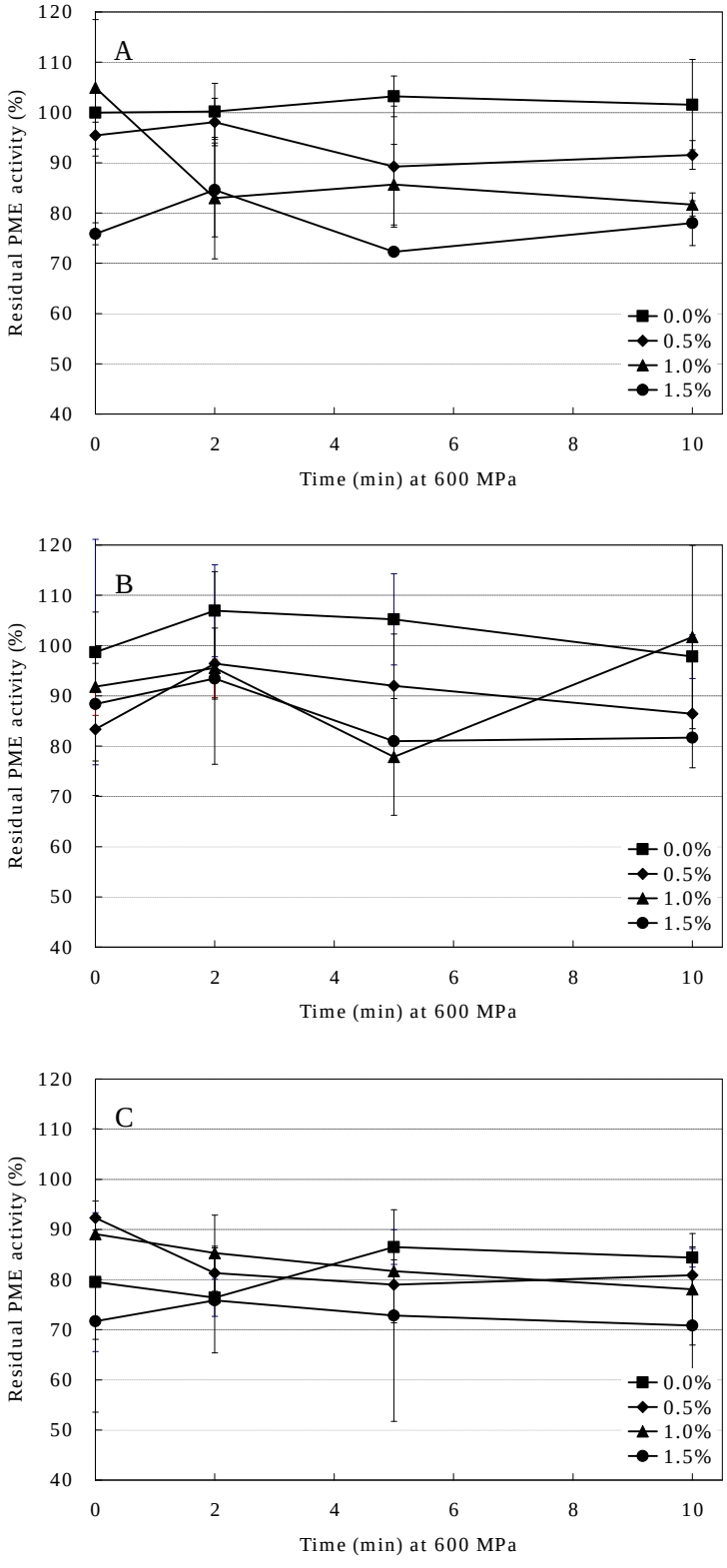


Figure 9: PME residual activity in UHP treated strawberries in groups A, B, and C based on PME activity in raw strawberries.

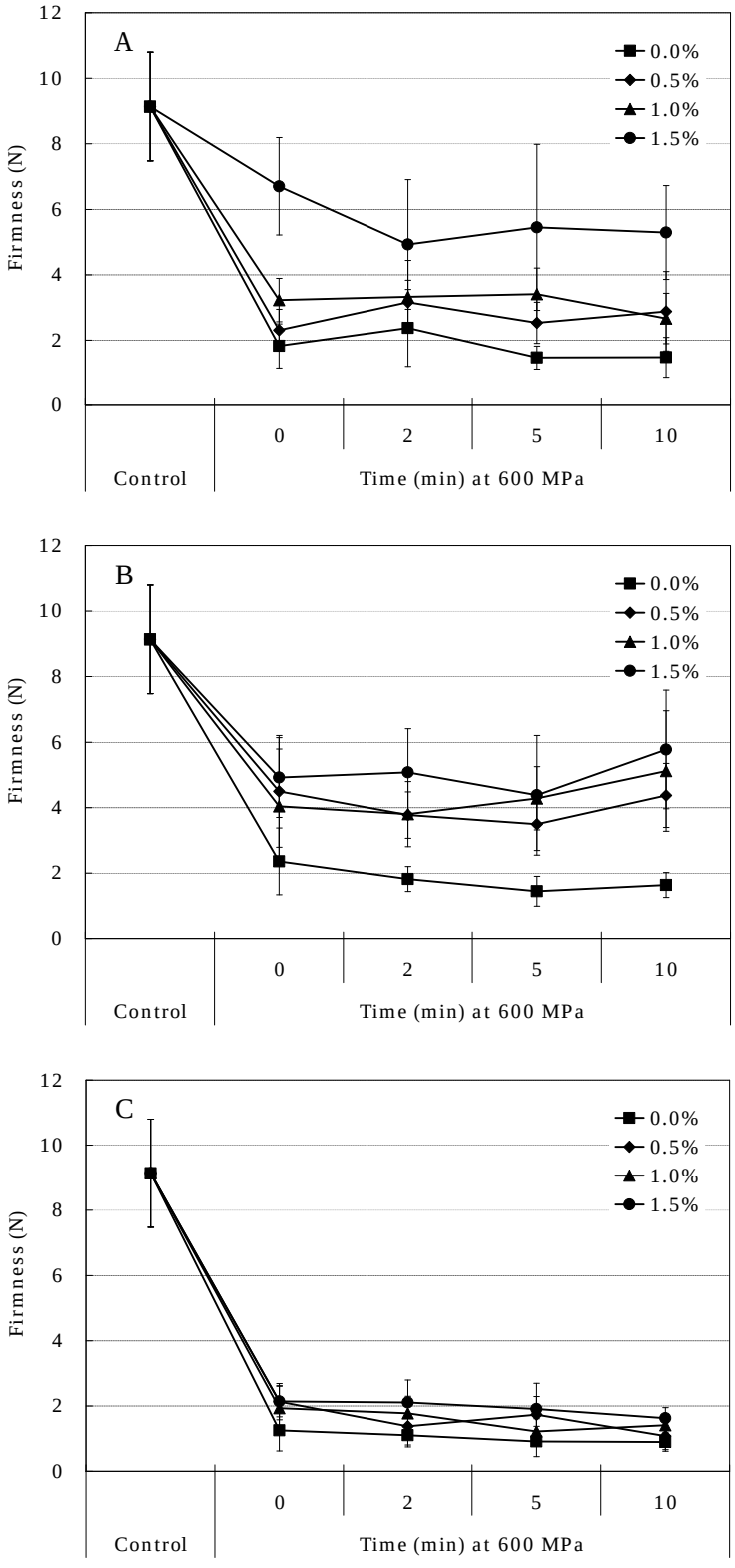


Figure 10: Firmness of raw strawberries and UHP treated strawberries in groups A, B, and C.

CHAPTER 6

Conclusions and Recommendations for Future Research

The conclusions from this research are extracted to provide overviews of the fluorescence oxygen probe method to determine polyphenol oxidase (PPO) activity and utilization of ultra high pressure (UHP) as a tool of processing fruits and vegetables to preserve natural color or increasing tissue firmness.

The fluorescence oxygen probe method determines PPO activity in reasonably short time when compared to the conventional spectrophotometric method. Assays of crude extracts or clarified extracts of fruits or vegetables with the fluorescence oxygen probe method provide diphenol oxidase activity for soluble and insoluble fractions of plant tissues. The comparison of patterns between increases in absorbance at 400 nm and oxygen consumption attributed to tyrosinase-catalyzed *p*-cresol oxidation resulted in the proposed predominant reactions dependent on reaction times.

Future research using the fluorescence oxygen probe instrument will include;

1) Applicability of the fluorescence oxygen probe method to determine monophenol and diphenol oxidase activities in plant sources other than apples;
2) Activity and localization changes of plant PPO during maturation or storage of plant tissues by determining PPO activities in both soluble and insoluble fractions; and
3) Comparison of the effects of antibrowning agents on PPO activity determined with the fluorescence oxygen probe and spectrophotometric methods.

The combined methods of adding papain and cysteine to the apple homogenates followed by UHP treatment is a potential application for color preservation of apple products. Papain required 1 mM EDTA and 10 mM cysteine to inhibit PPO activity in apple homogenates during incubation at 4 °C for 30 min. PPO activity in apple homogenates was effectively inhibited in the presence of papain and 25 mM cysteine during incubation at 25 °C for 30 min. More than 85% inhibition of PPO in 1) control apple homogenates or apple homogenates containing 2) 1 mM EDTA, 3) papain and 25 mM cysteine, or 4) papain, 1 mM EDTA, and 25 mM cysteine was achieved with incubation at 25 °C for 30 min followed by UHP treatment of 600 MPa at an initial temperature of 23 °C for 10 min. UHP treated apple homogenates containing at least 25 mM cysteine exhibited chartreuse color during storage at 4 °C

for 28 d regardless of residual PPO activity after UHP treatments. The addition of papain to apple homogenates absent cysteine increased barotolerance of apple PPO. Exogenous papain was baroresistant to experimental incubation-UHP treatments.

Future research selecting combined papain and UHP treatments will include;

1) Determining thresholds of papain and cysteine concentrations in apple homogenates for acceptable color retention during shelf life;
2) Sensory evaluation of UHP treated apple homogenates containing papain and cysteine;
3) Inactivation of exogenous papain activity in apple homogenates by UHP treatments (probably combined with heat treatments) to produce enzyme activity free products; and
4) Elucidation of barotolerant apple PPO by examining molecular structures of PPO affected by papain in the absence and presence of cysteine.

The combined methods utilizing UHP treatments of strawberry wedges preheated to 60 °C is unlikely to improve firmness of strawberry fruit because of susceptibility of strawberry tissues to combined heat and UHP treatments. Strawberry pectin methylesterase (PME) was stable during UHP treatment at 600 MPa and an initial temperature of 23 °C for 10 min. UHP treatment is a potential processing method to enhance UHP treated strawberries by the addition of $CaCl_2$ to syrups. The selection $CaCl_2$ solutions at ambient temperature or heated to 60 °C as

syrups resulted in an enhancement of UHP treated strawberry firmness. Strawberry wedges heated in water at 60 °C for 6 min followed by immersing and packaging in syrups heated to 60 °C exhibited significant decrease in tissue firmness after the UHP treatments regardless of concentration of $CaCl_2$ in syrups.

Future research utilizing UHP treatment as a tool for increasing firmness of strawberries or other fruits will include;

1) Determinations of $CaCl_2$ concentration in syrups and UHP treatment condition to enhance UHP treated strawberry firmness considering microbial safety of the solid strawberry products;
2) Selection of fruit possessing larger endogenous PME activity as well as less susceptibility to combined heat and pressure compared to strawberries; and
3) The effect of the addition of exogenous PME to syrups including $CaCl_2$ on firmness of UHP treated fruit.