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CHARACTERIZATION OF POLYPHENOL OXIDASE AND ANTIOXIDANTS FROM PAWPAW (ASIMINA TRIBOLA) FRUIT

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ABSTRACT OF THESIS

CHARACTERIZATION OF POLYPHENOL OXIDASE AND ANTIOXIDANTS FROM PAWPAP (*ASIMINA TRIBOLA*) FRUIT

Crude polyphenol oxidase (PPO) was extracted from pawpaw (*Asimina triloba*) fruit. The enzyme exhibited a maximum activity at pH 6.5–7.0 and 5–20 °C, and had a maximum catalysis rate (V_{\max}) of 0.1363 s⁻¹ and a reaction constant (K_m) of 0.3266 M. It was almost completely inactivated when incubated at 80 °C for 10 min. Two isoforms of PPO (MW 28.2 and 38.3 kDa) were identified by Sephadex gel filtration chromatography and polyacrylamide gel electrophoresis. Both the concentration and the total activity of the two isoforms differed ($P < 0.05$) between seven genotypes of pawpaw tested. Thermal stability (92 °C, 1–5 min) and colorimetry (L^* a^* b^*) analyses showed significant variations between genotypes. Pawpaw fruit samples that were briefly heated (1–2 min) had improved color storage stability due to PPO inactivation. Moreover, antioxidants were extracted from the same seven genotypes of pawpaw fruit. All genotypes demonstrated significant ($P < 0.05$) radical-scavenging capability and inhibited lipid oxidation in a liposome system. Overall, these findings suggested that PPO-induced browning during pawpaw fruit processing may be minimized through controlling pH and temperature. All genotypes of the fruit also proved to be a good source of antioxidants.

Keywords: Pawpaw Fruit, Polyphenol Oxidase, Antioxidant, Browning, Thermal Stability

Caodi Fang

08/19/2007

CHARACTERIZATION OF POLYPHENOL OXIDASE AND ANTIOXIDANTS
FROM PAWPAW (*ASIMINA TRIBOLA*) FRUIT

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THESIS

Caodi Fang

The Graduate School

University of Kentucky

2007

CHARACTERIZATION OF POLYPHENOL OXIDASE AND ANTIOXIDANTS FROM
PAWPAW (*ASIMINA TRIBOLA*) FRUIT

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
Graduate Center for Nutritional Sciences
at the University of Kentucky

By

Caodi Fang

Lexington, Kentucky

Director: Dr. Youling L. Xiong, Professor of Food Science
and the Graduate Center for Nutritional Sciences

Lexington, Kentucky

2007

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Dedicated to my parents, uncles and aunts who give me the love, support and help.

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CHAPTER 1

INTRODUCTION

The pawpaw, *Asimina tribola*, also known as Poor Man's Banana, is the largest fruit tree native to east America. Pawpaw fruit has a rich history as an important food source to early European explorers and the Native Americans. In 1541, pawpaw was first formally described by an exploration group in the Mississippi River valley (Crabtree, 2004). Early settlers and some animals depended partially on pawpaw to sustain themselves during starvation (Peterson, 1991). Over 40 named pawpaw cultivars are available today. The pawpaw fruit is highly nutritious, with double the amount of vitamin C of apples or pears, higher amounts of potassium, phosphorus, magnesium, calcium and iron compared with typical fruits, as well as high levels of some essential amino acids (Peterson et al., 1982). The pawpaw has large commercial potential in the food market, and has been used to prepare pawpaw ice cream and bakery products.

There is an increasing trend in consumer selection for foods considered to be not only of high quality (color, flavor, and texture) but also of high nutrition value. To respond to the market demand for this selection, food processors make considerable efforts to improve fruit and vegetable quality from harvest through the end of the processing. In fruit and vegetable processing, endogenous enzymes can have a profound effect on the quality of fresh produce. One such endogenous enzyme is polyphenol oxidase (PPO),

which catalyzes oxidation of phenolic compounds. This enzyme is responsible for the production of dark pigments in fruits and vegetables, which impart the appearance of poor quality. Bruises incurred during harvest could release PPO into the cytoplasm of cells, thereby initiating the browning reaction and lowering the product quality. Thus, it is important to develop methods to control this enzyme activity to enable long-term product stability. Characterization of PPO from pawpaw fruit pulp, with emphasis on the effect of pH and temperature on the enzyme activity, is described in Chapter 3.

Like other climacteric fruits, i.e., fruits that exhibit enhanced respiration rates post-harvest, pawpaw fruit is sensitive to temperature and tends to have short shelf-life. Thermal processing is the most common method applied to control unwanted active enzyme activity and the growth of microbial organisms. However, this processing can also result in considerable loss of sensory and nutritional quality, such as, color deterioration and nutrient destruction. Thermal stability of pawpaw fruit PPO was investigated and is described in Chapter 4. Heating susceptibility of seven genotypes of pawpaw fruit at 92 °C was studied. The PPO activity of different genotypes was evaluated and the enzyme was quantified.

Many fruits are known to be good sources of natural antioxidants. Antioxidants play an important role in plant and animal health and their natural defense to illness. For humans, the potential health benefits of antioxidants from edible plant food include inhibiting lipid oxidation, scavenging radicals, reducing inflammation, suppressing tumor growth, prevention of obesity, etc. Antioxidants were extracted from pawpaw fruit pulp

and the radical-scavenging activity of the extracts from seven genotypes of pawpaw fruit are presented in Chapter 5.

The overall objective of my theses research was to investigate color stability and antioxidant activity of pawpaw fruit pulp. Polyphenol oxidase, the responsible enzyme for post-harvest browning, and total antioxidant activity of fruit pulp, were studied for the purpose of establishing optimum storage conditions and maximizing potential health benefits. The specific objectives were:

- 1) To characterize the PPO and to measure the PPO activity in pawpaw fruit pulp.
- 2) To investigate thermal susceptibility and variation in color stability of seven different genotypes of pawpaw fruit.
- 3) To evaluate the antioxidant activity of fruit pulp of seven genotypes of pawpaw.

CHAPTER 2

LITERATURE REVIEW

2.1. Pawpaw

2.1.1. Pawpaw biology

The Pawpaw (*Asimina triloba*) is the largest tree fruit native to United States. The *Asimina* is the only temperate climate genus of the tropical family Annonaceae. This family is famous for a number of fine fruit, including sugar apple (*Annona squamosa* L.), cherimoya (*Annona cherimola* Mill.), soursop (*Annona muricata* L.), atemoya (*Annona squamosa* x *A. cherimola*), soncoya (*Annona purpurea* Moc. & Sesse), custard apple (*Annona reticulata* L.), biriba (*Rollinia mucosa* Baill.), and ilama (*Annona diversifolia* Safford) (Callaway, 1993). Of the nine species of *Annonaceae* found in the United States, *Asimina Triloba* has the greatest commercial potential. There are more than 40 named varieties of pawpaw fruit, consisting of different amounts of fruit, a variety of different flavor, texture and appearances (Jones et al., 1999; Duffrin and Pomper, 2006).

The pawpaw fruit is usually a 3 to 15 cm long, 3 to 10 cm wide and 200 to 400 g weight edible fruit, with a shape that varies from oval to oblong (Fig. 2.1). This fruit contains two rows of almond-size seeds surrounded by yellow to orange-colored flesh, with the skin ranging from green to yellow when ripe (Wiese and Duffrin, 2003). Pawpaw grows indigenously in 26 states of the eastern United States, ranging from



Figure 2.1. Pawpaw cluster with ripe fruit (Scott, 1997)

northern Florida to southern Ontario and as far west as Missouri. A fresh and fully ripe pawpaw fruit has been described as having a strong, unique aroma which resembles a combination of pineapple, mango and banana. However, the aroma varies among varieties, with some fruits having an intense sweet flavor (Duffrin and Pomper, 2006).

From as early as 1982, Peterson et al. had evaluated nutritional content in pawpaw fruit. It was found that pawpaw has a high nutritional quality compared to apple, banana and orange. The fruit has a high protein content, as well as vitamins A and C, minerals consisting of Phosphorus, Calcium, Magnesium, Zinc, Manganese and Iron, and the essential amino acids. It is the fruit of the future.

2.1.2. Pawpaw fruit as food

Because of its intense aroma and sweetness, pawpaw fruit is widely accepted as fresh product and as an ingredient in a number of food products. As a fresh fruit, the pawpaw has a very short shelf life of only 2–3 days at room temperature and up to 2 weeks with refrigeration. There is commercial processing potential for pawpaw pulp in juice, ice cream, yogurt and baked goods. Consumer acceptability of pawpaw fruit and fruit products has been investigated during the past 10 years. Kentucky State University has one of the leading pawpaw horticultural research programs in the nation and is the site of the USDA National Clonal Germplasm Repository for Pawpaw *Asimina* species (Pomper and Layne, 2005).

2.2. Food Browning

In the food industry, browning is a serious detriment occurring during processing and storage, especially during manufacture of fruit, vegetable and meat product. Brown discoloration decreases the quality of food products due to changes in color, nutritional, sensory and flavor properties. Food browning results from several different processes that fall into two general categories: non-enzymatic reactions and enzymatic oxidation of phenolic and other compounds.

2.2.1. Non-enzymatic browning

Non-enzymatic browning is a chemical process yielding brown color in foods and occurring without enzymes being involved. There are three major forms of non-enzymatic browning: Maillard reaction, Ascorbic acid oxidation, and Caramelization.

2.2.1.1. Maillard reaction

The most common type of non-enzymatic browning is the Maillard reaction. First reported in 1912, Maillard browning involves a series of chemical reactions that occur when amino acids and reducing sugars are heated together (McEvily et al., 1992). The sugar interacts with amino acids, producing various aromas and imparting color to foods. The type of amine and reducing sugar influences the reaction rate as well as the products formed, which ultimately are brown melanoidin pigments (Willits et al., 1958). There are

3 basic phases of Maillard reaction: (1) Initial reactions involving generation of glycosyl-amino products followed by Schiff's base rearrangement to yield the Amadori product; (2) Intermediate reactions involving the formation of short-chain hydrolytic products, undergoing Stecker dehydration with amino acids to form aldehydes and condensation to aldols; and (3) Final reactions involving aldol condensation, polymerization, and the production of heterocyclic nitrogen compounds and melanoidins (O'Brien et al., 1998). Interestingly, the end products of Maillard reactions themselves appear to play an important role in preventing enzymatic browning. Their strong anti-browning effect was thought to be related to their antioxidant properties (Nicoli et al., 1991).

2.2.1.2. Ascorbic acid oxidation

Ascorbic acid oxidation leads to the destruction of vitamin C and the loss of nutrition in foods. This browning is the spontaneous thermal decomposition of ascorbic acid under both aerobic and anaerobic conditions. It was reported that over 80% of the browning of dried apple during storage packed under vacuum resulted from oxidative non-enzymatic reaction (Bolin and Steele, 1987).

2.2.1.3. Caramelization

Caramelization is the oxidation of sugar when heated, a process used extensively in the food industry for generating nutty flavor and brown color. Volatile chemicals are released during the reactions and produce the caramel flavor. The caramel brown color is used in a variety of beverages, such as soft drinks.

2.2.2. Enzymatic browning

Enzymatic browning is of significant importance in the fruits and vegetable industry. This kind of browning discoloration reduces the shelf life of many processed foods, and affects the production of dehydrated and frozen fruits and vegetables as well (Shewfelt, 1986; Huxsoll and Bolin, 1989). Although enzymatic browning is a cause of discoloration, it is a desired reaction in processes like tea and cocoa fermentation and in the formation raisin, date and prune products (Haard and Chism, 1996).

2.2.2.1. Polyphenol oxidase (PPO)

It is estimated that nearly 50% of tropical fruits are discarded due to quality defects resulting from enzymatic browning (Whitaker, 1996). The browning is mainly catalyzed by the enzyme polyphenol oxidase (1,2 benzenedio; oxygen oxidoreductase; EC 1.10.3.1) which is also known as phenoloxidase, phenolase, monophenol oxidase, diphenol oxidase and tyrosinase (Marshall et al., 2000).

PPO is a copper associated enzyme with two binding sites for phenolic substrates. It was first found to be a tetramer containing four atoms of copper per molecule in mushroom (Jolley et al., 1974). PPO is located in the chloroplast bound to thylakoid membranes (Golbeck and Cammarata, 1981). PPO is activated by releasing into the cytosol when plant tissues undergo physical damage such as bruising, cutting or blending. In the presence of atmospheric oxygen and PPO, monophenol is hydroxylated to *o*-diphenol (monophenol oxidase activity), and diphenol can be oxidized to *o*-quinones (diphenol oxidase activity), which then undergoes polymerization to yield dark brown polymers (Fig. 2.2).

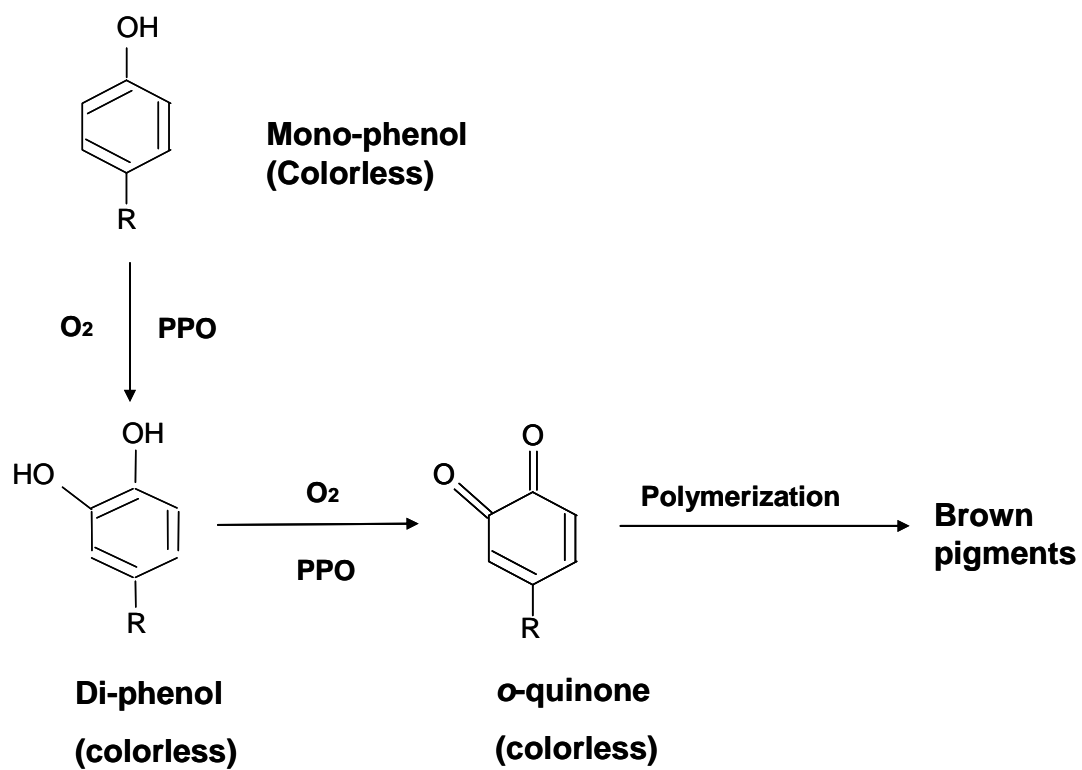


Figure 2.2. Schematic representation of oxidation reaction by PPO

Polyphenol oxidase is comprised of monophenol oxidase or diphenol oxidase. Early studies on plants, such as apple (Goodenough et al., 1983), avocado (Kahn and Pomerantz, 1980), eggplant (Perez-Gilabert and Carmona, 2000), grape (Ferrer et al., 1989), and potato (Ferrer et al., 1993), showed PPO to possess both types of activities, while those from lettuce (Heimdal et al., 1994), longan (Jiang, 1999), pineapple (Das et al., 1997), field bean (Paul and Gowda, 2000) and sunflower (Raymond et al., 1993) lack the hydroxylation properties and act only on *o*-diphenols. Diphenol oxidase is the most prevalent form of PPO (Yoruk and Marshall, 2003). Nicolas et al. (1994) found the ratio of monophenol to diphenol oxidase activity to be 1:10 to 1:40, when monophenol and diphenol oxidase co-existed in plants (Nicolas et al., 1994; Marshall et al., 2000). Diphenol oxidase has received more attention due to its high catalytic rate and its leading reactions to form quinones, which produce brown pigments, named melanin (Marshall et al., 2000).

The substrates for this reaction include simple phenols, such as catechol and gallic acid; cinnamic acid derivatives, such as dopamine; and flavonoids, such as catechin and epicatechin (Fennema, 1975). The oxidation products have potential to interact with food proteins leading to covalent condensation (Mathew and Parpia, 1971; Nicolas et al., 1994). Cross-linking reactions could change the structure, function and nutrition characteristics of food proteins (Yoruk and Marshall, 2003). The reason of reduction in nutrition value of food proteins is the interaction of quinones with the side chain of essential amino acids in food systems. The amine (-NH₂) and sulfide (-SH) groups of amino acids are mostly susceptible to reacting with quinones (Felton et al., 1992). Moreover, quinones formed during PPO oxidation reaction may undergo redox recycling,

which generates radicals and can damage DNA and proteins (Hill, 1992; Yoruk and Marshall, 2003). Selected properties of PPO from some plant sources, including optimum pH and temperature, substrates involved, and reaction constants, are summarized in Table 2.1.

2.2.2.2. Factors affecting PPO activity

The most important factors that determine the activity of PPO are the enzyme active site, prosthetic groups, pH and temperature.

2.2.2.2.1. Enzyme active site

Gene encoding PPO has been studied in a wide range of plants. Polypeptide sequence analysis revealed the presence of two highly conserved copper binding regions, containing histidine residues capable of binding prosthetic copper groups (Van Gelder et al., 1997). Yaar (2004) reported that ascorbic acid has a direct effect on PPO, especially in the presence of copper ion. Ascorbic acid and copper ions catalyze a free radical reaction which oxidized the histidine residues that act as a ligand to the active site coppers. Sulfides then bind to the sulphydryl group of the active site of the enzyme.

2.2.2.2.2. Prosthetic groups

Since copper is dispensable to the function of PPO, chemicals such as Ethylene diamine tetraacetic acid (EDTA) and Sodium azide would chelate copper at the active site, thereby rendering the enzyme less active. Bisulfate and thiols reduce copper from Cu^{2+} to Cu^+ , which then dissociates from the enzyme active site, causing complete PPO inactivation.

Table 2.1. Characteristics of PPO extract from some fruits and vegetables

Source	Optimum		Substrate	¹ K _m (mM)	Reference
	pH	T(°C)			
Potato	6.6	40	L-DOPA	32	Cho and Ahn, 1999
Artichoke	5.0	40	4-methylcatechol	11.6	Dogan et al., 2005
Sweet potato	4.5–6.5	30	chlorogenic acid	8	Lourencuo et al., 1992
Grape	5.0	25	Catechol	52.6	Rapeanu et al., 2006
Plum	6.0	20	Catechol	20	Siddio et al., 1992
Chinese water chestnut	6.5	40	Catechol	10.32	Lu et al., 2005
Yacon roots	5.0–6.6	30	Catechol	5.0	Neves and Silva, 2007
Litchi	7.0	70	4-methylcatechol	10	Jiang et al., 1997
Tobacco	7.0	40	Catechol	6.8	Shi et al., 2001
Blue berry	4.0		Caffeic acid		Kader et al., 1997
Bean sprout	9.0	40	Pyrogallol	84.6	Nagai and Suzuki, 2003
Strawberry	7.2	50	Catechol	11.2	Serradell et al., 2000
Banana peel	6.5	30	Dopamine	3.9	Yang et al., 2001

¹K_m = Michaelis-Menton constant

2.2.2.2.3. pH dependence

One of the most direct influences of enzyme activity is acidity. The pH can change the state of ionization of acidic or basic amino acids in the active site of enzymes. This leads to the alteration of enzyme as well as its substrate's tertiary structure. Changes in pH not only affect enzyme solubility but also influence the charge distribution of the enzyme, phenolic substrates and products (Serradell et al., 2000).

2.2.2.2.4. Temperature dependence

Temperature is a critical factor in fruit storage since it can affect fruit quality during development. PPO is generally heat susceptible. Previous research has shown that PPO is inactivated by heating $> 50\text{ }^{\circ}\text{C}$, but the heat treatment can produce undesirable colors and flavors (Nicoli et al., 1991; Martinez). There are two aspects of the temperature effect: heat effect and cold effect. Heat treatment is the most widely used methods due to its capability of inactivating enzymes and destroying microorganisms (Mashall et al., 2000). On the other hand, refrigerated temperature can reduce enzyme-catalyzed reaction, since low temperatures result in lower enzyme kinetic energy, slower enzyme/substrate mobility, and allows reduced chances of molecule collision. Freezing temperatures are also utilized in food long-term preservation and storage (Nunez-Delicado et al., 2005). The mechanism of enzyme inactivation at freezing temperature can be illustrated by several hypotheses. (1) Inhibition of enzyme and substrate reactions would be achieved by increasing the solutes concentration due to the semi-frozen state of the solutes and the change of solutes conformation. (2) Freezing temperature of $-20\text{ }^{\circ}\text{C}$ or lower allows pH changes because of the changes in buffer concentration, contributing to mobility

increases of the hydronium ion (H^+) from H_2O in ice. (3) Marshall et al. (2000) pointed out that perturbations of sulphhydryl groups are essential for the activity of some enzymes. At freezing temperature, the concentration of disulfide groups increases, enzymes undergo conformation changes, and oxygen concentration in ice rises, due to solubility changes, producing increased oxidation of sulphhydryl groups. Hence, a perturbation of sulphhydryl groups (-SH) ensues, increasing the propensity for SS-SH inter-changes (Fennema, 1975). (4) Removal of water during freezing changes water activity, eventually altering the microbial environment of the food system (Marshall et al., 2000).

2.2.2.3. Inhibition of PPO

PPO is distributed in almost all high plants, including papaya, apple, peach, banana, grape, mango, date, artichoke, chestnut, cabbage, and lettuce. In addition, PPO is found in shrimp and other seafood products (Rolle et al., 1991; Chen et al., 1997). Because those products have tremendous economic impact on the food industry, inhibition of PPO in seafood products has widely been studied (Yoruk and Marshall, 2003). PPO has been found to be relatively heat labile. Therefore, a thorough understanding of thermal treatments to control the action of PPO is crucial in an attempt to inhibit PPO activity in fruit and vegetable processing, including that of fruits and vegetables.

Thermal stability is the resistance to permanent change in properties caused by heat. Thermal treatment is generally considered to be the most effective way to inactivate PPO and, consequently, to prevent enzymatic browning (Weemaes et al., 1998). However, this method could result in considerable nutritional value as well as sensory quality losses in vegetable and fruits including breakdown of vitamins and amino acids, color and flavor

deteriorations (Lund, 1977; Sapers, 1993; Weemaes et al., 1997).

Thermal resistance of enzymes is largely dependent on environmental conditions such as pH (Weemaes et al., 1998) and the presence of sugar, salts and additives (Weemaes et al., 1997). Modified pH in food systems is known to aid thermal process efficiency (Earnshaw et al., 1995).

2.2.2.4. Methods to characterize PPO

There are a number of methods used to characterize PPO. This study utilized colorimetry, PPO extraction, PPO activity, gel filtration chromatography, and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.2.2.4.1. Colorimetry

Since customer selection of fresh vegetables and fruits largely depends on the appearance of the products, color measurement has gained much attention from food researchers and the industry (Ngo et al., 2007). To investigate color quality, it is critical to measure color and pigment concentration in fruit system. The instrumental specification of color utilizing the International Commission of Illumination (CIE) L^* a^* b^* system is mainly performed by the application of colorimetry (Fig. 2.3). The L^* value (lightness) represents a perfect reflecting diffuser. The maximum L^* value is 100, showing white; the minimum L^* value is 0, yielding black. The a^* value represents the red/green coordinate. A positive a^* indicates red, and negative a^* is green. The b^* value represents the yellow/blue coordinate. A positive b^* is yellow, while negative b^* is blue. Joshi (2001) stated that food samples are not flat, or perfectly transparent/opaque. Thus, the geometry

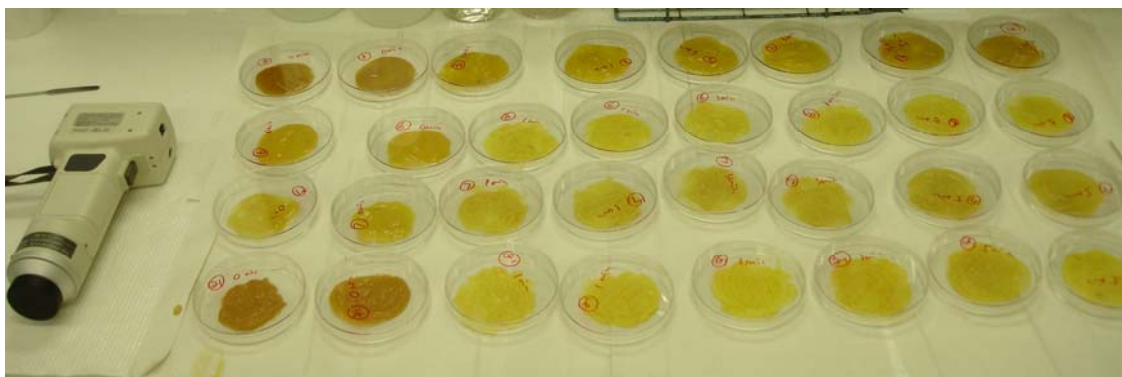


Fig. 2.3. A Minolta colorimetric measurement system (a colorimeter and samples in plastic Petri dishes)

and surface morphology of food samples, as well as the physical environment, can be influential of food colors.

2.2.2.4.2. Extraction

During the PPO extraction and purification process, releasing PPO from chloroplast membranes and removing endogenous phenolic compounds are of significant importance to reach a high purity and yield (Miyawaki, 2006). Phenol absorbents polyvinylpolypyrrolidone (PVPP), polyvinylpyrrolidone (PVP), and Amberlite XAD-4 are commonly used in the extraction of PPO for enzyme activity assays. These chemicals can prevent reaction between PPO and phenolic compounds during extraction. In addition, detergents, such as TritonX-100, are added to the extraction buffer for extracting PPO (Serradell et al., 2000; Nagai and Suzuki, 2001). Triton X-100 could break chloroplast membrane and release PPO into cytosol. Owusu-Ansah (1989) reported that Triton X-100 was effective for the partial purification of PPO.

2.2.2.4.3. Assay for PPO activity

The most common assay for PPO activity is spectrophotometric measurement of quinones formation at 400–500nm, depending on the substrates (Serradell et al., 2000; Yoruk and Marshall, 2003). For examples, *o*-quinones formed from epicatechol, pyrocatechin, caffeic acid and L-DOPA substrates exhibit an absorption maximum at 420, 400, 400, and 475 nm, respectively (Casado-Vela et al., 2006). In the plot of absorbance versus reaction time, the absorbance increases linearly in the initial 60 s and then decreases later (Chapter 3). The reason of the decrease in absorbance is the formation of

insoluble brown polymers called melanins. During the reaction with *o*-diphenol, PPO is inactivated by reacting with intermediate free radicals that act on copper in the PPO active site (Miyawaki, 2006).

2.2.2.4.4. Gel filtration chromatography

Gel filtration chromatography, also called size exclusion chromatography, molecular exclusion chromatography, or gel permeation chromatography, is a chromatographic method in which particles are separated based on their size (Fig. 2.4). It has been widely used not only to purify proteins but also to determine the sizes of macromolecules. In a typical chromatography setup, the stationary phase is composed of porous beads with a well-defined range of pore sizes. Larger molecules will be eluted with the mobile phase (elution buffer) faster, and proteins with smaller molecular weights will stay inside all the pores in the beads and be eluted later.

2.2.2.4.5. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a common technique used to separate proteins based on their mobility in an electric field (Laemmli, 1970). Reducing SDS-PAGE is commonly used in biochemistry, molecular biology, and genetics. In reducing SDS-PAGE, proteins samples are boiled in the presence of SDS and a reducing reagent such as dithiothreitol or β -mercaptoethanol, which can break disulfide bonds. Non-reducing SDS-PAGE is used for proteins whose native structures must be kept for further analysis.

In addition to SDS and reducing reagents, the protein samples subjected to

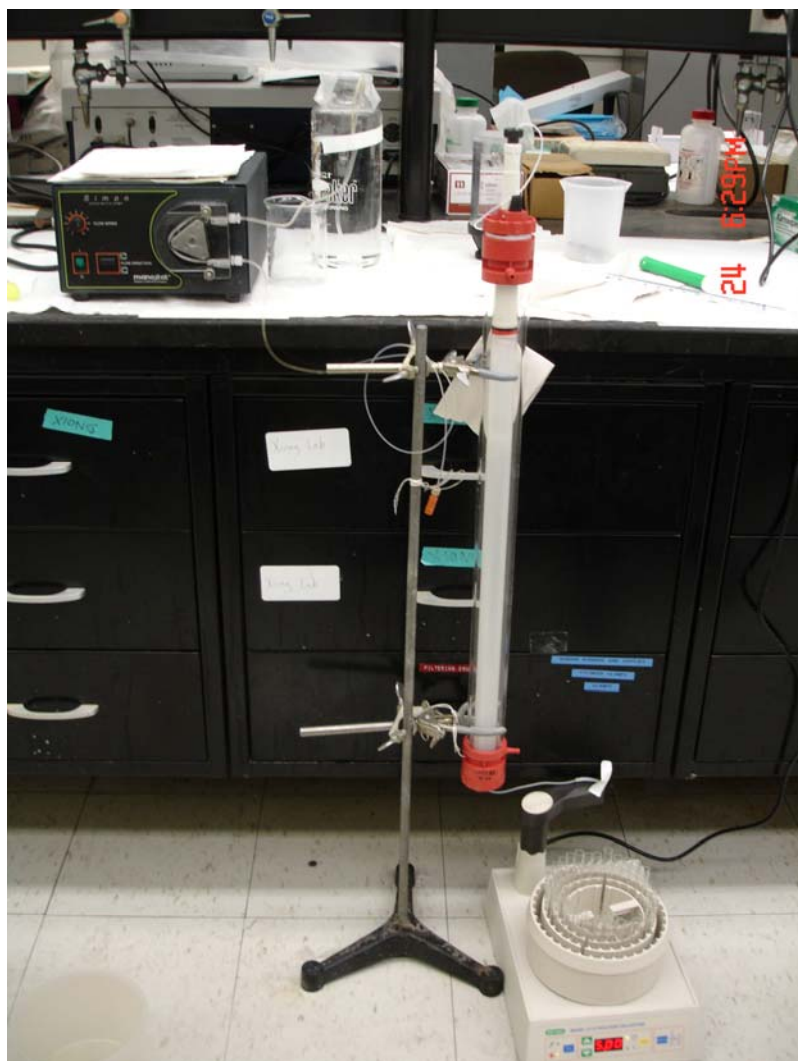


Figure 2.4. A typical gel filtration chromatography system (a pump, a column and a fraction collector)

SDS-PAGE also contains bromophenyl blue as a tracking dye. In addition, glycerol, which makes proteins fall to the bottom of the sample wells, and Tris-glycine buffers, which maintains the pH of the sample and running buffers, are required. Due in larger part to folding pattern differences, native proteins must be treated with SDS to render all protein molecules without a defined structure, enabling, separation by their size and not by their original shape. SDS acts to denature protein from secondary and tertiary structural level and yields proteins with only a primary structure. This then results in negative charges to protein in proportion to its mass (one SDS per two amino acids, or 1.4 g SDS/1.0 g protein). The gel is composed of a stacking gel and a resolving gel. Applying a voltage/current to the gel, the negatively charged protein-SDS micelles will migrate down towards the positive electrode and separated roughly by their size.

2.3. Lipid Oxidation

Oxidation of lipids is a major cause of quality degradation in natural and processed foods. Lipid oxidation produces rancidity, affecting flavor, color, texture and nutritional value of foods (Chaiyasit et al., 2007). The process of lipid oxidation is described as a complex sequence of chemical reaction between unsaturated fatty acids and active oxygen species (McClements and Decker, 2000; Chaiyasit et al., 2007).

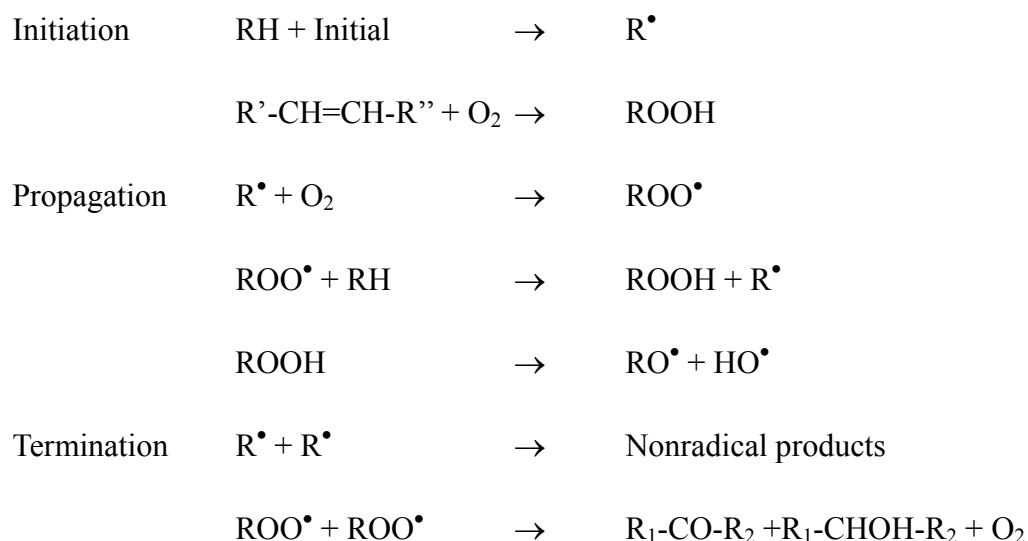
2.3.1. Mechanism of lipid oxidation

Theoretically, lipid oxidation is a free radical chain reaction between unsaturated triacylglycerols/phospholipids and singlet/triplet oxygen. However, lipid oxidation in

many systems is mediated by prooxidants (Cu^{n+} , Fe^{n+}), enzyme systems, or electron excitation (Kanner et al., 1987; Kubow, 1992). Classical studies (Kanner et al., 1987; Kubow, 1992; Nawar, 1996; Chaiyasit et al., 2007) have established the principle of lipid oxidation that involves three stages:

1. Initiation – Formation of free radicals
2. Propagation – Free-radical chain reactions
3. Termination – Formation of non-radical products

During peroxide generation, free radicals are formed. The major initial reaction products are hydroperoxides. The mechanism can also be shown as schematic reaction in (Kanner and Rosenthal, 1992).



2.3.2. Measurements of lipid oxidation

There are several analytic ways that can be used to measure lipid oxidation products. Common assessments of lipid oxidation are shown in Table 2.2. Peroxide Value (PV) and

Table 2.2. Common assay for lipid oxidation detection

Principle	Assay	Reference
Lipid substrate loss	Gas chromatography	Slater, 1984
Oxygen consumed for oxidation	Oxygen uptake assay	Slater, 1984
Generation of peroxides, hydroperoxides	PV assay	Kanner and Rosenthal, 1992
Formation of malonaldehyde	HPLC; TBA at 532 nm; Fluorescence at 553 nm	Esterbauer et al., 1984
Formation of conjugated dienes	OD at 234 nm	Recknagel and Glende, 1984
Formation of carbonyl compounds	GC-MS; HPLC	Esterbauer and Zollner, 1989
Formation of free fatty acids	Titration; electric conductivity	Laubli et al., 1986

thiobarbituric acid (TBA) tests are among the most commonly used methods. Physical measurements include spectrophotometric techniques, such as measurement of conjugated dienes at 234 nm.

2.3.2.1. Peroxide method

This parameter measures the amount of peroxides and hydroperoxides (primary products) generated during oxidation of unsaturated fats. Hydroperoxides and peroxides oxidize aqueous iodide to iodine which can be titrated with thiosulfate solution and starch as end-point indicator. However, because peroxides are able to quickly form from secondary oxidation products, and potential additional oxidation of iodide by dissolved oxygen, this approach may be of less value.

2.3.2.2. Thiobarbituric acid method

The Thiobarbituric acid (TBA) method is now the most commonly used method to detect lipid oxidation. The malonaldehyde formed as a result of lipid oxidation is reacted with TBA to form a pink pigment (TBARS) that can be monitored at 532 nm. A number of substrates have been used in the determination of TBARS, including plant and animal tissue samples, liposome, LDL, linoleic and other fatty acids (Kanner and Rosenthal, 1992).

2.3.2.3. Conjugated diene method

In the early 20th century, Gillam and colleagues discovered that stored fats develop an absorption peak around 234 nm (Dormandy and Wickens, 1987). Until the 1960s, monitoring diene conjugation emerged as an effective technique for lipid oxidation measurement (Antolovich et al., 2002). In lipid system, UV spectrophotometry is a direct detection method followed by conjugated dienes formation. When fatty acids are oxidized to generate hydroperoxides, the double bonds in these unsaturated fatty acids become conjugated (Kanner and Rosenthal, 1992).

2.4. Antioxidants From Edible Plants

The importance of oxidation in the body and food system has been widely investigated (Antolovich et al., 2002). Oxidative metabolism is of significant important for cell survival. The side effect of this physiological process is the formation of free radicals and reactive oxygen species (ROS) that can cause DNA, RNA, protein or cell accumulative damage. The body does, however, have protective mechanism of antioxidants to interact with these products.

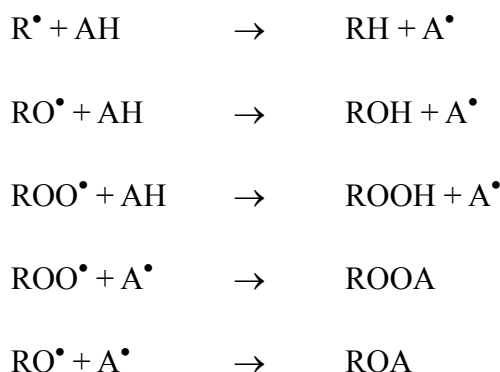
Antioxidants are chemicals used to control oxidative reaction and decrease the adverse effect of reactive species in biological system. Owing to the low level of natural antioxidants in body, in order for the appropriate function of free radical scavenger, additional antioxidants must be supplied from dietary intake (Naidoo, 2005). Within

biological system, there are four general categories of antioxidants (Prior et al., 2005):

1. Large molecules, for example, ferritin.
2. Enzymes, for example, superoxide dismutase, catalase.
3. Small molecules, for example, polyphenols, ascorbic acid, tocopherols, carotenoids.
4. Hormones, for example, estrogen, angiotensin.

2.4.1. Mechanism of antioxidant action

The two main mechanisms of antioxidant action are: Interrupt the free radical chain reaction (primary); be preferentially oxidizing (minor). An antioxidant can interfere with the free radical and the intermediate products generated during the oxidation process. The schematic reaction occurred as follows:



2.4.2. Extraction of antioxidants

It is widely accepted that plant materials contain high amount of antioxidants as phenolic compounds, including simple phenols, polyphenols, benzoic acid derivatives,

flavonoids, stilbenes, tannins, lignans, and lignins (Shahidi and Naczki, 2004; Xu and Chang, 2007). Different solvent systems have been used to extract antioxidants from vegetables and fruits. The common extraction solutions are water, methanol, ethanol, and acetone (Sun and Ho, 2005).

2.4.3. Evaluation of antioxidant activity

Antioxidant activity cannot be determined directly but rather by the inhibition effect of antioxidants on oxidation (Antolovich et al., 2002). Table 2.3 shows the diverse array of assays as well as solvent systems that can be used for these determinations.

2.4.3.1. Radical-Scavenging methods

There are two radical-scavenging methods used to evaluate antioxidant activity: the ABTS assay and the DPPH assay.

2.4.3.1.1. ABTS assay

The procedure based on reduction of the generation of green-blue [(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] $\text{ABTS}^{\bullet+}$ chromophore through the reaction of ABTS with potassium persulfate (Fig. 2.5). The accumulation of $\text{ABTS}^{\bullet+}$ can be inhibited by the presence of an antioxidant in the reaction solution, on a time scale dependent on the antioxidant activity (Antolovich et al., 2002). The antioxidant activity is indicated by its ability of H^+ donation which is expressed by comparison with a standard

Table 2.3. Extraction of antioxidants from fruits and vegetables and antioxidant activity measurements

Food	Extraction Solvent	Antioxidant Activity Assay	Reference
Strawberry	70% acetone	Methy-linoleate oxidation	Kahkonen et al., 1999
Black currant	70% acetone	Methy-linoleate oxidation	Kahkonen et al., 1999
Cranberry	70% acetone	Methy-linoleate oxidation	Kahkonen et al., 1999
Apple	70% acetone	Methy-linoleate oxidation	Kahkonen et al., 1999
Carrot	80% methanol	Methy-linoleate oxidation	Kahkonen et al., 1999
Tomato	70% acetone	Methy-linoleate oxidation	Kahkonen et al., 1999
Oat	80% methanol	Methy-linoleate oxidation	Kahkonen et al., 1999
Cherry	Methanol+0.01%HCL	ORAC; TEAC	Blando et al., 2004
Spearmint	95% ethanol	ABTS	Arumugam et al., 2006
Potato	Water	β -carotene oxidation	Al-Saikhan et al., 1995
Basil	80% ethanol w/t 0.1% HCL)	TEAC; FRAP	Juliani and Simon, 2002
Pineapple	70% methanol	Liposome oxidation; β -carotene oxidation	Hassimotto et al., 2005
Eggplant	70% methanol	Liposome oxidation; β -carotene oxidation	Hassimotto et al., 2005
Pepper	80% methanol	Ethanol–linoleic acid oxidation; DPPH	Yamazaki et al., 2007

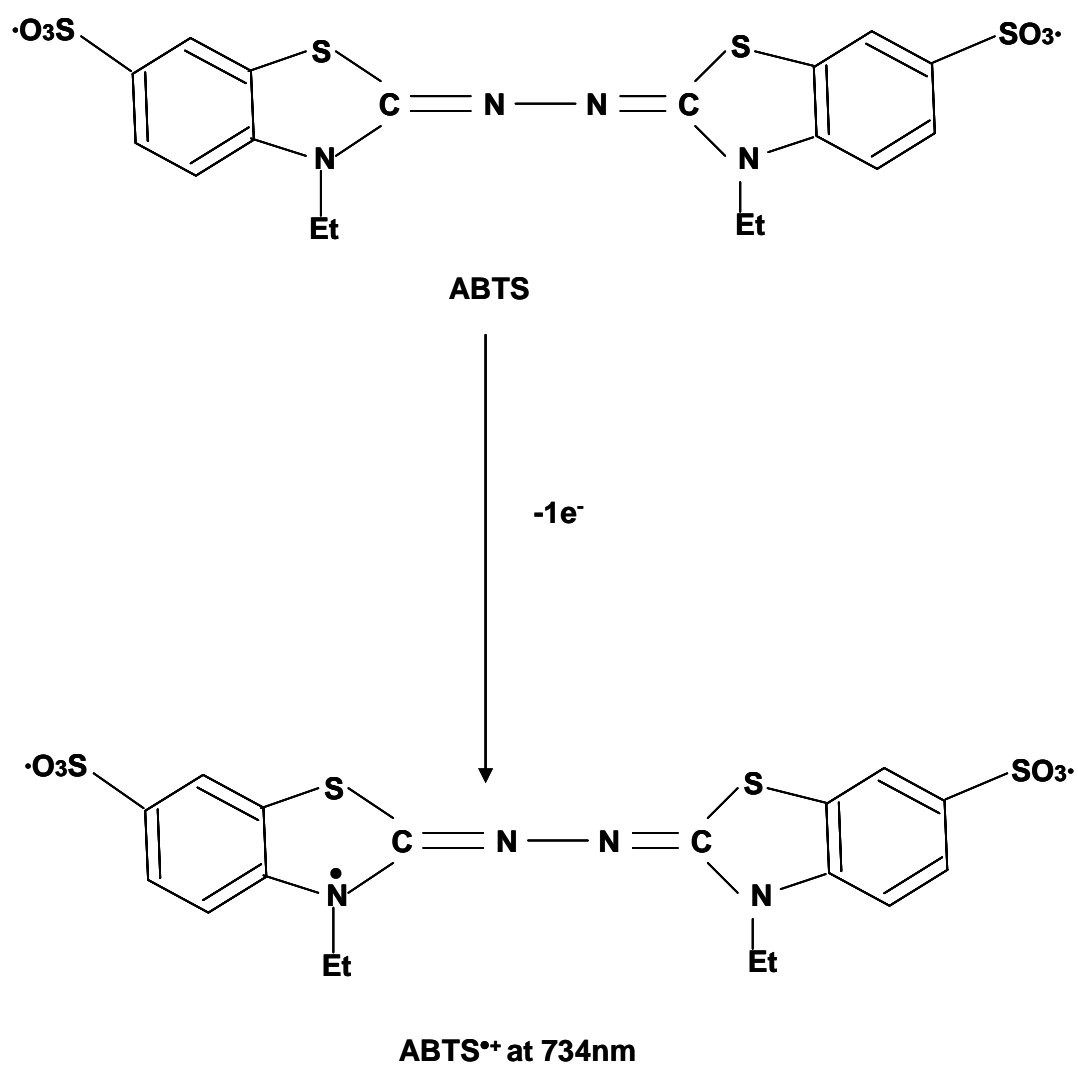


Figure 2.5. Schematic representation of oxidation of ABTS to $\text{ABTS}^{\bullet+}$

antioxidant, trolox, which is an analogue of tocopherol.

2.4.3.1.2. DPPH assay

The 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) is one of the few stable organic nitrogen radicals, and has a purple color. The radicals absorb at 517 nm. Antioxidant activity can be determined by monitoring the decrease in the absorbance. The result is reported as the amount of antioxidant utilized to decrease the initial DPPH concentration by 50%. The assay is simple and rapid; however, the interpretation is difficult when the test samples have maximum absorption in the range of UV-light that overlaps with DPPH at 517 nm (Prior et al., 2005).

2.4.3.2. Metal-chelation methods

There are several metal-chelating methods used to evaluate antioxidant activity. The Phycoerythrin assay is described below.

2.4.3.2.1. Phycoerythrin assay

The highly fluorescent protein phycoerythrin (PE) has been used to assess the effectiveness of antioxidants against free radicals. The radicals are produced from an ascorbate- Cu^{2+} system at copper-binding sites on PE. Antioxidants protect against damage by chelating Cu^{2+} necessary for site-specific radicals formation (Antolovich et al., 2002). The antioxidants' capability is examined by the retardation of the loss of

fluorescence.

2.4.3.3. Reducing power methods

There are a number of reducing power methods used to evaluate antioxidant activity, such as FRAP assay and the copper reduction assay.

2.4.3.3.1. FRAP assay

The Ferric Reducing Antioxidant Power (FRAP) method is based on the reduction of a ferroin analog at acidic condition. Tripyridyltriazine $\text{Fe}(\text{TPTZ})^{3+}$ is reduced to $\text{Fe}(\text{TPTZ})^{2+}$, generating a blue color, the intensity of which is measured at 595 nm. Reduction/Oxidation (Redox) reactions and resultant changes from radicals to ions can end as radical chains. Reducing power thus reflects the ability of antioxidants to modify the redox reactions in a system. This reaction detects antioxidants with a redox potential of $< 0.7 \text{ V}$ (the redox potential of Fe^{3+} -TPTZ) (Prior et al., 2005).

2.4.3.3.2. Copper reduction assay

This assay is based on the reduction of Cu^{2+} to Cu^{1+} by the action of antioxidants. Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) can form complex with Cu^{1+} in a 2:1 ration, yielding a chromophore with maximum absorption at 490 nm. The reaction rate and concentration of antioxidants are calculated from the generation of bathocuproine- Cu^{1+} complex in the system (Prior et al., 2005).

CHAPTER 3

EXTRACTION AND CHARACTERIZATION OF POLYPHENOL OXIDASE IN PAWPAW (*ASIMINA TRILOBA*) FRUIT

3.1. Summary

Crude PPO was extracted from pawpaw (*Asimina triloba*) fruit pulp, partially purified by gel filtration, and electrophoresed for molecular weight (MW) determination. The enzyme activity was assayed by monitoring the absorbance (420 nm) of the PPO extract-catechol mixtures over time. The PPO activity varied with the pH and was the highest at pH 6.5–7.0. The crude enzyme exhibited a maximum activity at 5–20 °C, but, a brief exposure to 40–80 °C resulted in a rapid decline or complete loss of the enzyme activity. The enzyme kinetics based on the reaction rate in the linear region (0–60 s) showed a V_{\max} of 0.1363 s⁻¹ and a K_m of 0.3266 M for the crude PPO. The PPO existed as two isoforms with MWs of 28.2 kDa and 38.3 kDa, respectively.

3.2. Introduction

Pawpaw (*Asimina triloba*) fruit is a climacteric fruit grown in eastern United States (Archbold and Pomper, 2003). With its intense flavor resembling a combination of banana, mango and pineapple, and its high phytonutrient content, pawpaw fruit is being used in value-added products such as ice creams, yogurts, and baked foods (Pomper and Layne, 2005). However, pawpaw fruit pulp is susceptible to brown discoloration, suggesting that polyphenol oxidase (PPO) might be involved.

The physiology and biochemistry of pawpaw fruit associated with ripening, especially during postharvest storage, have not been thoroughly investigated. McGrath and Karahadian (1994) have shown that as pawpaw fruit ripens, the content of soluble solids content increases and the flesh softens. The latter process appears to result from an enhanced activity of enzymes, such as polygalacturonase, endo-(1→4) β -D-glucanase, endo- β -1,4-mannanase, and pectin methylesterase, that degrade the cell wall (Koslanund et al., 2005). Furthermore, the volatile compound production increases rapidly, a result of increased respiration and ethylene generation (Archbold and Pomper, 2003). Currently, there is no published study on the activity of PPO that may be present in pawpaw fruit pulp.

The enzyme PPO (EC 1.10.31) is found in the tissue of a wide range of fruits and vegetables and is responsible for the browning discoloration observed in these food materials (Dry and Robinson, 1994; Broothaerts et al., 2000; Chen et al., 2000;

Okot-Kotber et al., 2002; Leja et al., 2003; Yang et al., 2004; Casado-Vela et al., 2005). PPO catalyzes mono- and *o*-diphenol conversion to *o*-quinones during ripening and during postharvest handling, storage and processing (Richard-Forget et al., 1998; Nkya et al., 2003; Casado-Vela et al., 2005; Spagna et al., 2005; Selles-Marchart et al., 2006).

The activity of PPO varies between fruits and vegetables (Nunez-Delicado et al., 2005). PPO extracted from most plants and fruits is capable of oxidizing *o*-diphenol, while mushroom PPO can catalyze both mono- and *o*-diphenol oxidation (Cash et al., 1976). Because PPO-catalyzed oxidative reaction in fruits and vegetables can negatively affect the appearance, its presence in fresh products could reduce their shelf-life and consumer acceptance, and therefore, their economical value (Richard-Forget et al., 1998; Nunez-Delicado et al., 2005).

While pawpaw fruit pulp is known to be susceptible to brown discoloration, the possible involvement of PPO is not clear. Identification of PPO and its pH and temperature dependency in pawpaw fruit pulp is important because the information will be valuable for the development of treatment technology and storage conditions to inhibit undesirable browning reactions. The objective of this study was to establish the PPO activity in pawpaw fruit pulp under varying pH and temperature conditions, and to characterize its enzyme kinetics.

3.3. Materials and Methods

3.3.1. Materials

Pawpaw (*Asimina triloba*) fruit were harvested in the fall of 2005 at Kentucky State University Research and Demonstration Farm in Frankfort, Kentucky. Freshly prepared pulp homogenate (from approximately 100 pawpaw consisting of about 20 fruit each of the varieties Shenandoah, NC-1, 10-35, 1-23 and 1-68) were divided into 30 individual, equal-sized portions, placed in Zip-Loc plastic bags, sealed, and stored in a -20°C freezer before use within 4 weeks. Reagent grade catechol, polyvinylpyrrolidone, Amberlite XAD-4, and Triton X-100 were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

3.3.2. Crude enzyme extraction

Five crude PPO extracts were prepared using different, random bags of pawpaw fruit pulp, and each extract was used for an independent trial (replicate experiment). The crude enzyme was prepared by mixing 20 g of pawpaw fruit pulp with 30 mL of 0.2 M sodium phosphate ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) buffer (pH 6.5) containing 5% (w/v) polyvinylpolypyrrolidone (PVPP), 2% (w/v) Amberlite XAD-4, and 2% (v/v) Triton X-100 (Cheng and Crisosto, 1995). The mixture was stirred at room temperature for 5 min to extract PPO and subsequently chilled in an ice slurry for 5 min. It was then filtered through four layers of cheesecloth and centrifuged at $20,000 \times g$ for 20 min at 4°C . The

supernatant, which contained PPO, was kept at 0 °C (on ice) before subjecting to pH and temperature treatments and enzyme activity measurements. The supernatant crude PPO extract was also fractionated and subjected to molecular weights (MWs) determination.

3.3.3. Enzyme purification and separation

Solid ammonium sulfate was added to the supernatant of the crude PPO extract to attain an 80% molar saturation. After 2 h setting at 0 °C, the mixture was centrifuged at $20,000 \times g$ for 20 min. The precipitate was collected, redissolved in a small volume of 0.1 M sodium phosphate buffer (pH 6.5), and then dialyzed overnight against the identical buffer in a dialysis bag (MW cutoff < 12–14 kDa; dialyzing medium changed two times).

The dialyzed PPO (referred to as ‘partially purified PPO’) was subjected to fractionation at room temperature by low-pressure gel filtration chromatography with a Sephadex G-25 (Pharmacia, Piscataway, NJ) column according to Pena-Ramos et al. (2004). The chromatography column was packed in the laboratory using a 1.6 cm (dia.) \times 70 cm (l.) glass column with a thermal control jacket, a packaging reservoir (Pharmacia RK 16/26, Piscataway, NJ), a varistaltic pump (Monostat, Barrington, IL), and a fraction collector (Model 2110, Bio-Rad, Hercules, CA). The Sephadex G-25 gel was prepared by swelling the resin in an excess of elution buffer and heating the mixture for 1 h at 90 °C in an Isotemp 3013S refrigerated circulating waterbath (Fisher Scientific, Pittsburgh, PA).

To carry out the fractionation and PPO separation, partially purified PPO was diluted with 0.1 M sodium phosphate buffer (pH 6.5) to a 0.5 mg/mL protein solution, and 10 mL of the diluent was loaded to the Sephadex column. The varistaltic pump was set to allow a 1 mL/min flow rate of the elution buffer (0.15 M NaCl, 0.1 M sodium phosphate buffer, pH 6.5) through the column. Fractions of 4.0 mL aliquots were collected and the absorbance measured at 280 nm. Each of the fractions was also assayed for PPO activity at 420 nm. The fraction showing the maximum PPO activity was subjected to electrophoresis to identify the MW of the enzyme.

3.3.4. Electrophoresis

The crude PPO extract, partially purified PPO, and the fraction exhibiting the maximum PPO activity, were individually mixed (1:1, v/v) with an electrophoresis sample buffer (10% glycerol, 4% SDS, 0.5 M Tris, pH 6.8, 10% β -mercaptoethanol) and then heated in boiling water (100 °C) for 3 min. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) using a 15% polyacrylamide resolving gel stacked with a 3% polyacrylamide stacking gel in a Mini-Protean 3 Cell electrophoresis system (Bio-Rad, Hercules, CA). Electrophoresis was started with a 20 mA constant current, which was increased to 40 mA after the protein samples had entered the resolving gel. The gel was stained for proteins with Coomassie Blue.

To determine the MWs of protein bands, a cocktail protein standard with 9 marker

proteins (6.5–200 kDa) (Sigma Chemical Co., St. Louis, MO) was electrophoresed together with the PPO samples. MWs of sample protein bands were estimated from the regression line of log MWs versus the migration distance of standard proteins.

3.3.5. Assay for enzyme activity.

Because the gel filtration yielded only small quantities of partially purified PPO and the composition of the purified enzyme was similar to that of crude PPO extract (results described later), the PPO activity assay (pH and temperature effects as well as the enzyme kinetics) was carried out only with crude PPO extracts. The PPO activity was determined at 22 °C (unless otherwise specified) by the oxidation reaction of a phenolic compound at 420 nm (Cheng and Crisosto, 1995). The PPO extract was diluted 6 times (1:5, v/v) with a 0.1 M sodium phosphate buffer (pH 6.5). This degree of dilution allowed the absorbance values of the assay solutions to be within the spectrophotometric linear range, as established in our preliminary trials. In the PPO activity assay, 1 mL of the diluted enzyme solution was mixed with 4.6 mL of 0.1 M of appropriate buffer (pH 4.0–8.5). After 5 min equilibration at room temperature (22 °C), 0.4 mL of 0.2 M catechol (substrate) was added. The enzyme-substrate mixture was immediately transferred into a quartz cuvette and the absorbance at 420 nm was read at 15 s intervals up to 600 s. The PPO sample blank contained all the reagents except that the 1 mL of the diluted PPO solution was replaced by 1 mL of the 0.1 M sodium phosphate buffer (pH 6.5).

The absorbance (420 nm) of the assay solution was plotted against the reaction time

to demonstrate the enzyme kinetics, where the absorbance was found to increase linearly within the first 60 s. It was reasonable to assume that the amount of substrate converted in the linear range was proportional to the absorbance change. Thus, the activity of PPO can be expressed as the change in absorbance at 420 nm per second (instead of moles converted per unit time). With this treatment, the enzyme activity in the original extract equated the reaction rate (V) in the initial 60 s, which was calculated with the following equation (Eq. 1):

$$V (s^{-1}) = \frac{\Delta \text{Absorbance (420 nm)}}{60 \text{ s}} \times 6 \text{ (dilution factor)} \quad (1)$$

3.3.6. The pH effect

The pH optimum was determined over a pH 4.0–8.5 range. Specifically, 1 mL of the diluted (1:5, v/v) PPO extract was mixed with 4.6 mL of 0.1 M citrate acid buffer (pH 4.0, 4.5, 5.0, and 5.5) or with 4.6 mL of 0.1 M sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5). The PPO activity was then tested as described above.

3.3.7. The temperature effect

Two experiments were conducted to determine the temperature effect on PPO activity. In the first experiment, diluted (1:5, v/v) crude PPO extracts in 0.1 M sodium phosphate buffer (pH 6.5) were incubated for 10 min at 5, 10, 20, 30, 40, 50, 60, 70, or 80 °C in an Isotemp 3013S waterbath (Fisher Scientific, Pittsburgh, PA). Heated solutions were immediately chilled in an ice slurry and then centrifuged at 20 °C for 10 min to remove

particulates, if any. The supernatants were immediately subjected to PPO activity assay as described above (at 20 °C and pH 6.5).

In the second experiment, diluted (1:5, v/v) crude enzyme extracts in 0.1 M sodium phosphate buffer (pH 6.5) without heating were assayed for PPO activity at 5, 10, 15, 20, 30, 40, 50, and 60 °C isothermal temperatures. Specifically, the assay solution placed in the cuvette was maintained at the target temperatures during the entire enzyme activity test by circulating heating (cooling) water around the cuvette holder using an Isotemp 3016 refrigerated water circulator (Fisher Scientific, Pittsburgh, PA). The exact temperatures of the assay solutions were verified with a thermocouple. Before adding the PPO extract, the assay solution (containing phosphate buffer and catechol) was pre-warmed to the target temperatures. Preliminary tests indicated a lack of browning in the warm-up buffer-catechol solution (the solution turned brown only when a PPO extract was added). The absorbance at 420 nm was measured immediately after the PPO extract was mixed with the buffer-catechol solution as described above.

3.3.8. Enzyme kinetics

The reaction rate of PPO at a series of substrate concentrations was determined. Specifically, 1 mL of the diluted (1:5, v/v) enzyme extract was mixed with 4.6 mL of 0.1 M sodium phosphate buffer (pH 6.5). After the addition of 0.4 mL of catechol (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, or 0.40 M), the absorbance was immediately read at every 15 s intervals. Preliminary experiments showed that the absorbance increased linearly within

the initial 60 s. Hence, the reaction rate in the 0–60 s corresponding to each substrate concentration was calculated. The absorbance versus time, and the reaction rate versus substrate concentration, were plotted to fit the Michaelis-Menten equation and to derive the Michaelis constant (K_m) and the maximum velocity (V_{max}) (Whitaker, 1994).

3.4. Results and Discussion

3.4.1. PPO extraction and purification

The PPO, which catalyzes the oxidation of phenolic compounds into *o*-quinones in the cell, is generally associated with cell membranes (Tolbert, 1973). Thus, in the present study, the enzyme was extracted from pawpaw fruit pulp by means of a combination of the non-ion detergent PVPP, ionic anion exchange resin Amberlite XAD-4, and Triton X-100, a system that was reported to be effective, thus, widely used, to extract PPO (Cano et al., 1996; Gonzalez et al., 1999, 2000). The absence of brown discoloration in all the PPO extracts even upon heating (but without added catechol) indicated that the PVPP, which binds to phenols, was effective in removing phenolic compounds from the enzyme extract during its preparation (Ziyan and Pekyardimci, 2004). Catechol was reported to be an excellent substrate for measuring PPO activity (Cano et al., 1996) and hence, was employed in the present study.

Up to 110 fractions were collected from the Sephadex gel filtration of PPO samples. The crude PPO extract showed a relatively sharp peak (fractions 34 to 44) and a broad

peak made up of the rest of the fractions (Fig. 3.1A). However, only the sharp peak was associated with PPO activity, which was indicated by the absorbance at 420 nm in the enzyme assay. Thus, in addition to PPO, the crude enzyme extract contained other, small MW peptides and/or organic compounds that absorb UV light. Furthermore, Triton X-100 and Amberlite XAD-4 used in the enzyme extraction could contribute to the absorption.

In contrast, partially purified PPO displayed only a single peak (A_{280}) when eluted from the Sephadex G-25 column (Fig. 3.1B). When the PPO activity was specifically assayed, a single peak (A_{420}), which coincided with that of the A_{280} curve, was obtained. Therefore, the individual fractions that made up the protein peak (280 nm) were attributed to PPO. The correspondence of the maximum absorbance at 280 nm (for total protein) to that at 420 nm (for PPO) suggested that PPO was concentrated in the peak fraction, i.e., Fraction 38. The result also demonstrated that the purification procedure using saturated ammonium sulfate was effective in removing small MW substances. Solid ammonium sulfate is widely employed for PPO purification, and it is reported to concentrate PPO yielding a high activity for extracts from other fruits, such as loquat (Ding et al., 1998), banana peel (Yang et al., 2001), pineapple (Das et al., 1997), apple (Ni Eidhin et al., 2006), and chrysanthemum (Nkya et al., 2003).

SDS-PAGE was performed to identify PPO bands and to examine the purity of the enzyme extract. Crude PPO extracts (CE) contained a number of polypeptides, with a 55.5 kDa protein being the most salient component (Fig. 3.2). Because there were no definitive protein bands below 22 kDa, the substances present in fractions 45–110 of the

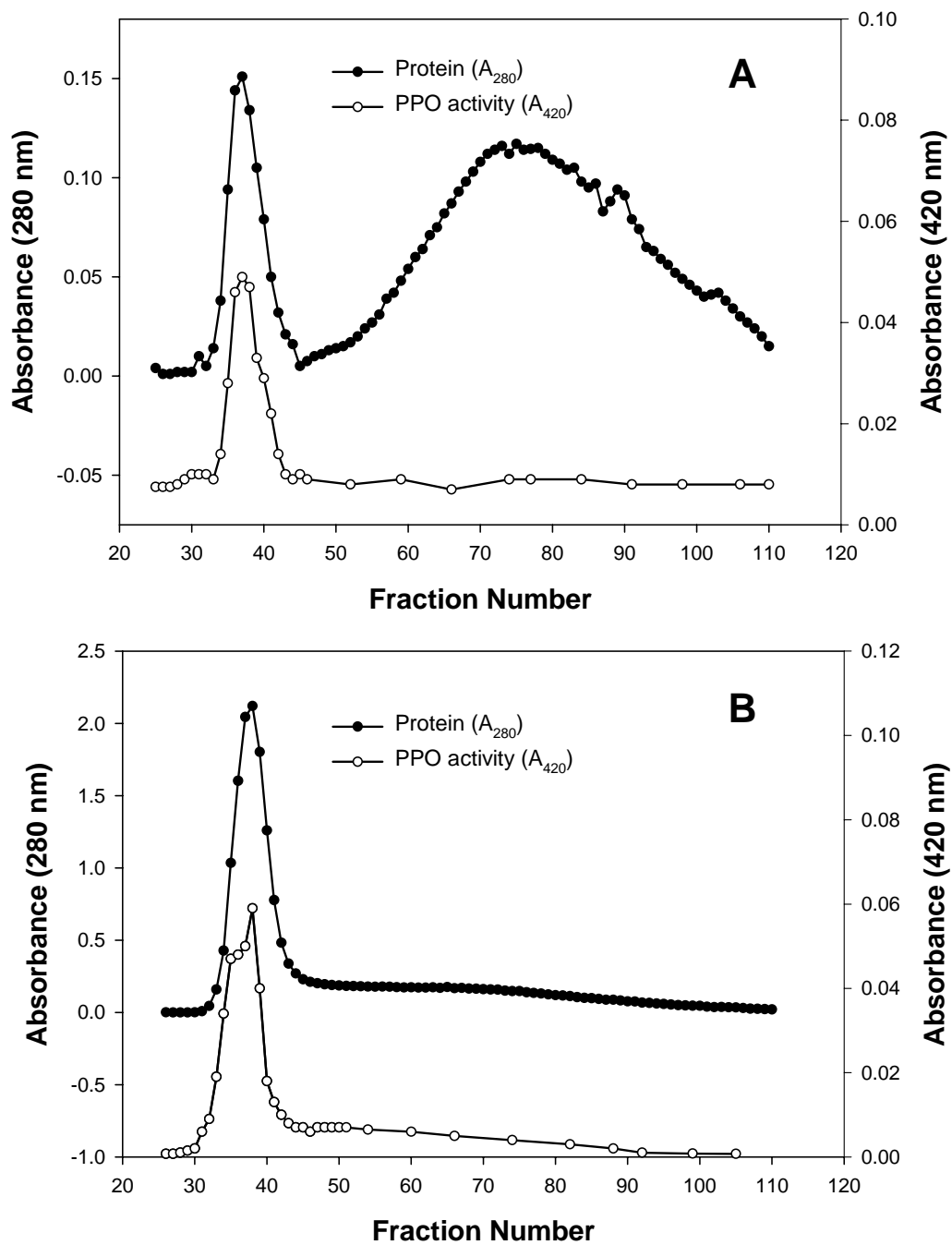


Figure 3.1. Representative elution profiles of crude PPO extract (A) and partially purified PPO (B) of pawpaw fruit pulp. The protein content (A_{280}) and the enzyme activity (A_{420}) in the eluents are plotted.

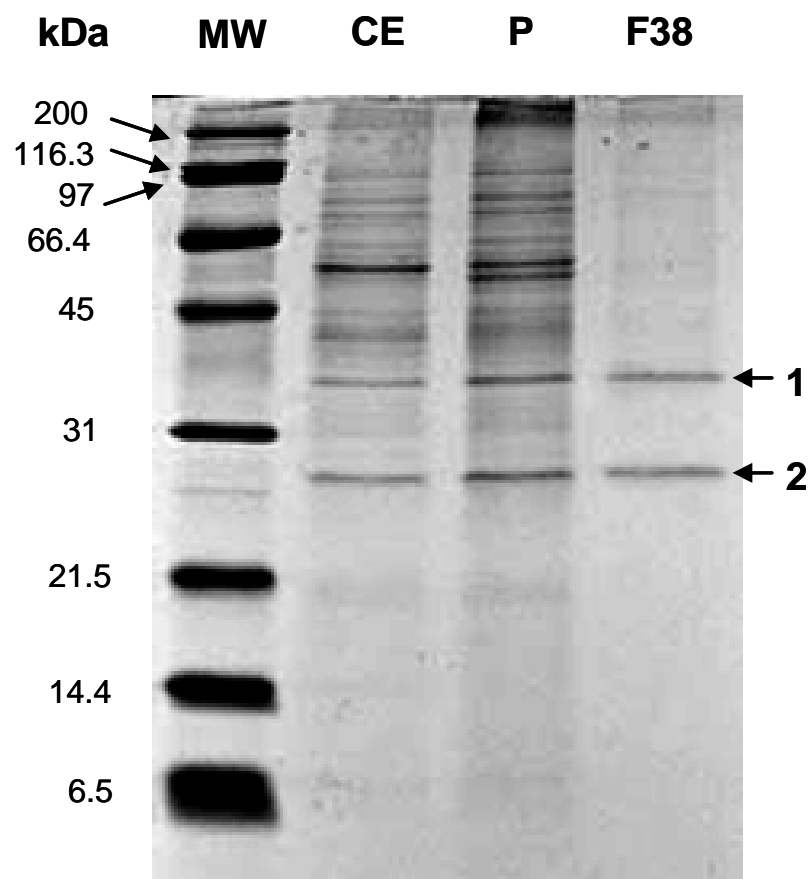


Figure 3.2. SDS-PAGE of crude and partially purified PPO of pawpaw fruit pulp (Lane MW = molecular weight standard; lane CE = crude PPO extract; lane P = partially purified PPO; lane F38 = fraction 38 from the Sephadex G25 column. Band 1 and band 2 are putative PPO isoforms).

crude PPO extract, which were responsible for the UV absorption (Fig. 3.1A), were likely non-protein materials. The partially purified enzyme preparation (P) also displayed a number of polypeptides, and the overall electrophoretic pattern was similar to that of the CE except that an additional polypeptide immediately below the 55.5 kDa band and a high MW protein (> 200 kDa) were present. Thus, the procedure used to purify PPO concentrated proteins rather than separated PPO from other proteinaceous components.

On the other hand, Fraction 38 (F38), which exhibited a maximum PPO activity (Fig. 3.1B), was quite homogeneous as it contained only two protein bands (1 and 2) (Fig. 3.2). Based on the linear regression ($Y = -0.0053 X + 1.9830$; $R^2 = 0.9996$) of \log MW (Y) versus migration distance (X) of standard proteins in the 14.4–66.4 kDa region, the MWs of the two PPO polypeptides were calculated to be 28.2 kDa and 38.3 kDa. These two protein bands, which also appeared in crude and partially purified PPO extracts, were assumed to be isoforms of the PPO enzyme. Proteomic or immunological analysis is needed to obtain a definitive identification. PPO isozymes have also been found to exist in other plant foods, such as Chinese chestnut (Lu et al., 2005), peach (Wong et al., 1971), and blueberry (Kader et al., 1997). Reported isoforms of PPO from plant and fruit sources ranged from 12 kDa to over 200 kDa in MW, but mostly within the range of 35–70 kDa (Van Gelder et al., 1997; Yang et al., 2001; Lu et al., 2005). The MW of pawpaw PPO found in the present study was smaller than those isolated from some common fruits and vegetables, including banana peel (41 kDa; Yang et al., 2001), head lettuce (56 kDa; Fujita et al., 1991), apple (65 kDa; Murata et al., 1992), and litchi (76

kDa; Jiang et al., 1999).

3.4.2. The pH effect

The crude extract of pawpaw fruit pulp exhibited significant PPO activity over a broad pH range (Fig.3.3). When the enzyme extract was mixed with the substrate, the solution instantly turned brown, which absorbed strongly at 420 nm. The absorbance increased linearly within the initial 60 s, displaying a first-order enzymatic reaction mode. The reaction reached an equilibrium in 3 to 10 min, depending on the specific pH. In the pH 4.0–6.5 range, the absorbance increased steadily within the 300 s assay time; at pH 7, it reached a plateau after 180 s; and between pH 7.5 and 8.5, the absorbance initially increased and then decreased. The pH 7.5–8.5 assay solutions showed precipitates toward the end of the enzyme assay, suggesting that pawpaw PPO was unstable at pH greater than 7.0 or that residual proteins present in the enzyme extract might have formed insoluble complexes with the reaction products, namely, oxidized catechol (quinone polymers). Since increased exposures of hydrophobic groups are expected for proteins dissolved in an alkaline solution, an enhanced hydrophobic protein-polyphenol association, which led to precipitation, probably occurred.

The dependency of PPO activity on pH followed a bell-shaped curve, and a maximum activity ($\sim 0.05 \text{ s}^{-1}$) was seen at pH about 7.0 (Fig. 3.4). This pH maximum coincided with the natural pH of pawpaw fruit pulp (pH 6.9). The common range of pH for maximum PPO activity in other fruits is between pH 6.0 and 7.0, including Spanish

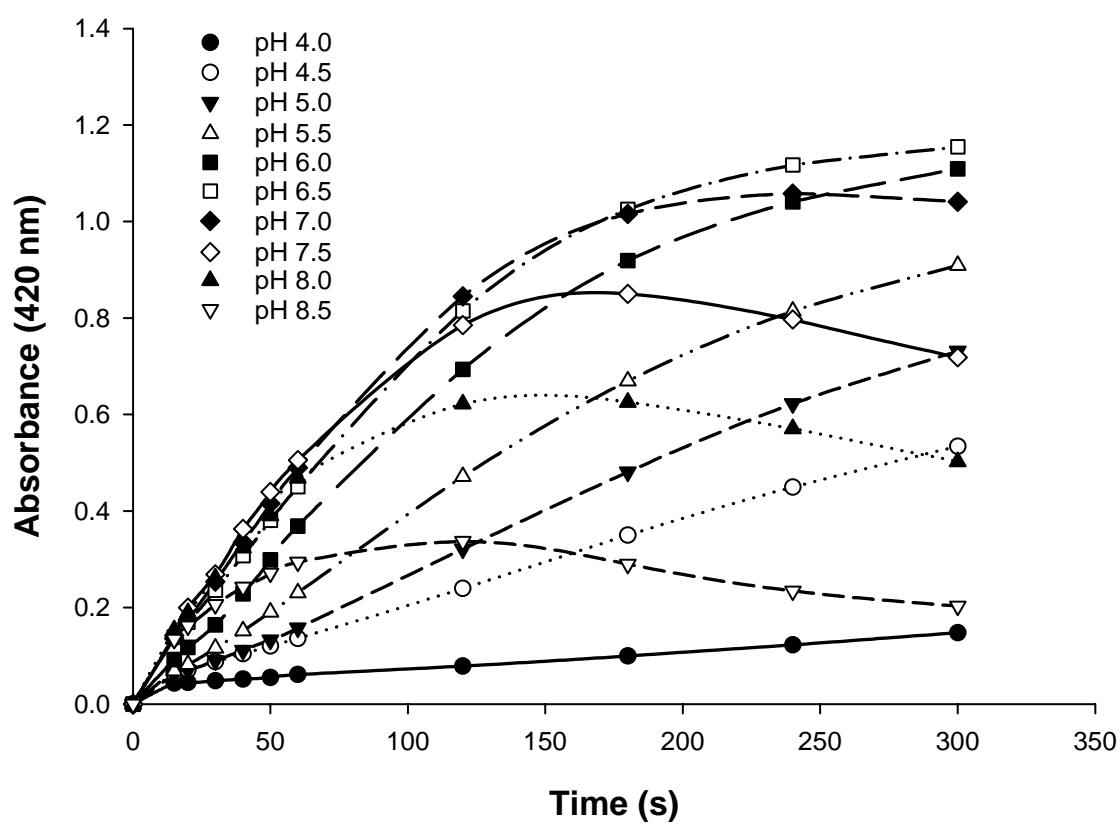


Figure 3.3. Influence of pH on the activity (absorbance of crude PPO extracted from pawpaw fruit pulp). The activity assay was conducted at 20 °C.

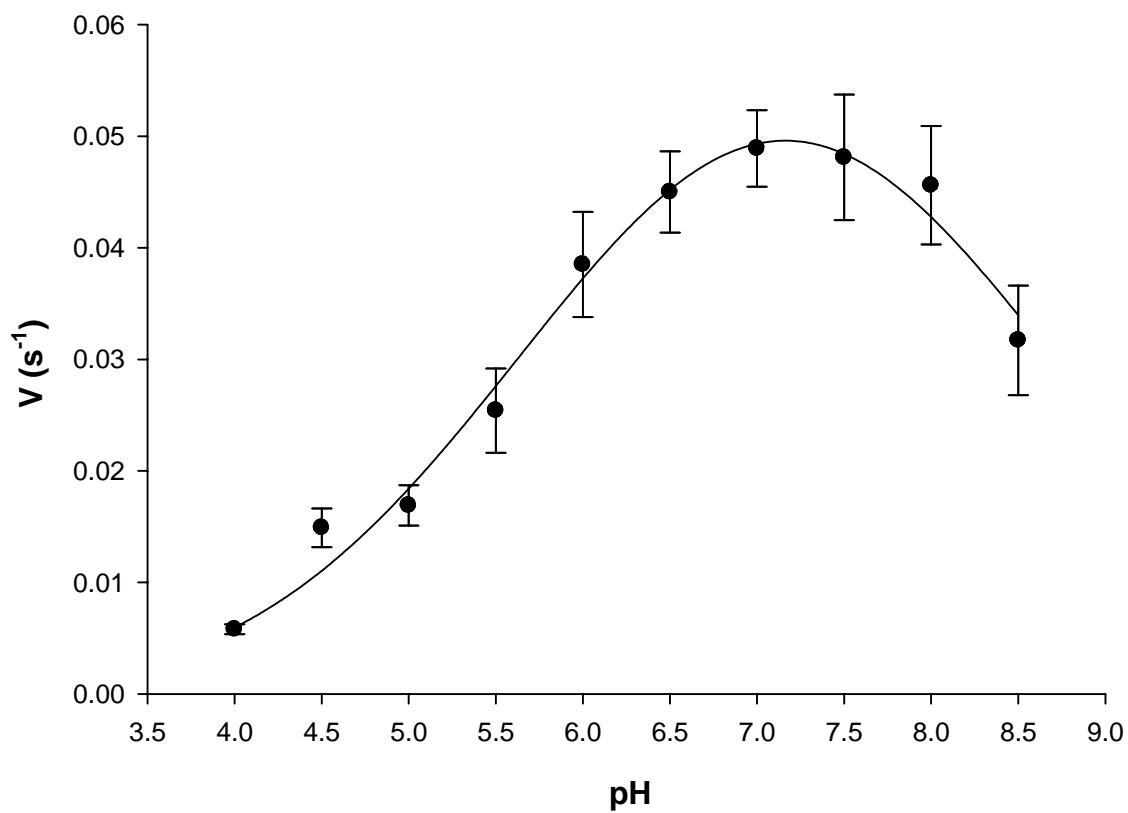


Figure 3.4. Identification of the optimum pH for crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 °C.

papaya (pH 6.5), a species that was closely similar to pawpaw (Cano et al., 1996). Other fruits susceptible to browning also show high activities of PPO at pH around 6.5–7.0, e.g., apples (Ni Eidhin et al., 2006), pear (Ziyan and Pekyardimci, 2004), banana peel (Yang et al., 2001), raspberry fruits (Gonzalez et al., 1999), and blackberry (Gonzalez et al., 2000). The residual PPO activity at pH 4.0 was about 13% of the maximum at pH 7.0.

3.4.3. The temperature effect

The activity of the crude PPO extract after a 10 min exposure to temperatures ranging from 5 to 80 °C was tested, and the results were depicted in Fig. 3.5 and Fig. 3.6. The enzyme extract incubated at 5–20 °C showed a similar, high activity ($\sim 0.065 \text{ s}^{-1}$), at 30 °C the activity started to decrease. The loss of PPO activity became remarkable at 40 °C, and, when the enzyme was heated at 70 °C for 10 min, more than 90% of the activity was lost. At 80 °C, essentially all the PPO activity diminished. The result demonstrated that crude PPO extracted from pawpaw fruit pulp could be readily inactivated by “pasteurization” temperatures. However, it was not clear whether PPO in the actual pawpaw fruit pulp, which had a high solid content and high viscosity, would be similarly affected by the heating (or blanching) temperatures as was the PPO extract.

The PPO assay conducted at constant (isothermal) temperatures (5–60 °C) revealed remarkable PPO activity when the temperature was 20 °C or less. Interestingly, there was no reduction in PPO activity when the assay temperature was lowered from 20 to 5 °C. However, it was noted that the assay solution (PPO and catechol in buffer) at $\sim 40 \text{ °C}$

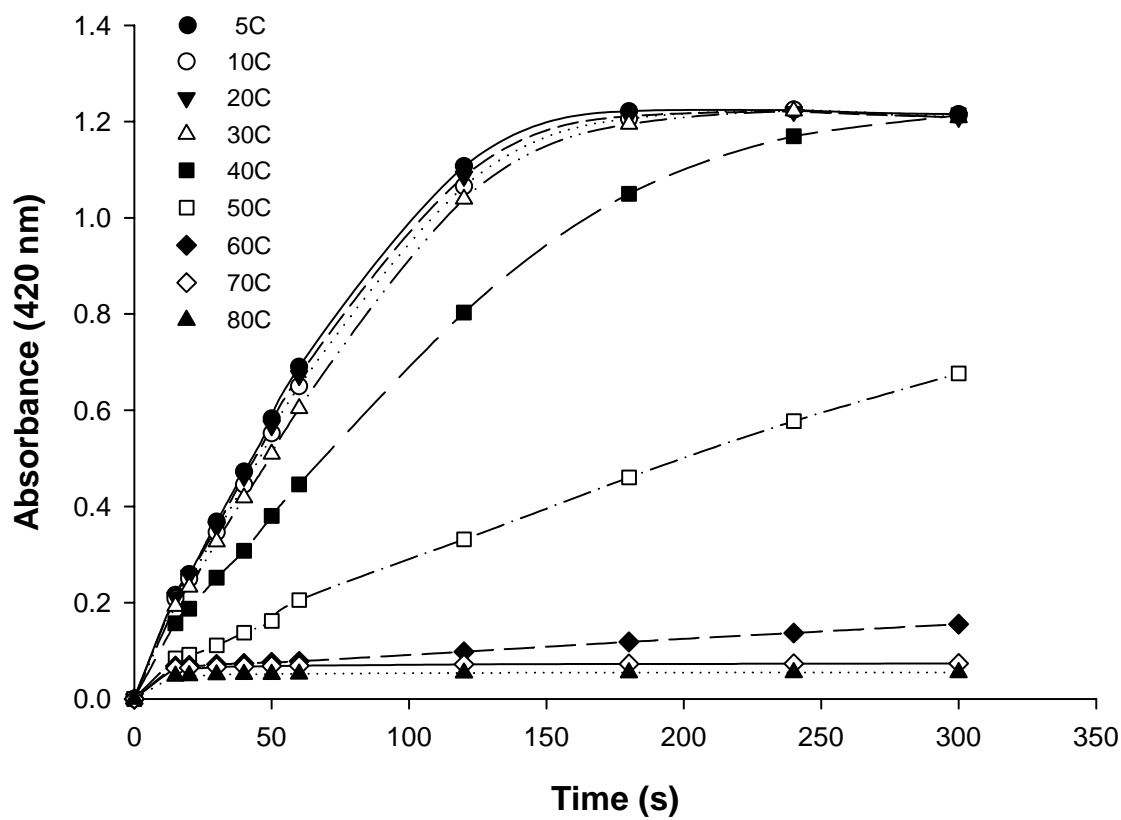


Figure 3.5. Effect of preheating (10 min) on the activity (absorbance) of crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 °C.

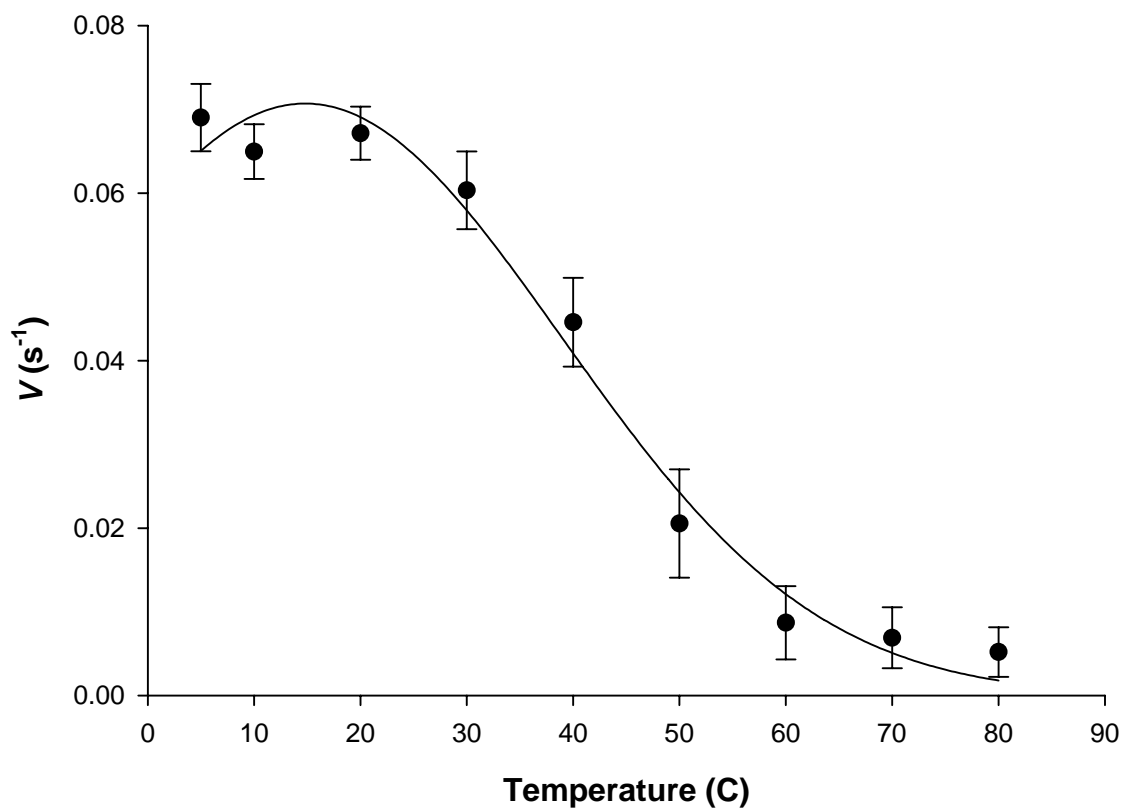


Figure 3.6. Influence of preheating (10 min) on the activity of crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 $^{\circ}\text{C}$.

formed flocculates almost instantly, which scattered the light thereby producing a falsely high absorbance (optical density) value. The flocculation was attributed to hydrophobic association of unfolded proteins. Because of the cloudiness problem, the results from this experiment were not presented. Previous research has shown the optimal temperature values of 25 °C for thymus PPO (Dogan and Dogan, 2004), 30 °C for Bramley's Seedling apple and banana peel (Yang et al., 2001; Ni Eidhin et al., 2006), 45 °C for grape (Lamikanra et al., 1992), and 70 °C for loquat fruit (Selles-Marchart et al., 2006). The high activity of pawpaw fruit pulp PPO seen at 20 °C was indicative of favorable PPO-catechol reactions that produced *o*-quinone compounds, whereas the reduced activity at higher temperatures probably resulted from thermally-induced irreversible conformation changes in PPO, i.e., denaturation. The reduced enzyme activity at temperatures above 30 °C in the present study was likely also related to protein-phenol (catechol) association.

3.4.4. Enzyme kinetics

The PPO enzyme kinetics was determined with a series of substrate concentrations (0–0.40 M). As expected, the absorbance at 420 nm increased with the concentration of catechol at equal reaction times (Fig. 3.7). The absorbance values at 60 s of reaction time (a linear region) were collected to generate the Michaelis-Menten plot (Fig. 3.8) based on the following equation (Eq. 2):

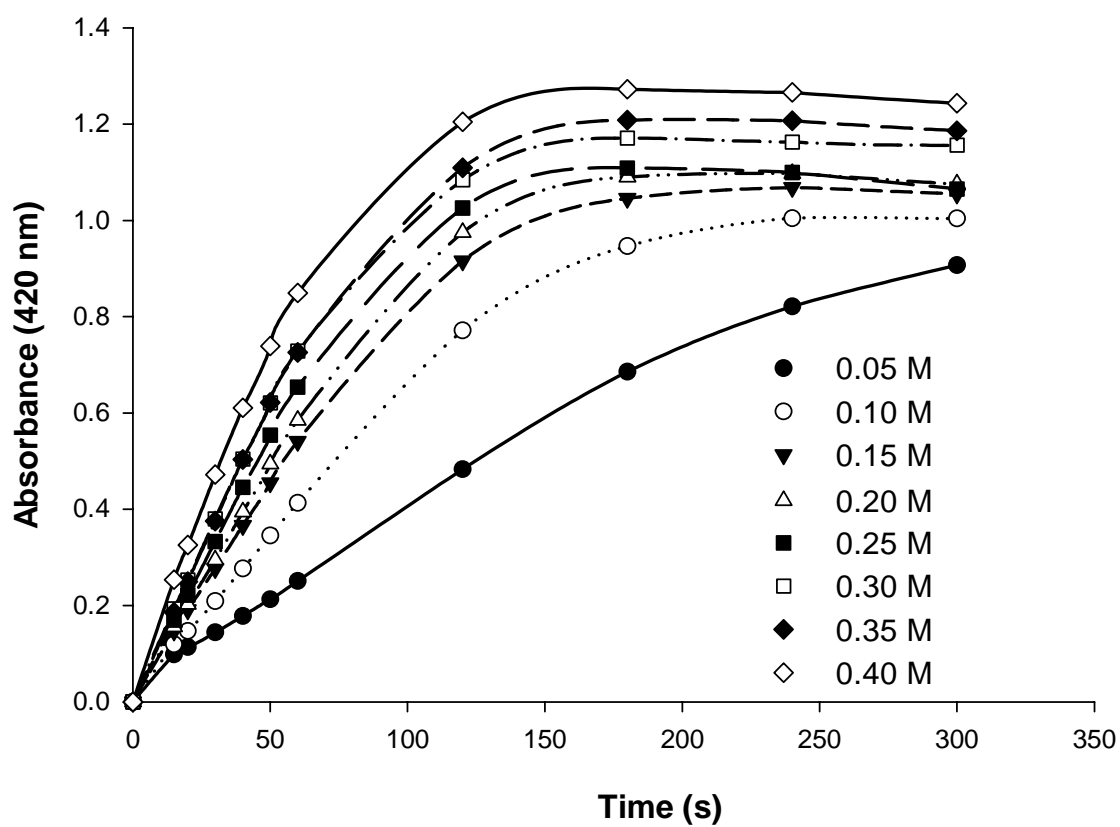


Figure 3.7. Effect of substrate concentration on the activity of crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 °C.

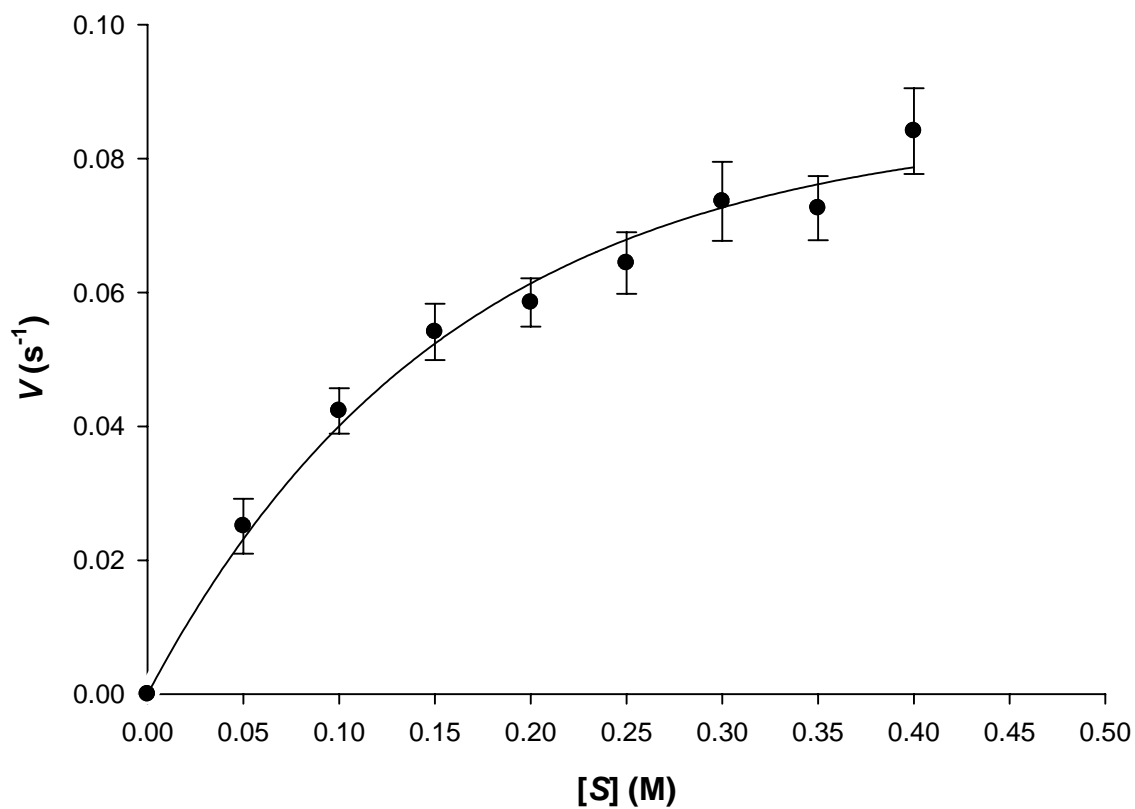


Figure 3.8. Michaelis-Menten plot of reaction rate versus substrate (catechol) concentration for crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 °C.

$$V = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (2)$$

where $[S]$ was the substrate (catechol) concentration, K_m was the Michaelis-Menten constant, and V_{\max} was the maximum enzymatic reaction rate. By rearranging Eq. 2 into Eq. 3, a Lineweaver-Burk plot (Fig. 3.9) was generated.

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (3)$$

The pawpaw PPO activity fit well with the Lineweaver-Burk equation, producing a linear $1/V_{\max}$ versus $1/[S]$ plot. The V_{\max} and K_m of the enzyme extract, based on the reaction rate in the linear range (0–60 s), were 0.1363 s^{-1} and 0.3266 M , respectively. The K_m for the PPO in pawpaw fruit pulp was comparable to K_m of PPO from Spanish papaya (Cano et al., 1996), thymus (Dogan and Dogan, 2004), apricots (Yemenicioglu and Cemeroglu, 2003), marula fruit (Mdluli, 2005), raspberry fruits (Gonzalez et al., 1999), and blackberry fruits (Gonzalez et al., 2000). However, the specific K_m value varies with fruit species, indicating different isoforms of PPO are produced by different plant species (Broothaerts et al., 2000).

3.5. Conclusions

The results from the present study demonstrated that pawpaw fruit pulp contained significant polyphenol oxidase activity. The enzyme apparently existed in two isoforms with molecular weights of 28.2 and 38.3 kDa. It exhibited the greatest activity at pH

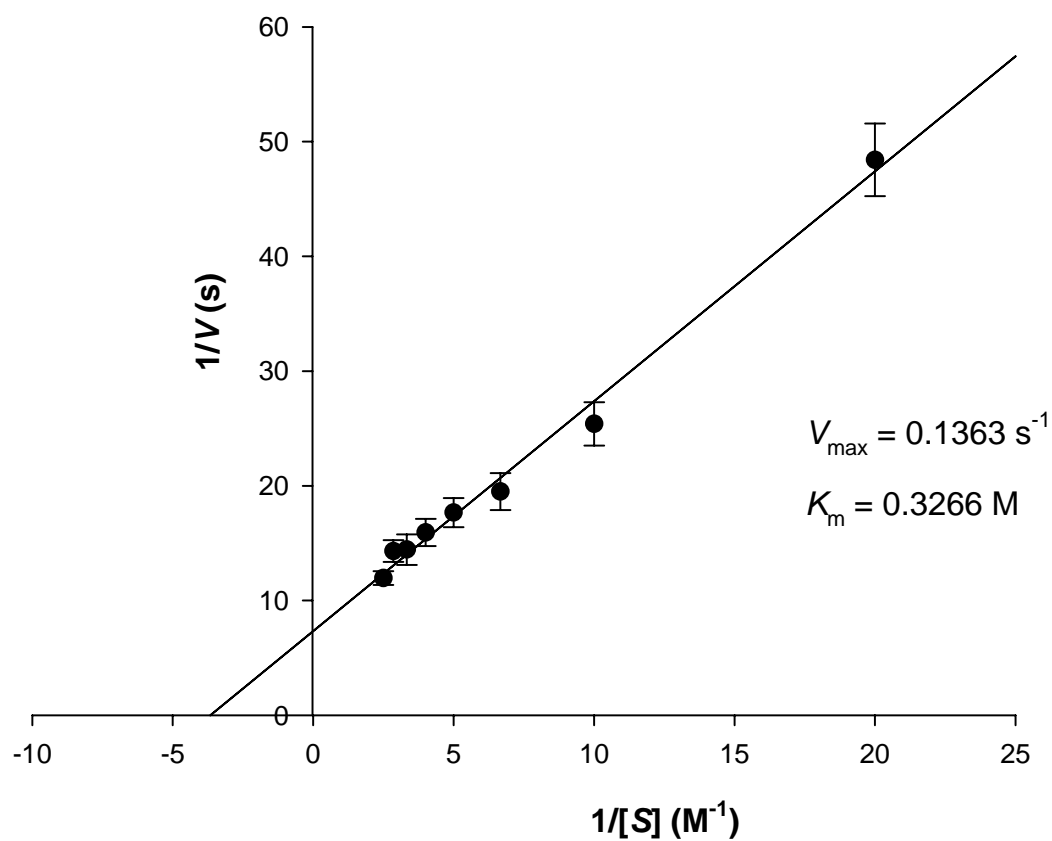


Figure 3.9. Lineweaver-Burk Plot for the activity of crude PPO extracted from pawpaw fruit pulp. The activity assay was conducted at 20 °C.

6.5–7.0 and in the temperature zone of 5–20 °C, but was susceptible to heating at above 40 °C. These findings suggested that PPO present in pawpaw fruit pulp is responsible for the brown discoloration during fruit pulp storage at ambient temperatures. The enzyme can be inactivated or inhibited by brief heating or pH adjustment thereby extending the shelf-life of the fruit pulp.

CHAPTER 4

ACTIVITY AND THERMAL STABILITY OF POLYPHENOL OXIDASE FROM DIFFERENT GENOTYPES OF PAWPAW (*ASIMINA TRILOBA*) FRUIT

4.1. Summary

Polyphenol oxidase (PPO)-catalyzed browning is an unwanted reaction for most fruit and vegetable products. In this research, PPO activity in fruit pulp of seven different genotypes of pawpaw was studied. Pulp homogenates were heated in water bath at 92 °C for 0, 1, 2, 3, 4 and 5 min. One set of samples was extracted for crude PPO and assayed for PPO activity. The second set was stored aerobically at 22 °C and the color measured with a colorimeter at 0, 1, 2, 3, 4, 5 and 20 h after the heat treatment. Additionally, PPO in each genotype was quantified by SDS-PAGE/gel band scanning. PPO activity decreased with heating time for all seven genotypes. In nonheated samples, genotype 11 exhibited the highest PPO activity and genotype 10 the lowest activity, with genotypes 3, 6, 7, 20 and 21 in the middle. The concentration of PPO in the seven cultivars was found in the range of 0.04–0.14 mg/g, and the specific PPO activity was 0.1–1.2 s⁻¹mg⁻¹. Color change (L^* , a^* and b^*) was evaluated at 20 °C for 0, 1, 2, 3, 4, 5 and 20 h. There were significant differences between genotypes as well as thermal treatments.

4.2. Introduction

Enzymatic browning is one of the biggest problems during the processing of fruits and vegetables due to the presence of endogenous polyphenol oxidases (PPO) (Yemenicioglu and Cemeroglu, 2003). PPO is present in most fruits and vegetables. This enzyme is a copper-associated oxidoreductase, catalyzing two distinct reactions involving phenolic substrates and oxygen, namely, the hydroxylation of mono-phenol to *o*-diphenol, and oxidation of *o*-diphenol to *o*-quinones. The second reaction yields a brown color. Apart from this color deterioration, the browning reaction is usually accompanied by the development of off-flavors and a reduction in nutritional value (Golan-Goldhirsh et al., 1984; McEvily et al., 1992). Thus, prevention of enzymatic browning is of great importance to the food industry.

Pawpaw is a nutritious tropical fruit, rich in protein, vitamin A and C, minerals and amino acids. A fresh and fully ripe pawpaw fruit has been described as having a strong, unique aroma which resembles a combination of pineapple, mango and banana (Duffrin and Pomper, 2006). However, the perishable nature of pawpaw fruit, combined with the lack of proper preservation methods, makes it difficult to fully market fresh pawpaw fruit in the present food industry.

Thermal treatment is generally considered as the most effective way to inactivate PPO and consequently, to prevent enzymatic browning (Weemaes et al., 1998). However, high-temperature processing could result in considerable nutritional quality as well as

sensory quality losses (e.g. destruction of vitamins and amino acids, and color and flavor deterioration) (Lund, 1977; Sapers, 1993; Weemaes et al., 1997).

Thermal resistance of enzymes is largely dependent on environmental conditions, such as pH (Weemaes et al., 1998) and the presence of sugar, salts and additives (Jolibert et al., 1994; Weemaes et al., 1997). Modified pH in food systems is known to aid thermal process efficiency (Earnshaw et al., 1995). Compared with apple, berries and other regular fruits, the influence of heating on pawpaw color stability has not been investigated.

Thermal processes cause the breakdown of fruit structures through disrupting the cell wall materials, thereby altering the product appearance. To evaluate color quality, it is necessary to quantify the pigments formed during processing (Ngo et al., 2007). Instrumental measurements of fruit color utilizing the CIE lab $L^* a^* b^*$ system is a conventional colorimetric method. Color degradation in fresh and processed strawberry products has been well studied using the colorimetric tool (Abers and Wrolstad, 1979; Bakker et al., 1992). The color stability of pawpaw fruit pulp during storage is largely unknown. The aim of this study was to assess thermal processing effect on PPO activity and color stability of pawpaw fruit pulp prepared from different genotypes.

4.3. Materials and Methods

4.3.1. Pawpaw materials

Seven genomic variations of ripe Pawpaw (*Asimina triloba*) fruit, randomly designated as genotypes 3, 6, 7, 10, 11, 20 and 21, were harvested in the fall of 2006 at the Kentucky State University Research and Demonstration Farm in Frankfort, Kentucky. For each genotype, pulp was prepared from 10–15 individual pawpaw fruit and mixed. The freshly prepared pulp homogenate was divided into 25 individual, equal-sized portions, placed in Zip-Loc plastic bags (18 cm × 20 cm), sealed, and stored in a –20 °C freezer. They were used within 4 weeks. Reagent grade catechol, polyvinylpyrrolidone (PVPP), Amberlite XAD-4, and Triton X-100 were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

4.3.2. Heating effect

Aliquots (15 g) of pawpaw fruit pulp from seven genotypes were placed in small Zip-Loc plastic bags (10.5 cm × 16.5 cm); after squeezing out the air, the bags were zipped. The pulp was flattened to a 1–2 mm thickness. Sample bags were submerged in a 92 °C circulated water bath and heated for 0, 1, 3, and 5 min (Devece et al., 1999). At the end of each of the above heating times, 4 bags were removed and chilled in an ice slurry for 10 minutes. One set of samples (2 bags as duplicate for each heating time) was extracted for crude PPO and assayed for PPO activity, and the other set (also 2 bags for

each heating time) was transferred to Petri dishes for color stability testing as described later.

4.3.3. Crude enzyme extraction

Crude PPO extracts were prepared from the fruit pulp of different genotypes of pawpaw, and each extract was used for an independent trial (replicate experiment). The extraction followed the procedure described by Cheng and Crisosto (1995) with some modifications. The crude enzyme was prepared by mixing pawpaw fruit pulp (1:1.5, v/g) with 0.2 M sodium phosphate buffer (pH 6.5) containing 5% (w/v) PVPP, 2% (w/v) Amberlite XAD-4, and 2% (v/v) Triton X-100. The mixture was stirred at room temperature for 5 min. It was then filtered through four layers of cheesecloth and centrifuged at $20,000 \times g$ for 20 min at 4 °C. The volume of the supernatant (which contained PPO) was measured for each genotype, and the supernatant was kept at 0 °C (on ice) for later enzyme activity measurement. The same crude PPO extract was also used for enzyme molecular weights determination as described below.

4.3.4. Enzyme activity measurement

The PPO activity was determined following the method described in Chapter 3. The PPO extract was diluted 6 times (1:5, v/v) with a 0.1 M sodium phosphate buffer (pH 6.5). This level of dilution allowed the absorbance values of the assay solutions to be within the spectrophotometric linear range, as established in our preliminary trials. In the PPO

activity assay, 1 mL of the diluted enzyme solution was mixed with 4.6 mL of 0.1 M of appropriate sodium phosphate buffer (pH 6.5). After 5 min equilibration at room temperature (22 °C), 0.4 mL of 0.2 M catechol (substrate) was added. The enzyme-substrate mixture was immediately transferred into a quartz cuvette and the absorbance at 420 nm was read at 10 s intervals up to 60 s. The sample blank contained all the reagents except that the 1 mL of the diluted PPO solution was replaced by 1 mL of the 0.1 M sodium phosphate buffer (pH 6.5). The absorbance (420 nm) of the assay solution at the initial 60 s in each treatment was collected to calculate PPO activity. The absorbance was found increasing linearly within the first 60 s as shown in Chapter 3.

4.3.5. Electrophoresis

Crude PPO extracts were individually mixed (1:1, v/v) with an electrophoresis sample buffer (10% glycerol, 4% SDS, 0.5 M Tris, pH 6.8, 10% β -mercaptoethanol) and then heated in boiling water (100 °C) for 3 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using a 12% polyacrylamide resolving gel stacked with a 3% polyacrylamide stacking gel in a Mini-Protean 3 Cell electrophoresis system (Bio-Rad, Hercules, CA). Electrophoresis was started with a 20 mA constant current, which was increased to 40 mA after the protein samples had entered the resolving gel. The gel was stained for proteins with Coomassie Blue. To determine the amount of PPO in the gel bands, egg white protein standard was electrophoresed together with the PPO samples.

4.3.6. PPO quantification

Images of destained SDS-PAGE gels were captured with a digital camera, and the PPO bands were quantitatively analyzed using the UN-SCAN-IT Gel™ digitizing software (Ver. 6.1, Silk Scientific Corp., Orem, UT) as described elsewhere (Xiong et al., 2007). The number of pixels in each whole PPO band was recorded and compared with total pixels of a known amount of egg white (EW) that was also electrophoresed alongside. Thus, the net amount of PPO in each extract, as well as in each g of fruit pulp, were readily obtained assuming that the amount of pixels was equivalent to the mass of protein. As shown in Chapter 3, two isoforms of PPO existed in pawpaw fruit. The ratio of the two isoforms in total pawpaw fruit pulp for each genotype was calculated from their respective pixels obtained from the SDS-PAGE patterns.

From the above-calculated amount of PPO (the two PPO isoforms combined), the activity of PPO ($\text{s}^{-1}\text{mg}^{-1}$) can be derived from the absorbance of the assay solution at 420 nm in the initial 60 s and the PPO amount in pawpaw fruit pulp as follows (Eq. 1).

$$\text{PPO activity} = \frac{v}{\text{Conc. (PPO)}} \quad (1)$$

$$v = \frac{\Delta \text{Abs}}{60 \text{ s}} \times 6 \text{ (dilution factor)} \quad (2)$$

$$\text{Conc. (PPO)} = \frac{\text{EW amount} \times (\text{PPO}_1 + \text{PPO}_2) \text{ pixel}}{\text{EW total pixel} \times 20 \text{ uL}} \quad (3)$$

where v was PPO reaction velocity calculated in Eq. 2. The concentration of PPO in each genotype was shown in Eq. 3.

4.3.7. Colorimetric measurement

Heated pawpaw fruit pulp samples were transferred into plastic Petri dishes [100 mm (dia.) \times 15 mm (depth)] and stored at room temperature (20 °C). The color of pawpaw fruit pulp was determined by using Chroma Meter CR400 (Konica Minolta Sensing, Inc. Japan) at 0, 1, 2, 3, 4, 5 and 20 h after the heat treatment. The values were described as lightness L^* , redness a^* , and yellowness b^* .

4.3.8. Statistical Analysis

The SAS program (SAS Institute, Cary, NC) was run for the colorimetric L^* , a^* , and b^* analysis; multivariate analysis of variance (MANOVA) was done to determine the heating and storage time effects. The STATISTIX 7.0 software (Analytical Software 2000) was used for other data analysis where a general linear model was employed. When the main treatment effects were found significant, differences between means were separated by the LSD test.

4.4. Results and Discussion

4.4.1. PPO activity in crude extracts

The pH 6.5 was used in all assay systems based on our previous results (Chapter 3) which showed that pawpaw fruit PPO had maximum activity at this pH. Figure 4.1 shows PPO activity in different genotypes as a function of reaction time at 20 °C. As expected, the

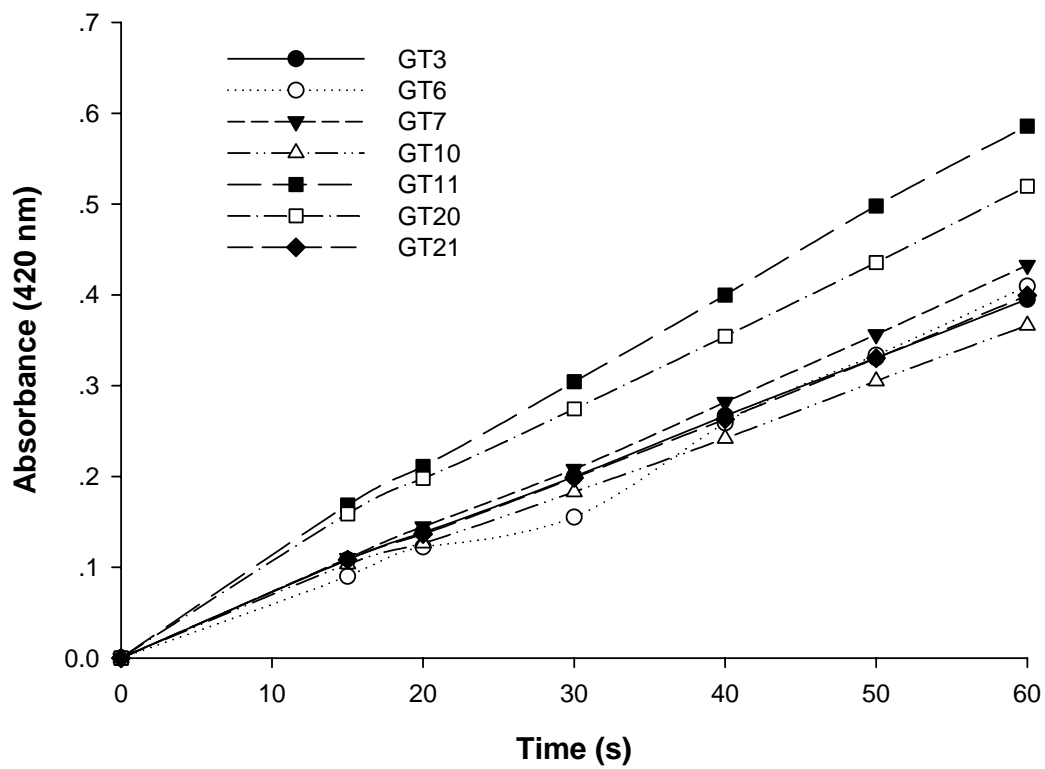


Figure 4.1. Activity of crude PPO extracts from fruit pulp of seven genotypes (GT) of pawpaw. The activity, expressed as absorbance at 420 nm, was assayed at 20 °C

absorbance of all seven genotypes exhibited a linear increase within the initial 60 s. The highest absorption was found in genotype 11, the lowest in genotype 10; and genome types 3, 6, 7, 20 and 21 were in the middle, indicating their PPO activities in this order.

4.4.2. PPO quantification

The electrophoretic patterns of PPOs in pawpaw crude extracts are displayed in Fig. 4.2. Egg white was used as a standard for comparison. An aliquot of 10 μ L of egg white solution (1 mg/mL protein) was loaded into the well. Each lane represented one genotype. Two isoforms, designated PPO₁ and PPO₂, with molecular weights of 38.3 kD and 28.2 kD, were identified in pawpaw crude extracts in our previous study (Chapter 3). It was apparent that these two bands had different contents in these seven cultivars. The PPO₁ isoform was ubiquitous in all varieties but was expressed with different intensities. Genotypes 6, 7, and 11 showed an appreciable PPO₁ band. PPO₂ was also detected in all genotypes except genotype 7, and it had the highest intensity in genotypes 6 and 11. There were significant differences between the seven genotypes and PPO isoforms ($P < 0.001$). PPO₂ had a significantly higher percentage compared to PPO₁ in genotypes 3, 6, 10, 11 and 21 ($P < 0.05$), and was similar to genotype 20 (Fig. 4.3A). In fact, the PPO₂ isoform was about 2 times more abundant than PPO₁ isoform in genotypes 6 and 11 (Fig. 4.3B).

Of the seven pawpaw genotypes, genotype 6 showed the highest PPO content ($P < 0.001$). Hence, genotype 6 was selected for further analysis to understand the structure of

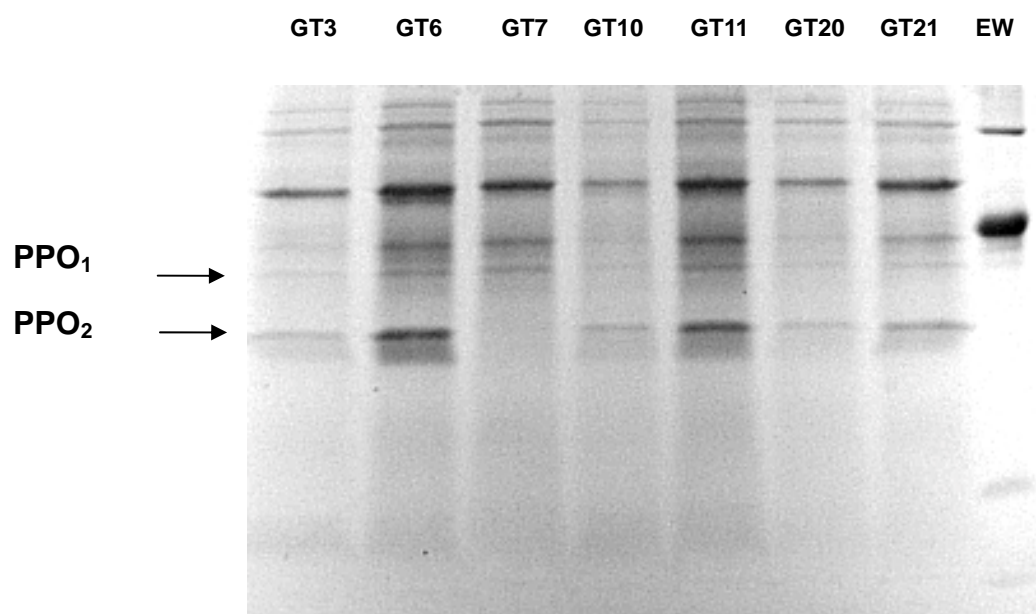


Figure 4.2. SDS-PAGE of crude enzyme extracts from seven genotypes (GT) of pawpaw fruit. EW = egg white (as standard). The two PPO isoforms are indicated.

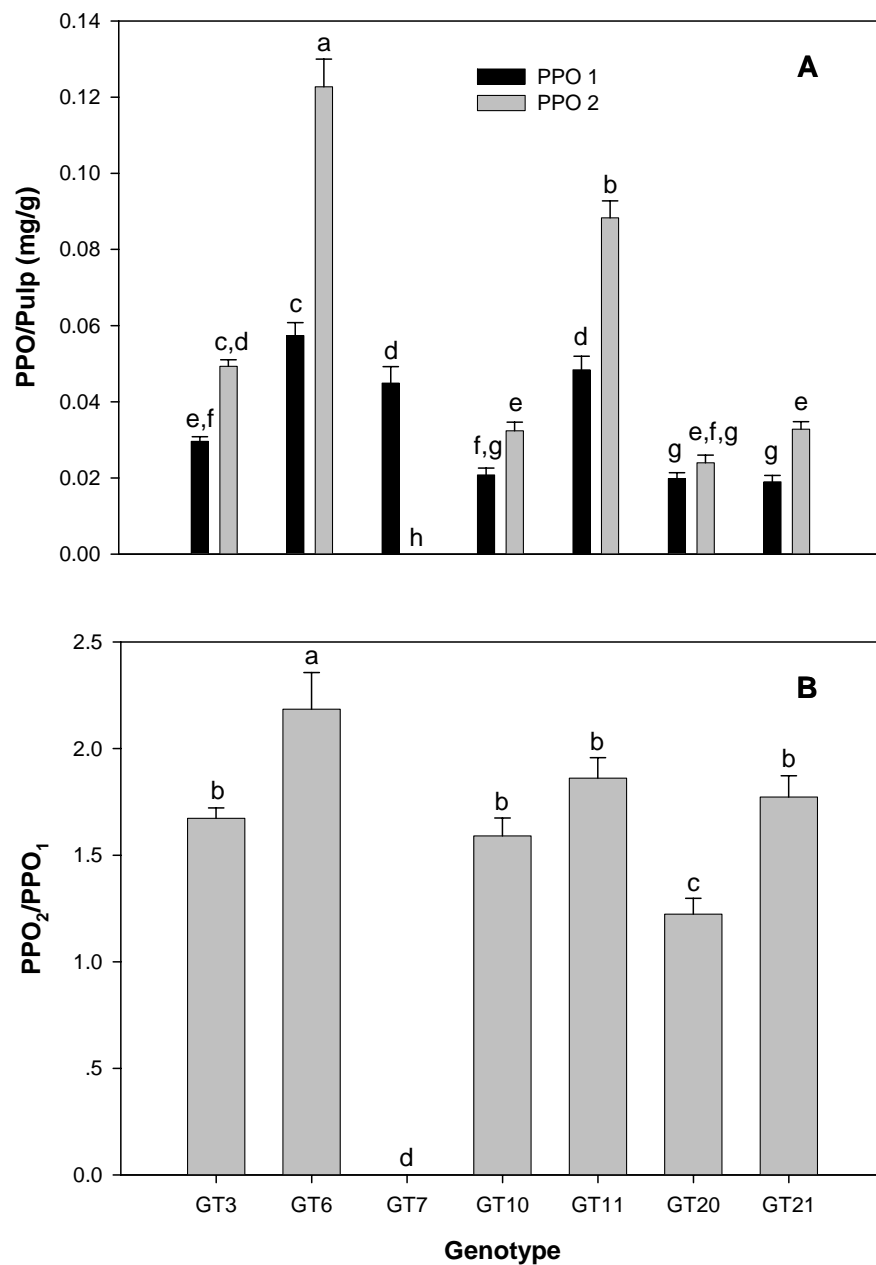


Figure 4.3. The concentration of PPO in pawpaw fruit pulp of seven genotypes (GT) (A), and the ratio of the two enzyme isoforms (PPO₂/PPO₁) (B). The symbols a–g indicate significant differences ($P < 0.001$) between genotypes and PPO isoforms.

PPO isoforms. Specifically, β -mercaptoethanol, a reducing agent that breaks the disulfide bonds, was used to treat the PPO samples. The two distinct PPO bands (PPO₁ and PPO₂) shown in the sample with β -mercaptoethanol also appeared in the sample containing no β -mercaptoethanol (Fig. 4.4). Thus, it can be concluded that PPO₁ and PPO₂ were indeed most likely two isoforms and not two enzyme subunits linked by disulfide bonds. The co-existence of two forms of PPO was not unique to pawpaw. Previous research has revealed a PPO doublet of 60 kDa and 69 kDa in purified potato extract (Partington and Bolwell, 1996). Harel and Mayer (1968) reported three isoforms of PPO from apple extract with MWs 30–40 kDa, 60–70 kDa and 120–130 kDa, respectively. Multiplicity of MW has also been observed in PPOs from avocado and banana (Mayer and Harel, 1979). The structure of PPO in some higher plants is more complex. For example, PPO in Indian pineapple fruit is a tetramer of identical subunits with a 25 kDa MW each (Das et al., 1997).

4.4.3. PPO thermal stability

The thermal activity of PPO is known to depend greatly on fruit types. To determine whether PPO from different pawpaw genotypes was equally sensitive to heat, crude PPO was extracted from preheated pawpaw fruit pulp, i.e., after heat treatment at 92 °C for 0, 1, 2, 3, 4 and 5 min, and then assayed for activity against catechol (Figs. 4.5 and 4.6). The activity of PPO was expressed as velocity divided by the PPO concentration. The PPO activity exhibited significant differences among seven genotypes. The highest initial

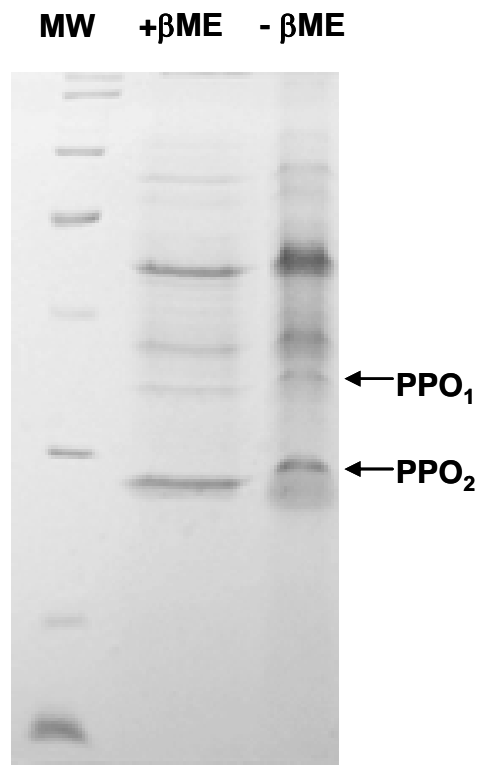


Figure 4.4. SDS-PAGE of crude enzyme extracts from genotype 6 of pawpaw fruit. +βME = sample with β-mercaptoethanol; -βME = sample without β-mercaptoethanol. The two PPO isoforms are indicated.

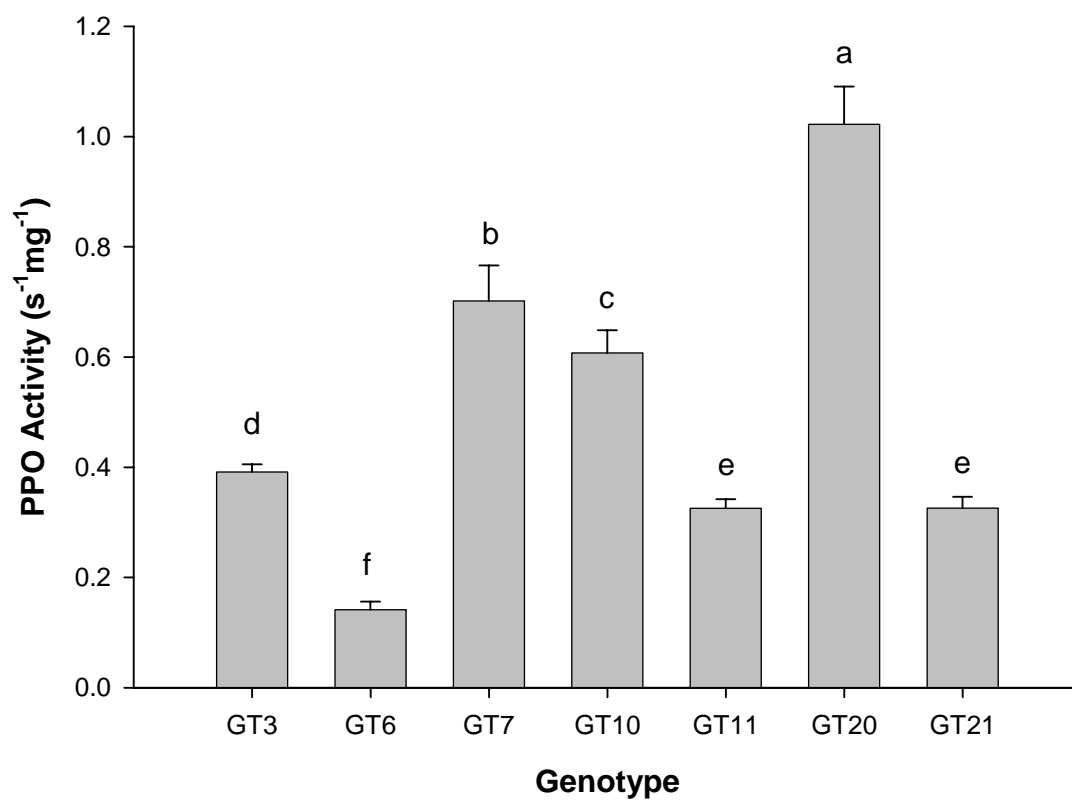


Figure 4.5. PPO activity (s⁻¹mg⁻¹) in seven pawpaw fruit genotypes (GT). The PPO activity was expressed as PPO reaction velocity per mg PPO in pawpaw fruit. The symbols a–f indicate significant differences ($P < 0.001$) between genotypes.

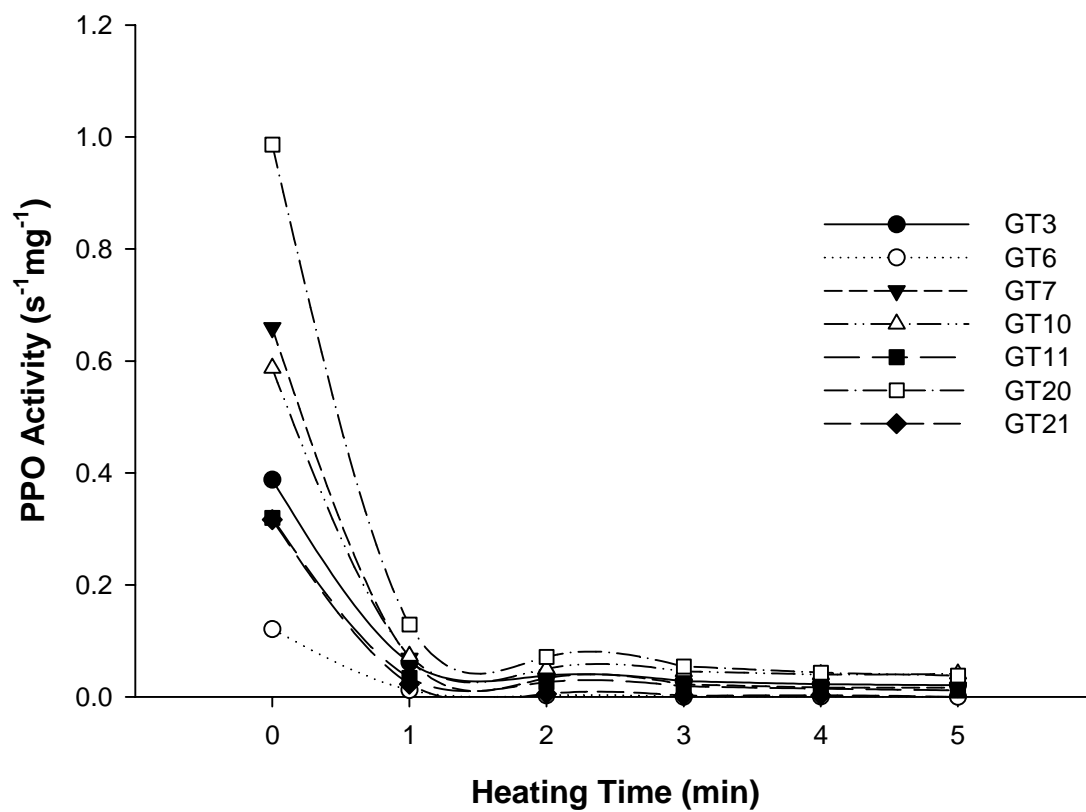


Figure 4.6. PPO activity in seven genotypes (GT) of pawpaw fruit after heat treatment at 92 °C for 0, 1, 2, 3, 4 and 5 min. The data shown in the plot were calculated from the absorbance values at 420 nm at 60 s.

activity of PPO in pawpaw fruit was found in genotype 20, which was about 7 times higher compared to genotype 6, followed by genotypes 7, 10, 3, 21 and 11.

It is noteworthy that, although genotype 6 had the most abundant PPO, the enzyme activity was the lowest. In contrast, although genotype 20 contained the least amount PPO, the enzyme exhibited the greatest activity ($P < 0.001$). The results indicated that the activity of PPO is inherent to the specific genetic variety within the big pawpaw superfamily.

Figure 4.6 illustrates the time for PPO inactivation after waterbath heating at 92 °C. The enzyme activity decreased markedly after the samples were heated for 1 min. It reached almost the baseline level after 1 to 2 min of heat treatment, indicating that PPO from pawpaw fruit was labile to heating. The heat treatment had the greatest effect on genotype 20 and the least effect on genotype 6. The thermal stability of pawpaw fruit was relatively low compared to other fruits. PPO in dates was reportedly inactivated by blanching in boiling water at 100 °C for 1.5 min (Mustapha and Ghalem, 2007). Devece et al. (1999) found that PPO from mushroom retained activity even after 6 min heating at 92 °C. Debowska and Podstolski (2001) claimed that PPO in vanilla did not show activity loss when incubated at 50 °C for up to 120 min. The drop in PPO activity in pawpaw fruit upon heating was likely due to a change in the rate-determining step, unfolding of the tertiary structure, exposure of active enzyme site, breakdown or destabilization of intracellular hydrogen bonds and salt bridges in the enzyme, or dissociation of the enzyme-substrate complex (Whitaker 1996).

4.4.4. Colorimetric measurement

The pawpaw fruit is susceptible to browning. During ripening, it undergoes tissue softening; the color and aroma also change. When the tissue is exposed to molecular oxygen, it can quickly turn brown due to the action of PPO. The original color of pawpaw fruit pulp (at harvest) is reddish-yellow. Thus, monitoring the loss of red color (a^*) and yellow color (b^*) serves as an objective tool to evaluate the color degradation during thermal processing. Because the change in a^* and b^* values is accompanied by a simultaneous change in L^* value (Ahmed et al., 2002), all the three colorimetric parameters were measured to indicate color changes after heat treatment of the seven genotypes of pawpaw fruit (Table 4.1).

The fresh samples of seven genotypes pawpaw fruit (nonheated and prior to storage) exhibited large variations, with a^* value ranging from -1.41 to 9.30 , b^* value ranging from 25.23 to 30.44 , and L^* value ranging from 45.49 to 58.16 . In particular, genotypes 6 and 7 were brighter, and genotypes 10 and 20 were darker, compared with others. Genotype 20 had high redness value (a^*), probably due to its highest PPO activity (Fig. 4.5). After 20 h of storage at $20\text{ }^{\circ}\text{C}$, the a^* values of genotypes 6, 7, 11 and 20 increased substantially. However, no significant a^* increase was observed in genotypes 3, 10 and 21.

Heat treatments brought about significant changes in brown color development during storage. The a^* values in all genotypes decreased ($P < 0.001$) when the pulp was heated for 1 and 3 min. However, they all increased at 5 min heating treatment. The reduction in

Table 4.1. Colorimetric evaluation of seven genotypes of fresh and heated pawpaw fruit pulp stored for different time periods¹

Storage time	Colorimeter value	Heating time	Genotype						
			3	6	7	10	11	20	21
0 h	<i>a</i> *	0 min	7.06	0.27	-1.41	9.30	0.82	8.81	6.13
		1 min	0.59	-4.93	-3.36	2.59	-2.95	1.30	-3.65
		3 min	0.16	-4.65	-2.39	1.51	-3.52	1.06	-4.00
		5 min	1.52	-4.10	-2.00	3.34	-2.51	1.25	-3.75
	<i>b</i> *	0 min	25.23	29.74	25.23	27.08	28.81	30.44	27.16
		1 min	30.57	25.75	23.19	30.47	28.27	30.15	30.37
		3 min	29.98	26.25	21.7	30.34	26.92	30.22	30.00
		5 min	27.90	24.61	20.98	28.34	25.65	29.04	29.19
	<i>L</i> *	0 min	45.49	58.16	54.65	49.48	51.63	53.91	52.05
		1 min	54.49	59.12	57.39	59.96	54.75	61.94	61.25
		3 min	54.92	59.61	56.27	60.75	54.34	62.05	61.28
		5min	53.22	58.19	55.68	58.38	53.47	60.98	59.22
1 h	<i>a</i> *	0 min	6.62	0.58	-1.23	1.16	9.75	9.01	6.76
		1 min	0.07	-4.76	-3.09	-2.74	2.50	1.14	-3.47
		3 min	0.37	-4.43	-2.45	-3.03	1.84	1.30	-4.17
		5 min	1.39	-4.11	-1.84	-2.10	3.05	1.68	-3.77
	<i>b</i> *	0 min	23.48	28.27	24.66	29.41	25.07	29.17	24.65
		1 min	29.42	25.2	23.17	27.35	29.54	29.38	31.15
		3 min	29.77	26.78	22.04	27.79	30.43	30.52	30.16
		5 min	27.61	24.21	20.68	25.65	27.38	28.81	29.03
	<i>L</i> *	0 min	44.17	56.9	54.19	51.83	47.27	52.30	49.26
		1 min	54.17	58.79	57.26	54.27	59.27	61.40	61.61
		3 min	54.71	59.84	56.49	54.57	60.97	62.60	61.40
		5 min	53.06	58.01	55.46	53.38	57.95	61.04	59.23

Table 4.1. (Cont'd)

2 h	<i>a</i>*	0 min	6.33	1.31	-1.02	10.17	1.52	9.74	9.74
		1 min	0.80	-4.55	-3.17	3.19	-2.75	2.05	2.05
		3 min	0.64	-4.51	-2.47	1.65	-3.22	1.44	1.44
		5 min	1.40	-4.07	-1.87	3.61	-1.87	1.53	1.53
	<i>b</i>*	0 min	21.82	27.17	23.96	23.51	28.62	28.27	23.76
		1 min	29.86	26.67	22.32	29.87	27.37	30.10	30.87
		3 min	30.21	26.51	21.32	29.39	26.93	29.81	29.16
		5 min	27.32	24.25	20.51	28.09	25.16	28.3	28.59
	<i>L</i>*	0 min	43.09	55.63	53.63	45.89	51.08	50.87	48.36
		1 min	53.89	59.50	56.83	59.35	54.25	61.68	61.61
		3 min	54.99	59.75	56.14	60.3	54.29	61.95	60.79
		5 min	52.88	58.09	55.32	58.23	52.94	60.85	58.77
3 h	<i>a</i>*	0 min	6.12	1.77	-0.62	10.15	1.67	9.49	6.85
		1 min	0.97	-4.49	-2.88	2.83	-2.61	1.76	-3.54
		3 min	0.87	-4.4	-2.47	1.91	-3.03	1.00	-4.14
		5 min	1.40	-3.86	-1.74	3.58	-1.88	1.70	-3.61
	<i>b</i>*	0 min	20.76	27.05	24.03	22.2	26.48	27.25	23.38
		1 min	29.39	26.28	22.00	29.58	27.10	29.4	30.62
		3 min	29.93	25.50	18.89	29.91	27.19	29.15	29.73
		5 min	27.38	24.69	20.52	27.86	25.65	26.05	28.85
	<i>L</i>*	0 min	42.09	55.32	53.57	44.49	49.11	49.76	47.90
		1 min	53.62	59.33	56.51	59.31	54.02	61.00	61.42
		3 min	54.72	59.19	55.82	60.62	54.3	64.95	61.11
		5 min	52.57	58.22	55.28	58.05	53.18	57.93	59.15
4 h	<i>a</i>*	0 min	6.85	2.19	-0.63	10.14	1.91	9.86	7.00
		1 min	-3.54	-4.37	-2.77	3.35	-2.42	1.79	-3.37
		3 min	-4.14	-4.41	-2.35	1.58	-3.22	1.72	-3.83
		5 min	-3.61	-3.92	-1.78	3.65	-1.87	1.40	-3.58
	<i>b</i>*	0 min	20.05	26.74	23.44	21.52	27.24	26.60	22.68
		1 min	28.45	26.14	22.46	29.55	27.54	29.29	30.46
		3 min	29.56	25.35	21.14	29.45	26.35	30.46	29.54
		5 min	27.10	23.71	20.07	27.83	25.41	25.07	28.93
	<i>L</i>*	0 min	41.45	54.95	53.11	43.92	49.97	49.08	46.82
		1 min	53.24	59.13	56.68	59.17	54.14	60.94	61.16
		3 min	54.50	59.21	55.97	60.37	53.86	62.27	60.89
		5 min	52.67	57.75	55.05	58.18	53.16	59.56	59.05

Table 4.1. (Cont'd)

5 h	<i>a</i>*	0 min	5.70	2.44	-0.37	10.01	1.79	10.10	6.53
		1 min	1.34	-4.47	-2.95	3.32	-2.45	2.24	-3.49
		3 min	1.01	-4.50	-2.08	1.55	-3.4	1.34	-3.82
		5 min	1.66	-4.03	-1.48	4.00	-1.81	2.01	-3.20
	<i>b</i>*	0 min	19.83	25.61	23.20	20.78	26.44	26.12	23.01
		1 min	29.31	25.54	21.40	29.26	26.91	29.73	30.72
		3 min	29.13	25.10	21.27	29.22	26.09	29.67	30.18
		5 min	26.77	23.75	20.69	28.33	24.88	29.20	29.5
	<i>L</i>*	0 min	41.36	53.88	52.93	43.02	49.34	48.50	47.39
		1 min	53.41	58.85	56.05	58.79	53.82	61.17	61.34
		3 min	54.12	59.05	55.88	58.84	53.74	61.91	61.23
		5 min	52.44	57.74	55.19	58.29	52.82	60.82	59.15
20 h	<i>a</i>*	0 min	5.79	5.18	1.11	9.29	3.88	10.43	7.84
		1 min	2.56	-3.94	-1.97	4.20	-1.50	3.50	-2.44
		3 min	1.52	-4.30	-1.17	2.03	-2.42	1.49	-3.89
		5 min	3.06	-3.70	-1.38	3.80	-1.35	8.93	-2.18
	<i>b</i>*	0 min	16.25	21.87	22.45	17.04	21.30	21.51	19.55
		1 min	27.89	25.07	21.96	27.10	26.45	28.18	28.60
		3 min	28.75	25.96	21.53	28.25	26.87	28.22	28.80
		5 min	26.89	24.32	20.31	26.30	25.17	27.19	27.80
	<i>L</i>*	0 min	38.59	49.45	51.72	39.66	44.48	43.60	42.65
		1 min	52.15	58.31	55.71	56.86	53.09	59.38	59.87
		3 min	53.68	59.47	55.55	59.32	53.66	60.63	60.44
		5 min	50.48	57.25	54.70	57.09	52.57	49.80	55.91

¹The main effects (genotypes, heating time, and storage time) were all significant ($P < 0.01$).

the a^* value by the short heating times (< 3 min) was due to thermal destruction of PPO (hence, inhibition of polyphenol oxidation) and other red-yellow pigments, while the a^* value increase after 5 min was probably the result of induced Maillard browning. Compared with a^* values, b^* and L^* values did not change significantly.

In order to visualize the thermal processing effect on color changes, the a^* , b^* and L^* values of the seven genotypes were combined, respectively, and the overall means were shown in Figs. 4.7, 4.8 and 4.9. All 3 figures depicted the same trend of color change as a result of different heating times. The a^* and L^* values of nonheated samples, which were substantially changed after 1 min of heating, gradually increased and decreased, respectively, during post-heating storage. The a^* change was consistent with the reduction in L^* values, i.e., a reduced brightness. From Fig. 4.8, nonheated pawpaw fruits showed significant decrease of the b^* value during storage. The reason of the less yellow color (more bluish) in nonheated samples could be attributed to the active PPOs. Nevertheless, less reduction of the b^* value were observed in heated samples.

It was possible that some biochemical degradation of coloring compounds occurred during heating and storage period. The a^* of samples heated for 1, 3 and 5 min had relatively small value when compared with nonheated samples, suggesting that PPO was largely inactivated after heating for 1 min at 92 °C. Results from this study showed an apparent correlation between PPO activity and the color change during the 20 h storage. This correlation indicated that the pawpaw pulp color was darker when the PPO activity was high. The availability of intact PPO and its substrate (phenolic compounds) in the

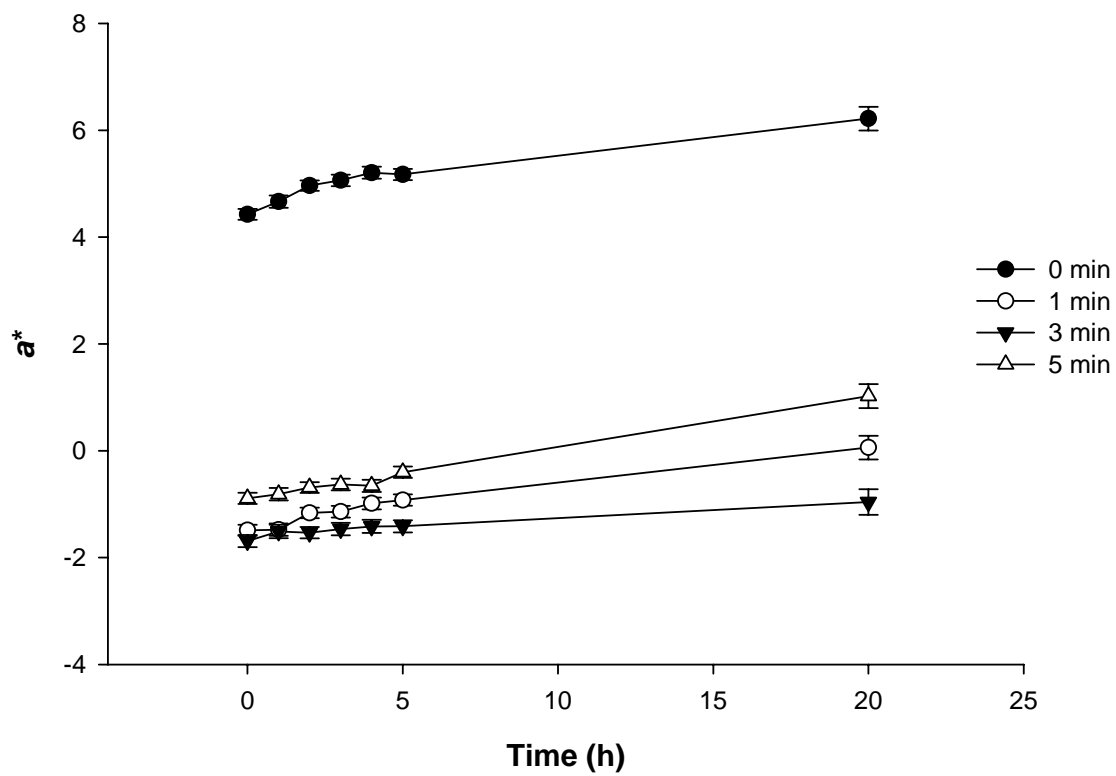


Figure 4.7. Effect of heat treatment (0–5 min) and storage time (0–20 h, at 20 °C) on the colorimetric a^* value of mixed pawpaw fruit pulp

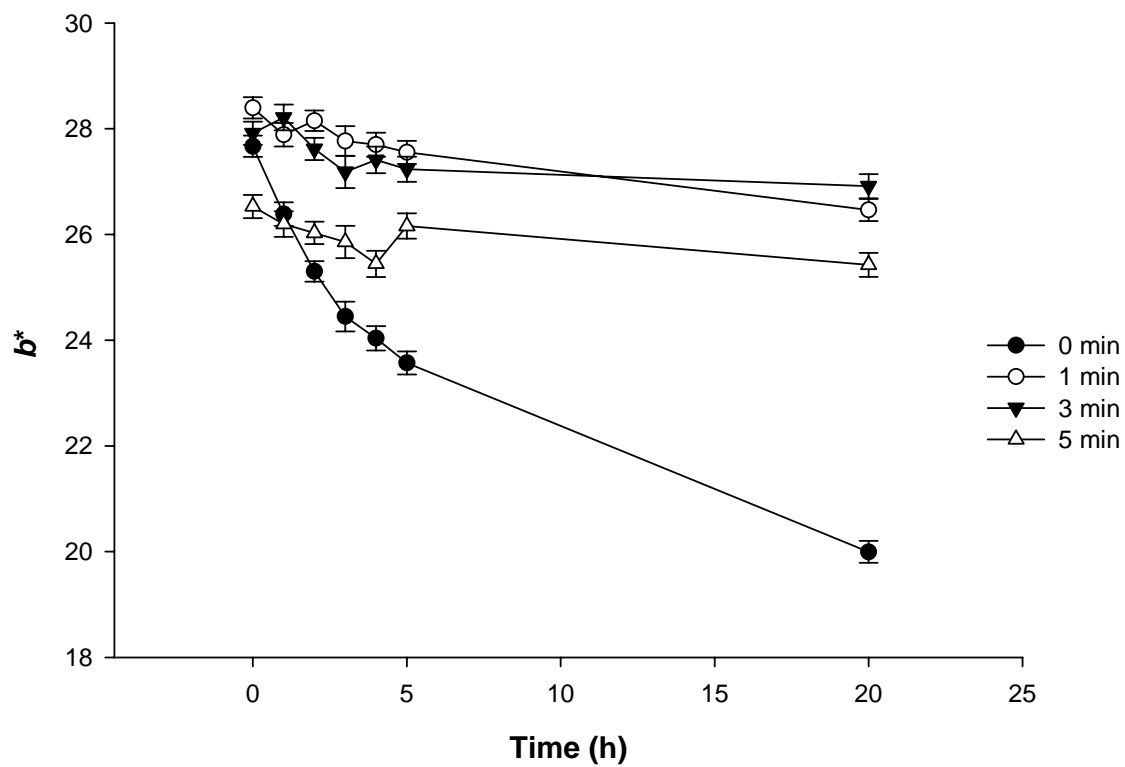


Figure 4.8. Effect of heat treatment (0–5 min) and storage time (0–20 h, at 20 °C) on the colorimetric b^* value of mixed pawpaw fruit pulp

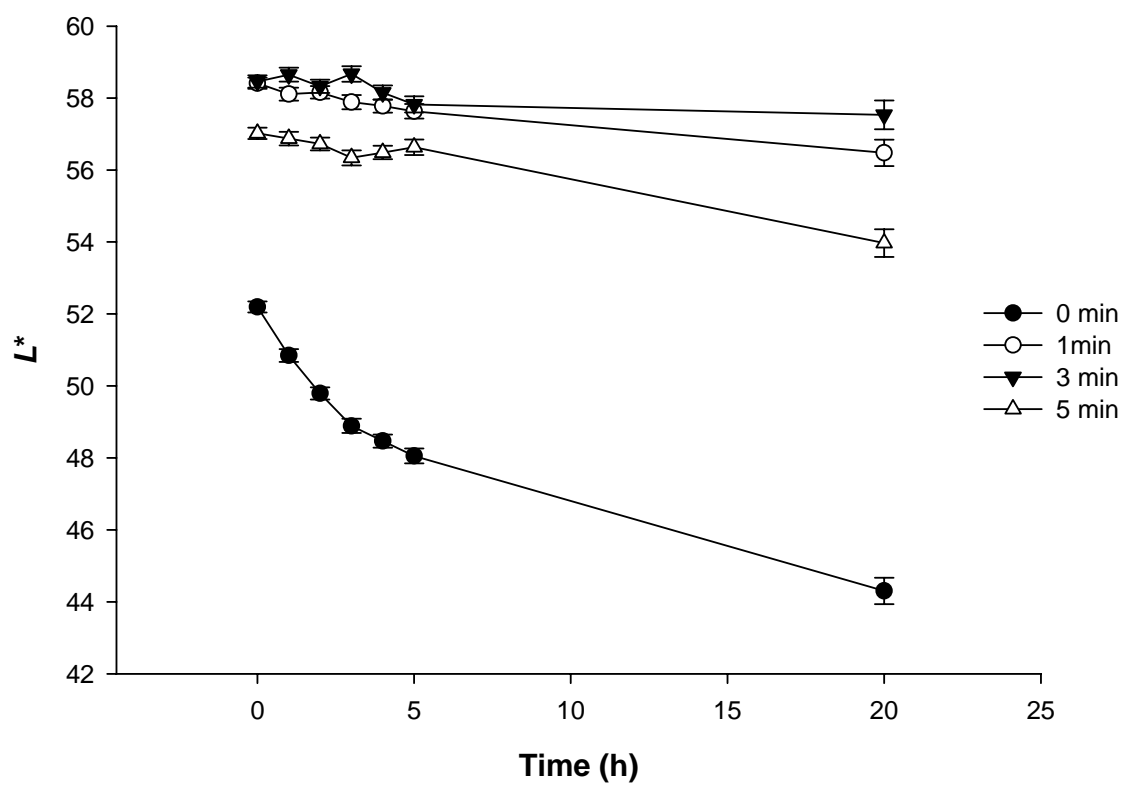


Figure 4.9. Effect of heat treatment (0–5 min) and storage time (0–20 h, at 20 °C) on the colorimetric L^* value of mixed pawpaw fruit pulp

fruit may determine the extent of the browning reaction (Qudsieh et al., 2002).

4.4.5. Relationship between PPO concentrations, PPO activity and visual color

Attempts were made to establish the relationship among the parameters studied for pawpaw PPO. Except in genotype 7, both PPO₁ and PPO₂ isoforms were related ($P < 0.01$) to the PPO total concentration (Table 4.2), suggesting that when PPO₁ was expressed, so was PPO₂. The total PPO activity (on an enzyme weight basis) was negatively proportional to the total PPO content. This indicated that the PPO activity did not increase with the expression of PPO in different genotypes of pawpaw fruit except genotype 7. Also, large negative correlations were seen between color parameters (a^* and L^*) and the PPO₁ and PPO₂ contents ($P < 0.05$). Ahmed et al. (2002) reported a significant correlation between the $a^* \times b^*$ values and total pigment concentration in papaya puree. However, Rocha and De Morais (2005) noted no correlation between PPO activity and color parameters in apples.

4.5. Conclusion

The findings from this part of the study demonstrated that diversity exists among pawpaw genotypes in PPO activity, and this is ostensibly responsible for the different levels of susceptibility of the fruit pulp to browning discoloration during storage. However, a brief heat treatment can effectively inactivate the enzyme, offering a

Table 4.2. Correlation coefficient (r) for PPO content, PPO activity, and a^* , b^* and L^* values of pawpaw fruit

	PPO₁	PPO₂	PPO	PPO	a^* 0h	b^* 0h	L^* 0h	a^* 20h	b^* 20h	L^* 20h
	cont.	Cont.	total	act.						
PPO₁		0.9898	0.9950	-0.6623	-0.9368	0.3457	0.4878	-0.8268	0.4974	0.7302
cont.		(P<0.01)	(P<0.01)		(P<0.01)			(P<0.01)		
PPO₂			0.9991	-0.7211	-0.9361	0.3243	0.5337	-0.8038	0.4839	0.7602
Cont.			(P<0.01)		(P<0.01)			(P<0.05)		(P<0.05)
PPO				-0.7332	-0.5330	0.4361	0.3878	-0.3252	0.2735	0.3027
total				(P<0.05)						
PPO					0.4039	0.0826	-0.0261	0.3559	0.1265	-0.0394
act.										
a^* 0h						0.0637	-0.5688	0.9063	-0.6914	-0.8535
								(P<0.01)		(P<0.01)
b^* 0h							0.5694	0.4411	0.4788	0.1393
L^* 0h								-0.2037	0.8775	0.8497
									(P<0.01)	(P<0.01)
a^* 20h									-0.4048	-0.6550
b^* 20h										0.8829
										(P<0.01)
L^* 20h										

convenient approach to preserving the shelf-life of pawpaw fruit pulp. PPO activity and concentration may contribute to the explanation of the discoloration in pawpaw fruit.

CHAPTER 5

ANTIOXIDANT ACTIVITY OF PAWPAW FRUIT

(*ASIMINA TRILOBA*) EXTRACT

5.1. Summary

The antioxidant activity of the pulp from seven cultivars of pawpaw fruit was investigated. Total antioxidants were extracted with 70% acetone, and the extracts were analyzed for radical-scavenging activity by the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay. The result showed that genotype 20 had a maximum radical-scavenging activity. Lipid oxidation at 37 °C was determined by the formation of conjugated diene hydroperoxides in a liposome model system. The result showed that genotypes 11 and 20 were strongly inhibitory of lipid oxidation. The findings suggested that pawpaw fruit antioxidant extracts could be used to improve oxidative stability of fat-containing foods.

5.2. Introduction

There is a growing interest in recent years in natural antioxidants present in vegetables and fruits, because these compounds are generally perceived to be safer than synthetic counterparts (Aida et al., 1998). Antioxidants are important in the prevention of diseases in both plants and animals by neutralizing reactive oxygen species. Dietary consumption of natural antioxidants (such as α -tocopherol, β -carotene, and ascorbic acid) has been linked to reduced health-related problems (Willet, 1991). In fruits, phenolic compounds make up a large bulk of antioxidants (Steinmetz and Potter, 1991). Because of their ability to donate electrons and/or protons, antioxidants can prevent or delay oxidation of a variety of food components. The mechanisms of antioxidant action include: (a) chelating metal ions, (b) scavenging free radical, (c) maintaining redox balance, (d) inducing phase 2 enzymes, and (e) reacting with molecular targets at high specificity (Packer, 2007).

The potential health benefits of pawpaw fruit have been demonstrated in several studies. Oberlies and coworkers (1997) showed that pawpaw extracts had the ability to kill tumor cells. McLaughlin et al. (2007) reported that pawpaw fruit extract can inhibit the production of ATP and reduce tumor sizes. A number of methods have been developed to measure total antioxidant activity in fruit (for review, see Prior et al., 2005). These assays differ in the generation of different radicals and/or target molecules and in the way by which to measure the end products. Different antioxidants act through

different mechanisms. Hence, a minimum of 2 methods are recommended for the evaluation of antioxidant activity (Pellegrini et al., 2003).

In the present study, the antioxidant activity of seven different pawpaw cultivars was assessed. Specifically, the effect of pawpaw fruit extract on lipid oxidation (which was induced by persulfate/copper in a liposome model system) was determined. A radical-scavenging assay and conjugated diene measurement were performed in an attempt to account for the antioxidant activity of pawpaw fruit extract that was illustrated in the liposome system. In addition, the antioxidant compounds in pawpaw fruit were detected by HPLC.

5.3. Materials and Methods

5.3.1. Materials

Seven genomic variations of Pawpaw (*Asimina triloba*) fruit were harvested in the fall of 2006 at the Kentucky State University Research and Demonstration Farm in Frankfort, Kentucky. Pawpaw fruit pulp samples were prepared as described in Chapter 4, Section 4.3.1. Reagent grade α -catechin, cupric acetate, L- α -Phosphatidylcholine, potassium persulfate, L-ascorbic acid and trolox were purchased from Sigma-Aldrich Inc. (St. Louis, MO). ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] was purchased from Fluka Chemicals, Steinheim, Germany Sigma Chemical Co. (Madrid, Spain).

5.3.2. Antioxidant extraction

The procedure described by Hassimotto et al. (2005), with some modifications, was followed. An aliquot of fruit pulp (10 g) was homogenized for 2 min in 25 mL of 70% acetone with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Then homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C. The residue was re-extracted with another 15 mL of 70% acetone under the same condition. The combined supernatants were concentrated in a rotary evaporator with a water bath (Rotavapor and Water Bath, Buchi, Brinkmann Instruments, Inc. Westbury, NY) set at 40 °C with a vacuum aspirator (Model B-169, Brinkmann, Sibata, Japan). The semi-solid extracts were placed in a glass jar, flushed with nitrogen gas, sealed, and then stored at –20 °C before use.

5.3.3. ABTS assay

The ABTS [(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid))] assay measures the ability of antioxidants to quench $ABTS^{\bullet+}$ in aqueous systems. Radical cation $ABTS^{\bullet+}$ was generated according to the method of Re et al. (1999) by oxidation of ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand in the dark at room temperature for 15 h before use.

The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. After adding 30 μ L of antioxidant extracts ($10 \times$ dilution) or trolox standards (final concentration 0, 2.5, 5, 10, 15 μ M, prepared in ethanol) to 3.0 mL diluted $ABTS^{\bullet+}$

solution (Abs = 0.7 ± 0.02), the absorbance at 734 nm was immediately measured and monitored up to 5 min. Each extract was analyzed in triplicate. The antioxidant activity of each sample was expressed as mmol of trolox per gram pawpaw fruit, and was calculated by the equation:

$$\text{TEAC (mmol/g)} = \frac{33.3 \times 10 \times a}{1000} \times 14 \text{ mL/10 g} \quad (1)$$

where 33.3 was a dilution factor originating from the protocol, 10 was the dilution times of antioxidant extract, 1000 was the unit conversion factor, and a was trolox equivalent ($\mu\text{mol}/30 \mu\text{L}$) calculated from the standard curve:

$$\text{Abs (734 nm)} = -0.003a + 0.6799 \quad (R^2=0.7088) \quad (2)$$

5.3.4. Liposome oxidation assay

The liposome was prepared, as described by Huang and Frankel (1997), from phosphatidylcholine with deionized water to a concentration of 0.8 mg/mL. Phosphatidylcholine was suspended in deionized water by sonicating for 40 min. An aliquot of 1 mL of pawpaw antioxidant extract (diluted 10 \times) was mixed with 4 mL of liposome solution. Deionized water (25 mL) was added to bring the final volume to 30 mL. After 30 μL of 3 μM cupric acetate was added, the sample solution was put into a shaking water bath (37 $^{\circ}\text{C}$) in the dark for 2 days. As comparison, 0.01% catechin and 0.01% ascorbic acid were tested as well. Inhibition against liposome oxidation was calculated at day 3 by measuring the formation of peroxidation products, namely, conjugated dienes, as described below.

5.3.5. Measurement of conjugated dienes

The conjugated diene measurement evaluates the reducing power of antioxidants to interrupt the hydroperoxides (or conjugated dienes) production in the liposome system. The formation of conjugated dienes was measured optically at 234 nm. Because the solvent acetone also absorbed strongly at this UV wavelength, two controls were set up. One was liposome with cupric acetate without sample extracts as “sample control”; and the other was sample extracts with cupric acetate without liposome as “background control”. Antioxidant activity was calculated as inhibition capacity:

$$\% \text{ Inhibition} = \frac{A_1 - (A_s - A_2)}{A_1} \times 100 \% \quad (3)$$

where, A_1 was the absorbance of sample control (liposome + cupric acetate), A_s was the absorbance of conjugated dienes formed in test samples (liposome + cupric acetate + extract), and A_2 was the absorption of antioxidant extracts at 234 nm as background control (cupric acetate + extract).

5.3.6. HPLC analysis

To characterize the type of antioxidants contained in pawpaw fruit extracts, a high performance liquid chromatograph (HPLC) was performed. The system was equipped with an ESA Model 584 Solvent Delivery Module Pump and a multi-channel coulometric electrochemical detector (ESA CoulArray Coulometric Electrode Array Detector, ESA, Inc., Chelmsford, MA). This system was suitable for analyzing electroactive compounds. Antioxidant compounds were identified based on their electrochemical activity versus a

known library (ESA Inc. website), which is reflected on their retention time (RT) and/or the ratio of the peaks at different potentials on the chromatogram.

In this study, only genotype 21 was analyzed. Duplicate samples from genotype 21 extract were filtered through a 25-mm nylon membrane Syringe filter. The filtrate was subjected to HPLC. A Supelco LC-18 column [4.6 mm (dia.) \times 250 mm (l.); 5 μ m (porosity)] was used. The mobile phases were: (A) water that contained 25 mM sodium acetate and 25 mM citric acid methanol (95:5, v/v); and (B) water that contained 25 mM sodium acetate and 25 mM citric acid methanol-ACN (20:40:40, v/v/v). The optimal gradient condition was established by linearly increasing the initial conditions of 25% B with 75% A to 100% B over 24 min, holding at 100% B for 16 min, returning to initial conditions of 25% B with 75% A, and holding for 20 min. The flow rate was 1.75 mL/min and responses were recorded at applied potential of 50, 150, 250, 350, 450, 550, 650, 750 mV.

5.4. Results and Discussion

5.4.1. Antioxidant activity

There are a number of components in plant tissues that can be broadly classed as antioxidants. The activity of each component may depend upon the extraction system applied (Al-Ismail and Abrjai, 2004; Vuorela et al., 2004). In this study the antioxidant activity of water and acetone extracts were evaluated by means of ABTS

radical-scavenging and hydroperoxide-inhibiting in a liposome system.

5.4.1.1. Scavenging effect on ABTS^{•+}

A number of methods have been developed to measure the antioxidant capacity of biological systems. Among them, TEAC is perhaps the most widely used. The assay involves the generation of green/blue ABTS^{•+} chromophores when ABTS reacts with potassium persulfate. It has a maximum absorption at a wavelength 734 nm (Miller and Rice-Evans, 1994; Re et al., 1999). The total antioxidant activity of pawpaw fruit extracts, measured as absorbance at 734 nm, was converted to trolox equivalent antioxidant capacity (TEAC). A lower absorbance and a higher TEAC value would indicate greater antioxidant activity of the testing sample.

Extracts from the seven genotypes of pawpaw fruit followed the same trend in the color change, thus, TEAC increases, during the assay (Figs. 5.1 and 5.2). A rapid reduction in absorption was observed within 1 min after antioxidant extracts were added to the ABTS^{•+} solution. Genotypes 3, 10, and 20 showed a strong ABTS^{•+} chromophore reduction, indicating that they were capable of neutralizing ABTS^{•+} radicals. The TEAC values ranged from 98 to 468 mmol/g in the 5 min assay time. The radical scavenging ability of the seven genotypes followed the order of: GT20 > GT10 > GT3 > GT6 > GT21 > GT7 > GT11. Previous research had shown that the TEAC value in banana, coconut, dragon fruit and passion fruit were 1,800, 1,530, 685, and 591 mmol/g, respectively (Okonogi et al., 2007). The vegetables broccoli, mushroom, potato,

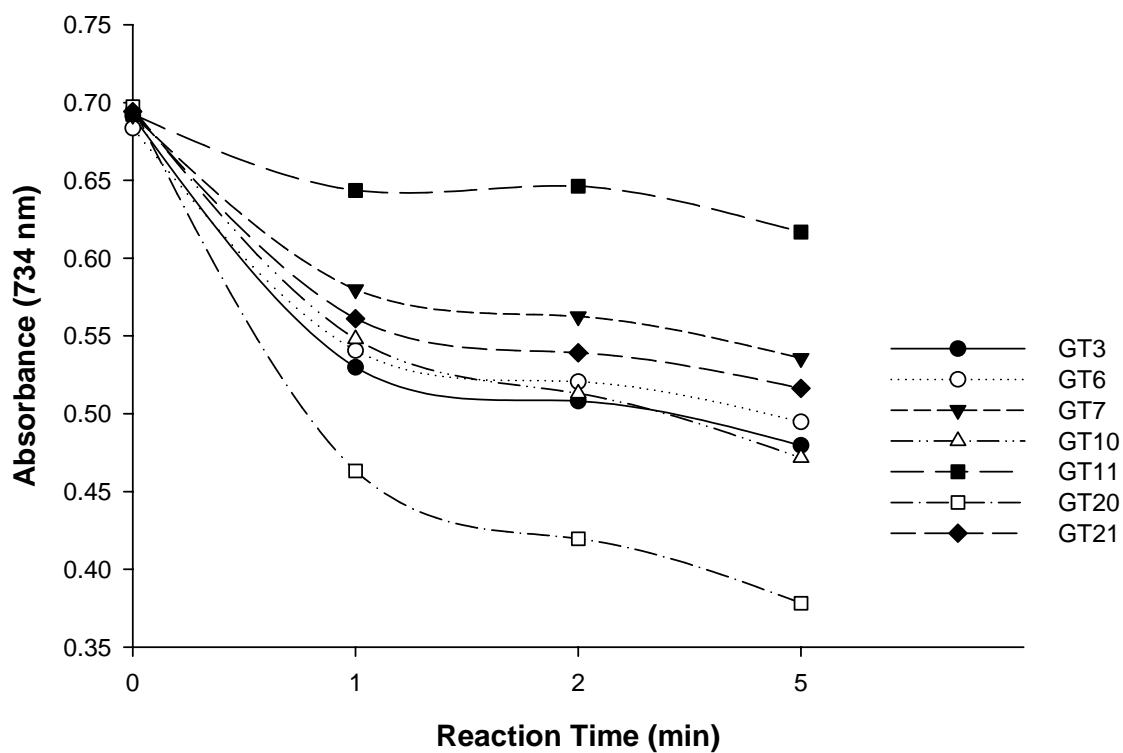


Figure 5.1. Radical-scavenging activity (absorbance at 734 nm) of antioxidant extracts versus reaction time for seven genotypes (GT) of pawpaw fruit. The extracts were diluted 10 times.

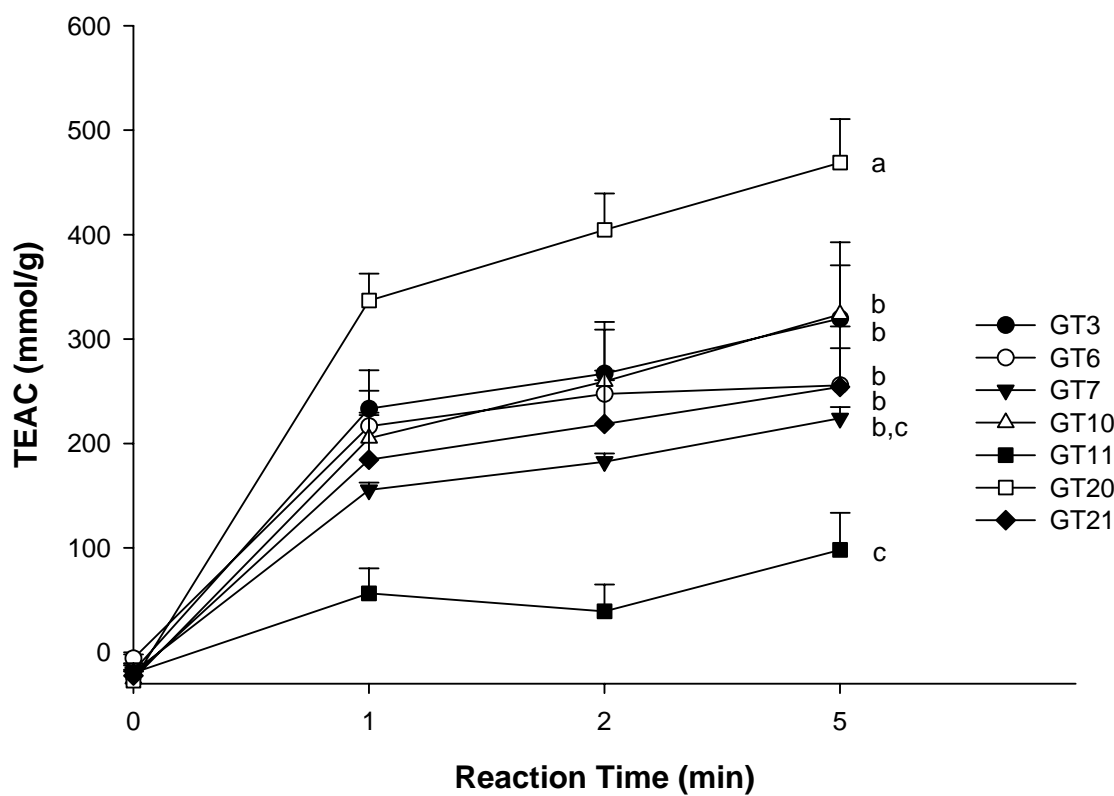


Figure 5.2. TEAC (mmol/g) of the antioxidant extracts from seven genotypes (GT) of pawpaw fruit (with positive error bars). The symbols a–c indicate significant differences ($P < 0.05$) between genotypes.

pumpkin, pinach and tomato were found to have much lower antioxidant activity with 3.04, 4.93, 0.8, 3.71 and 8.49 $\mu\text{mol/g}$ fruit fresh weight (Pellegrini et al., 2003).

5.4.1.2. Inhibition of liposome peroxidation

In the liposome oxidation system, the seven genotypes of pawpaw fruit showed different antioxidant activities. The absorbance of the seven genotypes at 234 nm was plotted in Fig. 5.3. The difference between the absorbance of a test extract and that of individual background control indicated that conjugated dienes formed from liposome oxidation. A smaller difference suggested a stronger antioxidant activity by an antioxidant extract sample. When the liposome system was oxidized with 3 μM copper solely, the production of conjugated dienes (thus, hydroperoxides) in the control sample was quite high. The presence of pawpaw fruit antioxidant extracts, as well as catechin (0.01%) and ascorbic acid (0.01%) used for comparison, suppressed the diene formation (Fig. 5.3)

To more clearly illustrate the antioxidant effects of the pawpaw fruit extracts, the percent inhibition of lipid oxidation in the liposome system was plotted. As seen shown Fig. 5.4, percentage oxidation inhibition ranged from 54% to 87%, with the relative effects in the order of: GT11 > GT20 > ascorbic acid > GT7 > catechin > GT3 > GT21 > GT6 > GT10 (Fig. 5.4). Hence, at their respective concentrations tested, the pawpaw fruit extracts were more or less comparable to the two common plant antioxidants. However, the order of antioxidant potential shown in the conjugated diene test was inconsistent

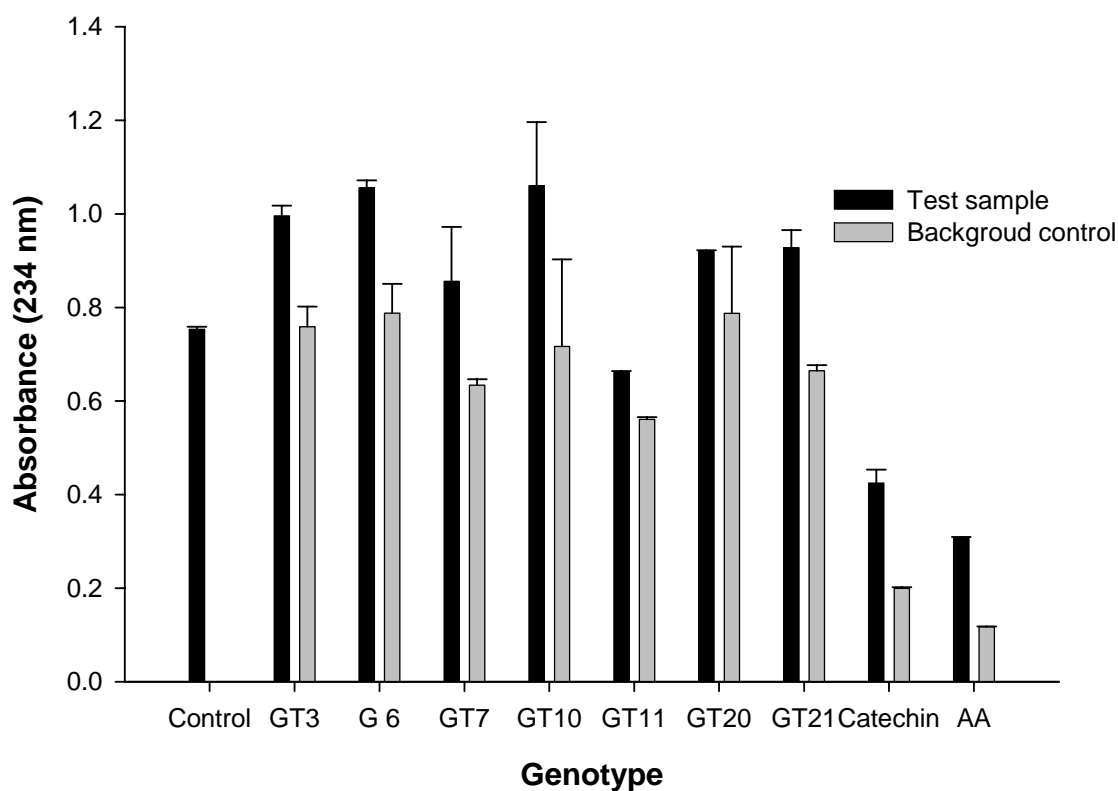


Figure 5.3. Conjugated diene measurement (A_{234}) of a liposome oxidizing system containing antioxidant extracts from seven genotypes (GT) of pawpaw fruit. Control = “sample control” (liposome + cupric acetate); black columns = “test samples” (extracts + liposome + cupric acetate); gray columns = “background control” (extract + cupric acetate). AA = ascorbic acid.

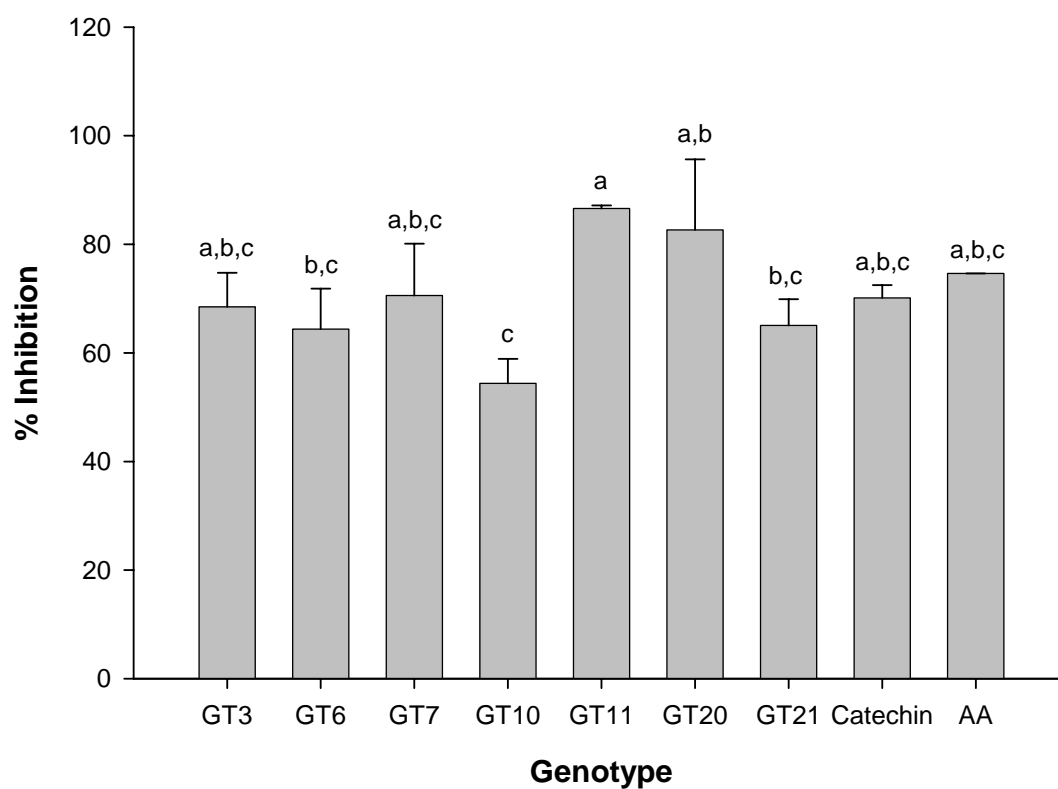


Figure 5.4. Inhibition of conjugated diene formation for antioxidant extracts from seven genotypes (GT) of pawpaw fruit. The standard error bars are indicated. AA = Ascorbic acid. The symbols a–c indicate significant differences ($P < 0.05$) between genotypes.

with that of the ABTS assay (Figs. 5.1 and 5.2). The cause was not clear, but may be inherently related to the test methods. The conjugated diene method measures the early product of lipid oxidation without regard to the mechanism of oxidation initiation (singlet oxygen, free radicals, metal ions, etc.). The presence of pawpaw fruit extracts could have prevented the loss of electrons or the addition of molecular oxygen to lipid unsaturated double bonds. On the other hand, the ABTS assay utilizes an indicator radical which may be neutralized via two mechanisms: direct reduction through electron transfer or radical quenching through proton transfers (Prior et al., 2005). It was thus difficult to interpret without a more in-depth study to reveal more detailed information about the composition of antioxidants being tested (Prior et al., 2005). For example, gas chromatography-mass spectrophotometry could be done to investigate the antioxidant chemicals in pawpaw fruit extract in the future.

5.4.2. HPLC

For a demonstration purpose, the antioxidant extract of genotype 21 was subjected to HPLC to separate the specific compounds. Three salient peaks (retention times RT = 1–2, 8 and 51 min, respectively) and a number of minor ones were detected in the fruit extract (Fig. 5.5A). The peak at RT 1–2, as well as all the small peaks, also appeared in the sample blank (Fig. 5.5B, extract solution only), suggesting that chemicals contained in the extraction solution contributed to the background signals detected by the HPLC detector. Thus, only two peaks (RT 8 and 51) could be attributed to

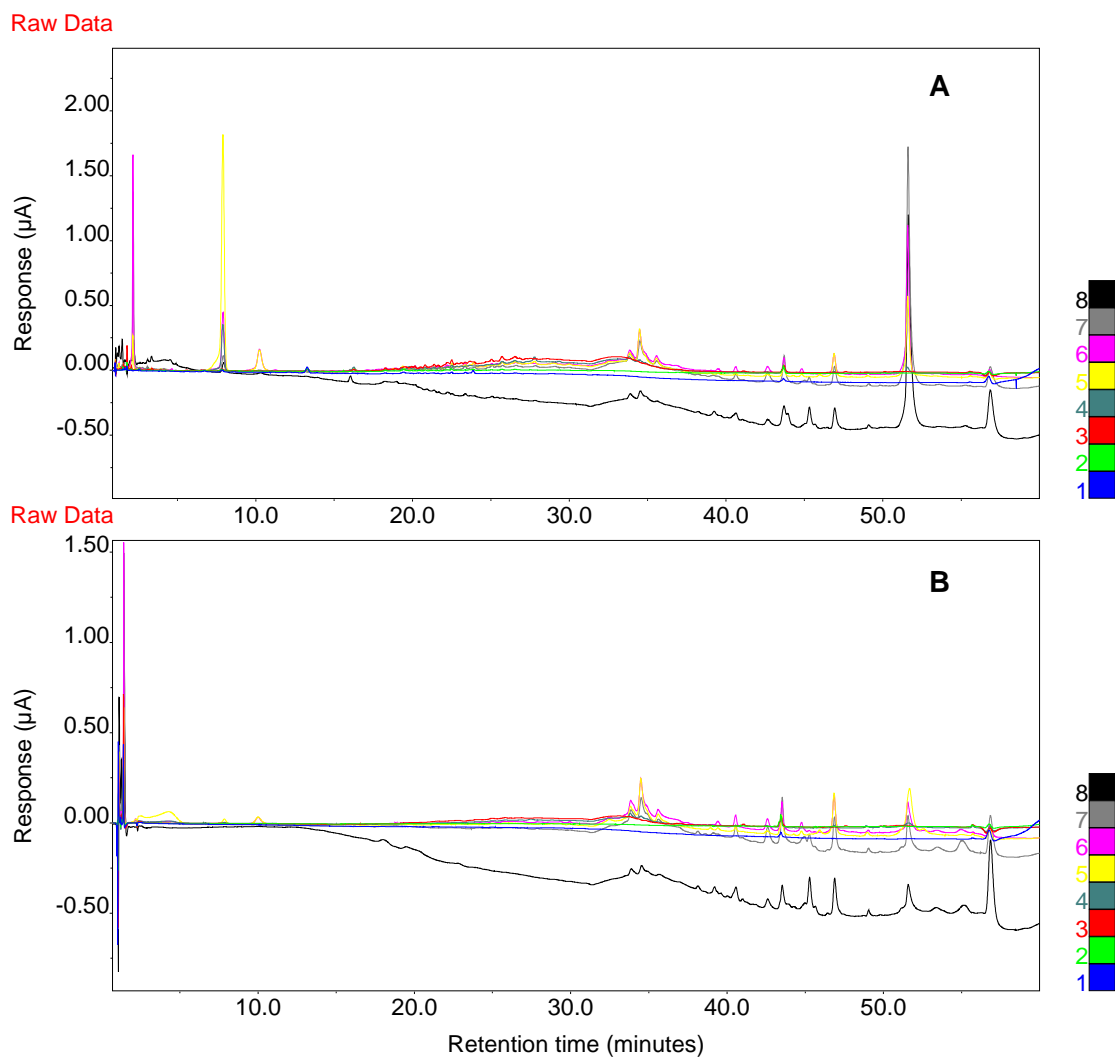


Figure 5.5. HPLC chromatograms of antioxidant extract from genotype 21 of pawpaw fruit. The chromatograms were recorded at 50, 150, 250, 350, 450, 550, 650, and 750 mV (shown as blocks 1–8). A: extract from genotype 21; B: blank (extraction solution only). The peaks were not identified.

antioxidants. However, neither peak was identifiable based on the theoretical values in the library provided by ESA. It is possible that both compounds were not common components documented in the library. Since more organic compounds are expected from pawpaw fruit, as with many other fruits (Gil, et al., 2000; Proestos, et al., 2005; Netzel et al., 2006), it is possible that the level of other antioxidant compounds was below the detection limit of the coulometric detector used. Thus, future direction of this experiment should be to apply a broader range of potentials to the electrodes to enhance the detection sensitivity. An improved detection could also be accomplished with a more concentrated sample solution.

5.4.3. Relationship between antioxidant activity PPO activity and visual color

The ABTS^{•+} radical scavenging ability of all genotypes was correlated to PPO activity, which has been reported in Chapter 4. A weak correlation ($r = 0.6610$; $P < 0.10$) was obtained (Figure 5.6; Table 5.1). However, the ABTS^{•+} radical scavenging activity did not show a significant correlation with the total PPO content (Table 5.1). It was plausible during fruit ripening, a high antioxidant intensity was established through some protection mechanism to counteract the strong rise in PPO activity, not the total PPO content *per se*. The presence of strong antioxidant activity in pawpaw fruit was probably a manifestation of the fruit's defense against other oxidizing enzymes and compounds. Moreover, there was a significant correlation between a^* value (redness) and TEAC value ($P < 0.05$) (Fig. 5.6; Table 5.1), suggesting that the reduction of the a^* value shown

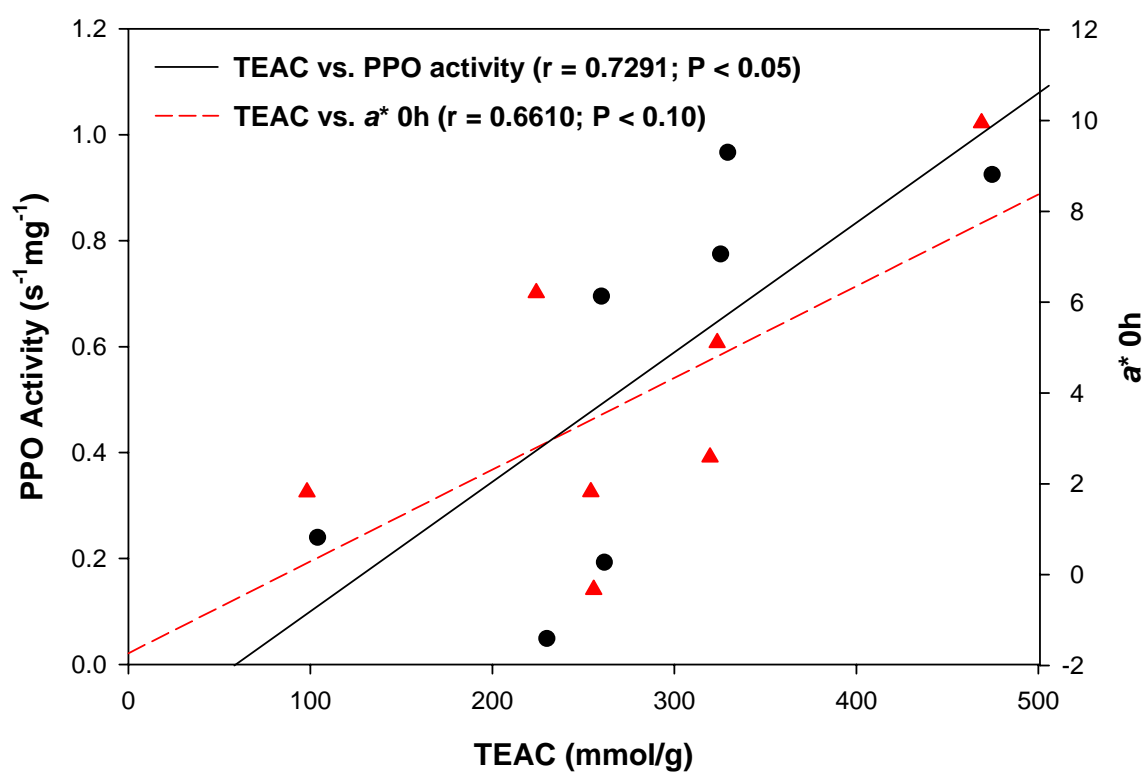


Figure 5.6. Correlation plots for PPO activity, TEAC and *a** 0h values. Circle shows TEAC vs. PPO activity; triangle shows TEAC vs. *a** 0h.

Table 5.1. Correlation coefficient (r) for antioxidant activity (inhibition of CD, TEAC), PPO content, PPO activity, and a^* , b^* and L^* values of pawpaw fruit

	Inh.	TEAC	PPO ₁	PPO ₂	PPO	PPO	a^* 0h	b^* 0h	L^* 0h	a^* 20h	b^* 20h	L^* 20h
	CD		Cont.	Cont.	total	act.						
Inh.		-0.1509	0.2165	0.1036	0.1265	0.2637	-0.2477	0.4086	0.1243	-0.1765	0.5219	0.1828
CD												
TEAC			-0.6180	-0.6061	-0.4974	0.6610	0.7291	0.2083	-0.0864	0.7336	-0.2391	-0.3343
						(P<0.1)	(P<0.05)					
PPO₁				0.9898	0.9950	-0.6623	-0.9368	0.3457	0.4878	-0.8268	0.4974	0.7302
cont.				(P<0.01)	(P<0.01)		(P<0.01)			(P<0.01)		
PPO₂					0.9991	-0.7211	-0.9361	0.3243	0.5337	-0.8038	0.4839	0.7602
Cont.					(P<0.01)		(P<0.01)			(P<0.05)		(P<0.05)
PPO						-0.7332	-0.5330	0.4361	0.3878	-0.3252	0.2735	0.3027
total						(P<0.05)						
PPO							0.4039	0.0826	-0.0261	0.3559	0.1265	-0.0394
act.												
a^* 0h								0.0637	-0.5688	0.9063	-0.6914	-0.8535
										(P<0.01)		(P<0.01)
b^* 0h									0.5694	0.4411	0.4788	0.1393
L^* 0h										-0.2037	0.8775	0.8497
											(P<0.01)	(P<0.01)
a^* 20h											-0.4048	-0.6550
b^* 20h												0.8829
												(P<0.01)
L^* 20h												

5.5. Conclusions

In the current study we extracted antioxidants from pawpaw fruit of seven genotypes and performed two antioxidant activity assays. This was the first report on antioxidant properties of extracts from different genotypes of pawpaw fruit. It was observed that the activity of pawpaw fruit extracts varied greatly between genotypes, suggesting that synthesis of antioxidants is inherent to the specific pawpaw cultivars. The ABTS radical scavenging assay result was not correlated with that of conjugated diene measurement in an oxidizing liposome system, indicating that further research is required to fully characterize the antioxidants from pawpaw fruit.

CHAPTER 6

OVERALL CONCLUSIONS

This thesis describes the isolation and characterization of polyphenol oxidase (PPO) from pawpaw fruit and antioxidant activity of pawpaw fruit extracts. The PPO present in most of the seven genotypes consisted of two isoforms with molecular weights of 28.2 and 38.3 kDa. This enzyme exhibited great activity at pH 6.5–7.0 and at temperature from 5 to 20 °C, but lost almost all its activity at above 80 °C. These findings proved the hypothesis that PPO was responsible for brown discoloration of the fruit tissue when damaged or exposed to molecular oxygen during storage and processing. For a maximum storage stability and shelf-life, it is recommend that pawpaw fruit or fruit pulp be kept at sub-freezing temperatures.

For a clear understanding of the thermal stability of pawpaw fruit PPO, crude extracts of the enzyme, prepared from seven genotypes, were heated at 92 °C for various time periods. PPO activity differed among pawpaw genotypes, but, it was completely inactivated when the fruit pulp was heated at 92 °C for > 1 min. The remarkable heat susceptibility of PPO makes thermal treatments a feasible means to control brown discoloration in pawpaw product processing. The actual activity of PPO and its heat sensitivity were inherent to the specific genetic variety within the big pawpaw superfamily. Colorimetric determination was conducted to interpret the relationship

between PPO thermal stability and pulp discoloration. Thermal treatments at 92 °C significantly decreased the darkness of the fruit color, which coincided with the inactivation of PPO at this temperature.

This study also established that pawpaw fruit from different genotypes contained considerable amounts of antioxidants, comparable to some other fruits as reported in the literature. Both the radical-scavenging test and the liposome oxidation measurement indicated the presence of antioxidants in pawpaw fruit, and free radical quenching appeared to be a main mechanism for the oxidation inhibition. Correlation analysis revealed a positive relationship between the antioxidant activity and the native red color (a^* value) of pawpaw fruit, and a negative relationship between antioxidant activity and PPO activity. However, the type of antioxidants is not clear and should be determined in future research.

Overall, this thesis research demonstrated that pawpaw fruit possesses both the oxidizing enzyme polyphenol oxidase and antioxidant compounds. The presence of the latter was presumably the manifestation of pawpaw to maintain a redox balance in the fruit cells. The existence of antioxidants also suggests that pawpaw fruit can be marketed and consumed as a health-beneficial functional food, in addition to its present use as fresh fruit or as a food ingredient. Whether pawpaw fruit also has medicinal effects, e.g., anti-tumor and anti-inflammation, remains to be investigated.

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