POLYPHENOL OXIDASE INHIBITOR(S) FROM GERMAN COCKROACH *(Blattella germanica)* EXTRACT

By

PAUL JUSTIN KURT GROTHEER

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2008

© 2008 Paul Justin Kurt Grotheer

In loving memory of my grandfather Charlie M. Rowden (1917-2006) and my grandmother Elvira Meyn Grotheer (1914-2006).

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. Maurice Marshall, for his support and friendship throughout my research. Without his encouragement this study could not have been completed. I would also like to thank my committee members, Dr. Amy Simonne, Dr. Steven Valles, and Dr. Allen Wysocki, for all their help and guidance throughout my research.

I would also like to thank my parents, Col (Ret) Kurt and Dr. Priscilla Grotheer, for their constant love and support. They have always believed in me, especially at times when I had trouble believing in myself. I also want to acknowledge their constant financial support during both my undergraduate and graduate degrees. I would also like to thank my sister, Laura, for always being there for me when I need to talk, and always being supportive of the decisions I make.

Lastly I want to thank the friends I made at the University of Florida. In particular, I thank some of my roommates who have become my closest friends: Michael Robuck, Robby Etzkin, and Aaron Vieira. In addition I acknowledge good friends Timothy Darby and his wife Brittany, Charles Angeles, and Gary McClain, and all the other friends I have made in Gainesville over the years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

POLYPHENOL OXIDASE INHIBITOR(S) FROM GERMAN COCKROACH *(Blattella germanica)* EXTRACT

By

Paul Justin Kurt Grotheer

May 2008

Chair: Maurice R Marshall
Major: Food Science and Human Nutrition

Enzymatic browning causes millions of dollars in losses yearly to the food industry by discoloration of fruits and vegetables. The Food and Drug Administration's (FDA) banning of sulfites in 1986 has created a large field of research in search of natural, effective and economic inhibitors of enzymatic browning. The objective of this research was to demonstrate inhibition of plant(s) polyphenol oxidase (PPO; EC 1.10.3.1) using inhibitor(s) from a whole body extract of German cockroach, *Blattella germanica*.

German cockroach samples were homogenized and extracted in a 0.1 M Na_2PO_4, pH 6.5 buffer and centrifuged for 15 min at 10,450xg, 4ºC. The extract was then filtered and centrifuged again at 105,000xg for 1 hr, filtered, and the supernatants collected. Apple and potato PPO were extracted from an acetone powder. Reaction mixture contained 2.45 mL buffer, 0.3 mL 0.5 M catechol, 0.2 mL inhibitor or control buffer, and 0.05 mL PPO. Activity was determined spectrophotometrically at 420 nm. Crude inhibitor(s) was characterized based on temperature stability, pH extraction profile, incubation time, dialysis, ultrafiltration, treatment with proteases and kinetic analysis.

Crude cockroach extract inhibited 60-70% of apple PPO activity and 15-25% of potato PPO, but it was not effective on banana and mushroom. Inhibition in the reaction mixture

occurred fairly rapidly. The inhibitor(s) was stable when stored at refrigerated temperatures as well as freeze-thaw cycling, but upon boiling at 100ºC up to 60 min, inhibition was totally inactivated. Further characterization showed that the inhibitor(s) was unstable to changes in extraction pH, being most stable at pH 6.5. The percent inhibition dropped drastically when the pH moved in either direction. Incubation time was found to affect the inhibitor(s) when the inhibitor and PPO were allowed to sit up to 30 minutes, however when the inhibitor and substrate were incubated there was no effect on inhibition. Ultrafiltration and dialysis studies showed that the unknown inhibitor(s) has an apparent molecular weight (MW) larger than 100,000 nominal molecular weight limit (NMWL). Kinetic studies using Lineweaver-Burk showed the inhibitor(s) displayed non-competitive inhibition on apple PPO.

Successful identification of inhibitor(s) of PPO from German cockroach would be useful to the fruit and vegetable segments of the food industry, due to the losses they incur from enzymatic browning. Further studies should be conducted to purify and identify the unknown inhibitor(s).

CHAPTER 1
INTRODUCTION

Discoloration of fruits and vegetables by enzymatic browning causes millions of dollars in losses yearly to the food industry (Whitaker and Lee 1995). This browning is associated with the enzyme polyphenol oxidase (PPO; EC 1.10.3.1). It is a copper-containing enzyme, which catalyzes the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols into *o*-quinones (diphenolase activity) using oxygen as the primary oxidant (Lax and Vaughn 1991; Whitaker 1994; Lerch 1995; Yoruk and Marshall 2003). The resulting quinones undergo further nonenzymatic condensation reactions leading to the formation of undesirable dark brown melanins (Sapers 1993). The catalytic action of PPO has a large effect on the quality of several fruit and vegetable crops, which can result in the alteration of color, flavor, texture, and nutritional value (Yoruk and Marshall 2003).

Enzymatic browning commonly occurs in fruit and vegetable crops after harvest during handling, processing and storage (Jang and others 2002). Intact plant cells do not undergo browning because phenolic compounds in vacuoles are separated from PPO in cytoplasm (Yemenicioglu and others 1997). Severe browning of plant products occurs when PPO and substrate come into contact due to stress conditions from subcellular decompartmentalization and oxygen penetration (Vamos-Vigyazo 1981).

Enzymatic browning is considered an undesirable reaction in raw fruits and vegetables due to the change in color and development of off-flavors, as well as loss of nutritional quality from the breakdown of vitamins (Murata and others 1995; Weemaes and others 1997). Apples and potatoes are popular crops worldwide, with apple production exceeding 85 million tons/y (Becker and others 2000; Yoruk and others 2004). In the United States, apple production is valued at more than $1.6 billion dollars annually, while total potato production was estimated at

424 million hundredweight (cwt) in 2004 (Miller 2005; USDA 2006). Historically, sulfites have been used to slow and/or prevent browning. FDA's banning of sulfites in 1986 has created a large field of research in search of natural, effective and economic inhibitors of enzymatic browning.

PPO is found in insects as well as plants (Sugumaran 1998). Insect PPO is considered to play a key role in important physiological processes in insects such as cuticular tanning and sclerotization, as well as in wound healing and defense reactions against foreign pathogens (Tsukamoto and others 1992; Sugumaran and Nellaiappan 2000; Wang and others 2004). It seems possible that natural inhibitors within insects regulate this development. Previous literature has demonstrated endogenous inhibitors in insects such as the housefly (Tsukamoto and others 1992; Daquinag and others 1995, 1999). Sugumaran and Nellaiappan (2000) isolated a heat-labile, high molecular weight (380000) glycoprotein inhibitor of PPO from the larval cuticle of tobacco horn worm, *Manduca sexta*. In addition, Yoruk and others (2003) discovered a new natural apple PPO inhibitor(s) from housefly, *Musca domestica* L. that was stable to heat, but lost all but 10% PPO inhibition after ultrafiltration with a 100,000 NWML membrane.

Preliminary studies in our laboratory evaluating insects found a new natural plant polyphenol oxidase (PPO) inhibitor(s) from German cockroach (*Blattella germanica*). Crude inhibitor(s) isolated via buffer extraction inhibited the activites of apple and potato PPO at pH values of 5.5 and 6.0, respectively. Heat treatment showed that inhibition was totally inactivated. Ultrafiltration and dialysis studies showed the unknown inhibitor(s) was larger than 100 kDa. There is no previously known research examining the applicability of inhibitors from German cockroach to food systems. The objectives of this study were to provide evidence for the existence of plant PPO inhibitor(s) in German cockroach and to demonstrate inhibition of

plant PPO activity using these inhibitor(s). Characterization of crude inhibitor(s) included temperature stability, pH extraction profile, incubation, dialysis, ultrafiltration, treatment with proteases and kinetics.

CHAPTER 2
LITERATURE REVIEW

Enzymatic Browning

Polyphenol oxidase (PPO; 1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) causes enzymatic browning in food products by catalyzing the conversion of natural phenolic compounds to quinones (Nicolas and others 1994; Yoruk and Marshall 2003; Yoruk and others 2004). PPO is a generic name for a group of enzymes frequently referred to as tyrosinase, polyphenol oxidase, phenolase, catechol oxidase, cresolase, or catecholase. PPO is capable of catalyzing reactions for several phenols to produce *o*-quinones (Whitaker 1994). The catalytic action of PPO has a large effect on the quality of several fruit and vegetables, which can result in the alteration of color, flavor, texture, and nutritional value (Yoruk and Marshall 2003). Those are four attributes considered by consumers when making food choices. Browning commonly occurs in fruit and vegetable crops after harvest during handling, processing and storage (Lee and others 2001; Jang 2002).

PPO is most likely present in all plants, but is in especially high concentrations in mushrooms, potatoe tubers, apples, peaches, bananas, and avocados (Sanchez-Ferrer and others 1993b; Whitaker 1994). These crops are especially susceptible to browning reactions. It is estimated that over 50 percent of fruit losses occur due to enzymatic browning (Whitaker and Lee 1995). On the contrary, some cases of enzymatic browning are considered desirable, for example during the harvest of dates and in flavoring of beverages such as tea, coffee, and cocoa (Le Bourvellec and others 2004).

In addition to its general occurrence in plants, PPO also occurs in aquatic foods, primarily in crustaceans such as lobster and shrimp (Simpson and others 1988). Aquatic organisms rely on PPO to impart important physiological functions for their development, such as hardening of the

shell (sclerotization) after molting, as in crustaceans such as shrimp and lobsters. PPO is also responsible for wound healing. The mechanism of wound healing in aquatic organisms is similar to that of plants in that the compounds produced as a result of the polymerization of quinones exhibit both antibacterial and antifungal activities. However, PPO-catalyzed browning of the shell after harvest has an adverse effect on both the quality and consumer acceptability of these products (Marshall and others 2000). Although black spot formation does not affect the nutritional quality of seafood, it is perceived as spoilage by consumers (Chen and others 1992).

Biochemical Foundation of Enzymatic Browning

Enzymatic browning is a two-step process. The first step involves the enzymatic oxidation of monophenols (monophenolase activity) or *o*-diphenols (diphenolase activity) to yield *o*-quinones. In the second step, the *o*-quinones undergo condensation to form dark brown melanins (Sapers 1993; Whitaker 1994, 1995; Sanchez-Ferrer and others 1995). Figure 2-1 shows the reaction for enzymatic browning and subsequent nonenzymatic condensation .

The involvement of copper as a prosthetic group of PPO is essential for its activity. Monophenolase activity is always coupled to diphenolase activity; however, diphenolase activity is not always preceded by monophenolase activity (Whitaker 1994). In the two general reactions, phenols and oxygen are the substrates and BH_2 represents an *o*-diphenolic compound, which acts as an election donor. In the absence of an *o*-diphenol (BH_2), there is a characteristic lag or induction period in the monophenolase reaction prior to attaining a steady state rate. The duration of the lag period depends on the substrate concentration, the pH, and the presence of catalytic amounts of *o*-diphenol (Sanchez-Ferrer and others 1993c).

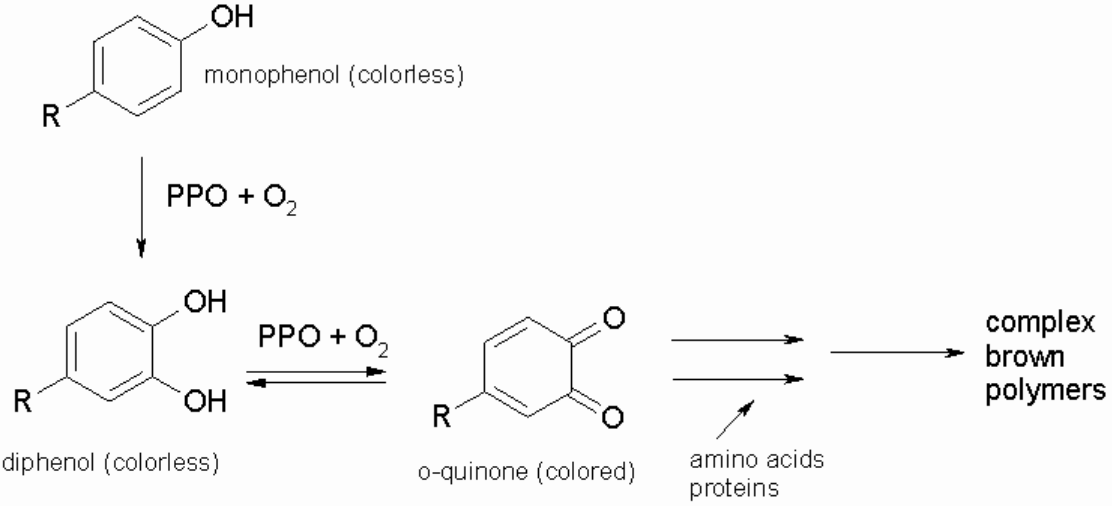

Figure 2-1. Reaction for enzymatic browning (Adapted from Walker, J.R.L. 1977. Enzymatic Browning in foods: Its chemistry and control. Food Technol. NZ, 12: 19-25)

Polyphenol Oxidase (PPO)

A large field of research shows that PPO is found throughout nature (Whitaker 1994). PPO is widely distributed and can be found in animals, plants, and microorganisms (Whitaker and Lee 1995). PPO is located in the plastids of all tissue types of species that contain the enzyme (Sherman and others 1995). Generally, PPO is associated with pigmentation of tissues, acting as protection in animals, while the role of the enzyme is not known with certainty in higher plants (Whitaker and Lee 1995).

Mechanism of PPO

The mechanism of the catalytic action of PPO was deduced based on the geometric and electronic structure of the copper active site (Wilcox and others 1985; Solomon and others 1992). The active site of the enzyme consists of a bi-nuclear copper cluster in the ligand field with three conserved histidine residues existing in three forms: met-PPO (Cu^{+2}), deoxy-PPO (Cu^{+1}), and oxy-PPO (Cu^{+2}) (Gandia-Herrero and others 2005). The resting form of the enzyme is thought to be met-PPO, which is kinetically capable of oxidizing diphenol but not monophenol substrates (Solomon and others 1992; Solomon and Lowery 1993; Gandia-Herrero and others

2005). Oxidation of one molecule of catechol to *o*-benzoquinone takes place to reduce met-PPO to deoxy-PPO. The deoxy-PPO then reacts with dioxygen to produce an oxy-PPO intermediate (Solomon and others 1992). Oxy-PPO hydroxylates monophenols to generate *o*-diphenols and the met-PPO site. PPO is converted into deoxy-PPO and ready for another round without cycling through the resting form.

In the diphenol oxidation pathway, diphenolic substrates react with both oxy-PPO and met-PPO. Met-PPO is first reduced to its deoxy form leading to the formation of oxy-PPO, which then forms an *o* – diphenol-PPO complex. Finally, after the oxidation of catechol to *o*-benzoquinone, the enzyme is reduced to the resting form of met-PPO (Yoruk and Marshall 2003).

Kinetic data indicate that PPO first binds oxygen and later monophenol in an ordered sequential mechanism (Wilcox and others 1985). Therefore, the mechanism is thought to be an Ordered Sequential Bi Bi Mechanism (Whitaker 1994).

Plant PPO

PPO is present in most plant tissues (Sherman and others 1995) and is in especially high concentrations in mushrooms, potato tubers, peaches, apples, bananas, avocados, tea leaves, coffee beans, and tobacco leaves (Whitaker 1994). PPO has been extensively studied by scientists in food and plant science due to its adverse browning effect on plant products. The chemistry of PPO-catalyzed browning reactions in higher plants beyond the o-quinone step has only been elucidated in a few cases, because of insolubility of the products (Whitaker 1995). A sequence of biochemical reactions leads to the formation of melanin from the oxidation of phenolic amino acid tyrosinase. The products through indole-5,6-quinone are soluble in *in vitro* systems (Whitaker 1995).

The oxidation of phenols and formation of melanins are normal physiological processes of PPO, although the significance of the enzyme activity in living intact plant tissues has not been unequivocally determined (Vaughn and others 1988; Sherman and others 1995; Yoruk and Marshall 2003; Maki and Morohashi 2006). This is surprising given a century of intensive research as a result of the economic importance of the enzyme (Sommer and others 1994). It has been frequently suggested that PPOs have a defensive role against pathogens and pests (Vaughn and others 1988; Maki and Morohashi 2006).

Location of PPO has always been of special interest to researchers, because one must take into account its subcellular location in order to assign a physiological function to the enzyme (Mayer and Harel 1979; Vaughn and Duke 1984b). PPO is located in several subcellular fractions, including peroxisomes, mitochondria, and microsomes (Mayer and Harel 1979; Martinez-Cayuela and others 1989; Yoruk and Marshall 2003). Research on the inheritance of isozyme patterns in species and interspecific hybrids (Lax and others 1984; Kowalski and others 1990), suggests that PPO is a nuclear-coded enzyme. The widely distributed enzyme is found in the plastids of all tissue types in species possessing PPO activity (Vaughn and others 1988; Sherman and others 1995; Murata and others 1997). It is implausible that the presence of a different form of the enzyme in aplastidic tissues exists in tentoxin-treated plants lacking PPO activity. The toxin has been shown to inhibit the import of precursor PPO protein into plastids (Sommer and others 1994).

PPO has been described as tightly bound to thylakoids, but others have suggested it is located in plastid envelopes or the thylakoid lumen (Vaughn and others 1988; Sherman and others 1991; Sommer and others 1994). More recent research suggests that PPO is synthesized on cytoplasmic ribosomes and is practically inactive until the time it is integrated into the

plasmid (Yoruk and Marshall 2003). Molecular chaperones, a class of cellular proteins that facilitate correct folding and assembly of proteins, stabilize a partially unfolded protein and prevent its refolding. This allows the protein to maintain its competence for insertion into thylakoid membranes (Yalovsky and others 1992; Marques and others 1995; Yoruk and Marshall 2003).

The size of the native enzyme has also been in question. Higher molecular weight nuclear-coded precursor proteins that possess a transit peptide sequence at the amino-terminal end are directed to the chloroplast. These signal sequences are later removed by specific stromal peptidases during incorporation, producing a lower molecular weight mature protein (Sommer and others 1994; Koussevitzky and others 1998). Molecular weights of precursor PPO proteins are approximately 68 kDa, while the mature proteins are approximately 60 kDa (Yoruk and Marshall 2003).

Intense photochemical activity occurs in the thylakoid membranes of chloroplasts. The location of PPO in the thylakoid has led researchers to believe that PPO may play a role in photosynthesis of functional chloroplast (Vaughn and Duke 1984a; Vaughn and others 1988; Thygesen and others 1995). The association of PPO with photosystem complexes suggested a possible role in photosynthetic electron transport (Lax and Vaughn 1991; Sheptovitsky and Brudvig 1996). Tolbert (1973) suggested PPO plays a role in pseudocyclic photophosphorylation, which is ATP production with oxygen as a terminal electron acceptor (Vaughn and Duke 1984a; Sherman and others 1995). However, it appears the activity of photosystem II membrane-associated PPO is pH dependent, with an optima around pH 8.0. During light-induced O_2 evolution, the pH of the lumen is rather acidic. Therefore, it appears

PPO is important mostly for dark processes in the thylakoid lumen, when the pH is close to optimum for PPO (Sheptovitsky and Brudvig 1996).

A number of PPO genes from different plant species have now been isolated and characterized. Tomato PPO is routed to the thylakoid lumen in a two step process (Sommer and others 1994). First a 67 kDa precursor is imported into stroma and then processed to 62 kDa by stromal peptidases. Finally, the resulting intermediate is translocated into the lumen and processed to a 59 kDa mature protein. The ratio between the intermediate and mature forms depends on the plant species, age, and growth conditions (Sommer and others 1994). In some species, PPO genes are present as multigene families, while in others only a single PPO gene has been identified. However, all the PPO genes characterized so far are nuclear encoded (Cary and others 1992; Robinson and Dry 1992; Shahar and others 1992; Hunt and others 1993; Thygesen and others 1995).

Involvement of PPO in oxidation of phenolic compounds in various plants is its most common role (Yoruk and Marshall 2003). Severe oxidative browning does not occur in healthy intact plant tissues perhaps because PPO activity *in vivo* is limited by lack of phenolic substrates physically separated from the enzyme inside the vacuoles (Vaughn and Duke 1984a; Macheiz and others 1990). It is generally believed that senescence or injury results in destruction of the biological barriers between PPO and polyphenols and the enzyme is active only when it unites with its phenolic substrates (Vaughn and Duke 1984a; Vaughn and others 1988; Eskin 1990; Murata and others 1997). However, it is unknown whether other factors, in addition to disruption of membrane integrity, are involved in formation of such active enzymes for synthesizing or oxidizing polyphenols, or that affect the amount of active enzyme form and the level of enzyme activity during senescence, developmental stages or injury.

General Properties of Apple and Potato PPO

Apples are popular throughout the world, with world production estimated to be 42 million metric tons in the marketing year (MY) 2004/2005 (USDA 2005). In the United States, apple production is valued at more than $1.6 billion dollars annually (Miller 2005) with production estimated at 4.6 million metric tons in MY 2004/2005 (USDA 2005). According to Miller (2005), apples are regarded as the third most valuable fruit crop in the United States behind grapes and oranges. During the 2004/2005 marketing year (MY), apple production is expected to increase to about 4.3 million tons (USDA 2005), while apple consumption in the United States is expected to increase 10 percent.

Total potato production in the United States in 2005 was estimated at 424 million hundredweight (cwt), up 1 percent from the estimate made in the January Annual Crop Summary (USDA 2006). Cwt is the commonly accepted measurement for potatoes and equivalent to 100 lbs. According to USDA (2006), the value of U.S. all potatoes sold in 2005 is estimated at $2.76 billion, up 18 percent from the previous year.

Enzymatic browning has a large impact on the quality of both apples and potatoes, and their products, as it results in alteration of color, flavor, texture, and nutritional value (Sapers 1993; Nicolas and others 1994). Therefore, research that discovers ways to maintain the original color of these fresh plant tissues is of great importance to the food industry.

Substrate Specificity

The primary substrates of apple and potato PPOs are phenolic compounds (Table 2-1). The type of natural phenols and their relative concentrations vary widely from plant to plant source (Yoruk and Marshall 2003). Catechin, epicatechin and caffeic acid derivatives have been found to be common natural substrates of fruit PPOs (Macheix and others 1990). In apples, chlorogenic acid is the major phenolic compound in mature fruit while catechin is the major

phenolic compound in immature fruit (Murata and others 1995). Activity of apple PPO on tyrosine was shown to be much lower than on *o*-diphenol (Zhou and others 1993). In addition, catechin was reported to be a major cause of browning in apples (Murata and others 1995).

Table 2-1 shows the difference in activity of apple, potato, and banana PPOs for different types of substrates. PPO is most active with substrates that have a high specificity for the enzyme. Factors such as the nature of the side chain, number of hydroxyl groups and their position on the benzene ring have a large effect on the catalytic activity of the enzyme (Macheix and others 1990). It appears that substrate specificity of PPO is also dependent on species and cultivars. In addition, PPO isoforms in a tissue of interest may also demonstrate differential substrate specificities and variations in their relative activities towards monophenols and *o*-diphenols (Park and Luh 1985; Macheix and others 1990). Four PPO isoforms differing in substrate specificities were discovered in apple tissues (Harel and others 1965).

Table 2-1. Relative substrate specificity of PPOs from apple, potato, and banana

	Percent Activity		
Substrates	Apple[a]	Potato[b]	Banana[c]
4-Methylcatechol	181	93	
Catechol	100	100	34
Chlorogenic acid	102		5
d-Catechin	54		12
Caffeic acid		0.7	1
Pyrogallol	38	27	1
Dopamine	37		100
DL-DOPA	12	0.6	8
Tyrosine	3		

DOPA: Dihydroxyphenylalanine; References: [a]Zhou and others (1993) [b]Cho and Ahn (1999) [c]Yang and others (2000)

Effects of pH and Temperature

The catalytic activity of PPO is greatly influenced by changes in pH. This is because changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the enzyme's active site. Changes in the active site's conformation alter the binding of substrates, and/or catalysis of the reaction (Tipton and Dixon 1983; Whitaker 1994; Yoruk and Marshall 2003). Factors that influence the stability of an enzyme do so by affecting the secondary, tertiary, and/or quaternary structure of proteins. Therefore, irreversible denaturation of the protein and/or reduction of the stability of the substrate as a function of pH could also influence the catalytic activity of enzymes (Valero and Garcia-Carmona 1998; Yoruk and others 2003).

The optimum pH of PPO varies widely depending on the plant source, but is generally in the range of 4.0-8.0, (Table 2-2) (Fraignier and others 1995; Marques and others 1995; Sheptovitsky and Brudvig 1996; Yoruk and Marshall 2003). Apple PPO has been found to have an acidic pH. While a single pH optimum of 4.5-5.0 has been reported in some cases (Janovitz-Klapp and others 1989; Zhou and others 1993), others have reported two pH optima at around 5.0 and another around 7.0 (Stelzig and others 1972). The optimum pH value for PPO is affected by several experimental factors such as extraction methods, temperature, nature and concentration of the buffer, nature and concentration of the substrate, and purity of the source preparation (Stelzig and others 1972; Janovitz-Klapp and others 1989; Zhou and others 1993; Whitaker 1994).

The modulator sodium dodecyl sulfate (SDS) can also affect the pH optimum for PPO. The general behavior of apple PPO with changing pH was altered in the presence of SDS, with the activity inhibited at acidic pH and activated at pH above 5.0 in the presence of 3.5 mM SDS

regardless of substrate (Marques and others 1995). SDS also causes a shift in the pH optimum of PPO from low to a higher pH value with different values for different substrates.

The catalytic activity of PPO is also greatly influenced by temperature. On one hand at low temperature the kinetic energy of the reactant molecules decreases and thus corresponds to a slower rate of reaction (Lehninger and others 1993; Yoruk and Marshall 2003). On the other hand, high temperature caused disruption of the integrity of the three-dimensional structure of the enzyme molecule as well as alteration of the solubility of oxygen. Oxygen is a required substrate for the catalytic activity of PPO (Whitaker 1994).

Optimum temperature of PPO varies depending on the plant source (Table 2-3). Apple PPO activity has an optimum temperature around 30°C, while potato PPO has an optimum around 40°C (Zhou and others 1993; Cho and Ahn 1999). Optimum temperature of the activity is also affected by the substrate used in the assay. A study on Monroe apple PPO showed when the temperature was increased from 20 to 40°C, the relative activity of PPO decreased from 100 to 96%. When the temperature was increased from 50 to 70°C, the relative activity decreased from 87 to 6%, indicating rapid denaturation of the enzyme at higher temperatures (Zhou and others 1993).

Thermal inactivation of PPOs from several sources was shown to follow first-order kinetics (Lee and others 1983; Yemenicioglu and others 1997). A study was done on several cultivars of apple (Yemenicioglu and others 1997) examining heat inactivation kinetics at temperatures of 68, 73, and 78°C. Results showed that after an initial increase in PPO activity, which can be attributed to the activation of a latent form, activity decreased following a first order kinetic model. In addition, a variance in the thermal stability between the different cultivars of apple was seen.

Table 2-2. The pH optimum of PPO for different plant sources

Source of Enzyme	Substrate	pH Optimum
Apple[a]	Catechol	5.5
Apricot[b]	4-Methylcatechol	5.0-5.5
Cherry[b]	4-Methylcatechol	4.5
Grape[c]	4-Methylcatechol	3.5-4.5
Peach[b]	4-Methylcatechol	5.0
Plum[b]	4-Methylcatechol	4.0-5-5
Potato[d,e]	Chlorogenic acid	4.5-5.0 and 6.0-6.5
	Catechol	6.6
Strawberry[f]	Catechol	5.5
Sweet Potato[g]	Catechol	6.5

References: [a]Yoruk and others (2003) [b]Fraignier and others (1995) [c]Valero and others (1998) [d]Sanchez-Ferrer and others (1993a) [e]Cho and Ahn (1999) [f]Wesche-Ebeling and Montgomery (1990) [g]Lourenco and others (1992)

Table 2-3. Effect of temperature on PPO activity

Source of Enzyme	Substrate	Temperature Opt. (ºC)
Apple[a]	Catechol	30
Banana[b]	Dopamine	30
Grape[c]	4-Methylcatechol	25-45
Potato[d]	Catechol	40
Strawberry[e]	Gallic acid	50

References: [a]Zhou and others (1993) [b]Yang and others (2000) [c]Valero and others (1998) [d]Cho and Ahn (1999) [e]Serradell and others (2000)

Insect PPO

PPO is found in insects as well as plants (Sugumaran 1998; Marshall and others 2000). Insect PPO is considered to play a key role in important physiological processes in insects such as cuticular tanning and sclerotization, as well as in wound healing and defense reactions against foreign pathogens (Tsukamoto and others 1992; Sugumaran and Nellaiappan 2000; Feng and Fu 2004; Wang and Jiang 2004; Zufelato and others 2004). The general properties of PPO appear to be simlar for plant and animals; the enzyme acts upon a number of phenolic substrates and oxidizes them into quinone compounds (Sugumaran 1998; Yoruk and Marshall 2003).

In many ways, the molecular biology of PPOs from plants and insects is similar. Recent studies concerning the molecular cloning of PPOs from different species of insects have shown that there are 2 conserved copper-binding sites showing high sequence similarity to arthropod hemocyanins (Sugumaran 1998). On the other hand, insect PPOs differ from plant PPOs in that they lack signal peptide sequences, directing nuclear-coded precursor of plant PPOs to the chloroplast envelope for subsequent processing, as discussed above (Yoruk and others 2003). PPO is present as a soluble enzyme in the blood of insects, which is stored in an inactive proform, and activated via a number of mechanisms during a time of need (Sugumaran and Nellaiappan 2000).

The blood of insects is rich in potential phenoloxidase activity, exhibiting both monophenolase and diphenolase activities. However, the cuticle only contains diphenolase activity (Brunet 1980). All known PPOs, whether plant or insect, are capable of oxidizing *o*-diphenols, making diphenolase activity the most common function of the enzyme (Yoruk and others 2003). There is a large fluctuation of PPO activity during insect metamorphosis (Hara and others 1993).

The discovery of a quinone tanning process involving PPO goes back to early research by Pryor (1940) on the sclerotization of cockroach oothecae and insect cuticle (Hopkins and Kramer 1992; Sugumaran 1998; Suderman and others 2006). The cuticle, or exoskeleton, of insects is responsible for body architecture, allowing locomotion, and acting to protect them against pathogen attack and environmental stress (Andersen and others 1996; Moussian and others 2005). Insect cuticle is composed of structural proteins, chitin, enzymes, catecholamines, lipids, and minerals. The relative ratio of these components varies from insect to insect, cuticle to cuticle, and even region to region according to varying demands of function and growth (Andersen and others 1996; Sugumaran 1998). In the case of cockroaches, the egg case (ootheca) is totally devoid of chitin with 8.5% proteins, 5% diphenol and 1% lipids, while the adult cuticle of cockroaches has about 40% chitin, 50% protein, 5-10% catechol, and 1% lipid (Sugumaran 1998).

The main roles of PPOs in insects include sclerotization (hardening) and tanning of the insect cuticle (Hopkins and Kramer 1992; Anderson and others 1996; Sugumaran 1998). Sclerotization of the cuticle is a vital process for the survival of most insects, which protects susceptible soft parts of their bodies (Sugumaran and Ricketts 1995). Sclerotins are chemically modified protein-containing components of insoluble and stiff skeletal structures. In cockroaches, sclerotins occur in the ootheca and exoskeleton (Peter 2006).

Cuticular sclerotization involves the introduction of covalent crosslinks between proteins as well as chitin, which results in the formation of protein-protein and protein-chitin crosslinks (Sugumaran and others 1992). A combination of studies over the years indicates that quinones, quinone methides and semiquinones all act as sclerotizing agents (Sugumaran 1998). According to a unified mechanism, catecholamine sclerotizing agent's precursors are converted by cuticular

phenoloxidases to quinones that either participate in the quinone tanning process or serve as substrates for quinone isomerase that converts 4-alkylquinones to hydroxyl-*p*-quinone methides (Sugumaran 1998; Sugumaran and Nelson 1998). Interaction of the sclerotizing agents with certain side groups on cuticular components, such as protein and chitin, results in the hardening of the cuticle (Sugumaran 1998). Cuticles can vary in hardness, color, and flexibility depending on the sclerotization mechanisms and precursors used (Sugumaran and Ricketts 1995).

Insect PPO is also suspected to play critical roles in defense (color and camouflage), wound healing, and disease resistance (Sugumaran and Nellaiappan 2000; Chase and others 2000; Yoruk and others 2003). The onset of an infection stimulates a multifaceted defensive response in insects (Gillespie and others 1997). The response triggers an activation of the PPO zymogen present in the hemolymph. Secondary reaction products of PPOs appear to fulfill some functional roles in insects, just like they do in plants (Gillespie and others 1997; Sugumaran and Nellaiappan 2000; Yoruk and Marshall 2003). The oxidative polymerization of quinones forms insoluble melanin over wounds to prevent loss of blood from infected tissues (Gillespie and others 1997; Sugumaran and Nellaiappan 2000). Another important defensive role of melanization in insects is its action in pathogen sequestration (Gillespie and others 1997). Parasites and pathogens which are too large to be phagocytosed are encapsulated in a melanin coat in the insect's blood. This acts to not only limit the growth and development of the foreign invader, but also acts to create a physical barrier to prevent damage to the host (Chase and others 2000; Sugumaran and Nellaiappan 2000).

PPO is present throughout the life cycle of insects in order to perform these multiple functions. PPO in the open circulatory system of insects occurs in the inactive proenzyme form, and is then activated when needed (Hara and others 1993; Chase and others 2000; Sugumaran

and Nellaiappan 2000). The regulatory biochemical mechanisms of endogenous PPO activity include inactivation, polymerization, complexation with other proteins, and inhibition (Sugumaran and Nellaiappan 2000). It is suggested that PPO activity might also be regulated by intrinsic inhibitors throughout the course of development (Tsukamoto and others 1992; Daquinag and others 1995, 1999; Sugumaran and Nellaiappan 2000; Yoruk and others 2003). Tsukamoto and others (1992) isolated inhibitors from the pupal extracts of house fly and demonstrated inhibition on house fly PPO. The potent house fly inhibitors were found to be heat-stable low-molecular weight (approximately 3000 to 4200) peptides with an inhibition constant in the nM range (Tsukamoto and others 1992). Further, Sugumaran and Nellaiappan (2000) have isolated a high molecular weight (380000) glycoprotein inhibitor of PPO from larval cuticle of the tobacco horn worm, *Manducta sexta*, and proposed that the presence of endogenous PPO inhibitor(s) would ensure direct control of PPO activity in insects. The specific endogenous PPO inhibitor(s) of *Manducta sexta* inhibited both insect and plant PPOs. Additionally, Yoruk and others (2003) isolated inhibitor(s) from the common house fly (*Musca domestica*). The isolated inhibitor(s) were found to be fairly heat-stable and low-molecular weight. The inhibitor(s) from *Musca domestica* inhibited apple PPO.

Inhibition of Enzymatic Browning

Enzymatic browning causes millions of dollars in crop losses every year (Whitaker 1996; Kim and others 2000). The economic impact of these losses in fruits, vegetables, and seafood has spurred a large amount of research set to control the reaction in order to extend product shelf-life (Yoruk and Marshall 2003). Inhibitors of enzymatic browning caused by PPO fall into six groups based on their mode of action according to McEvily and others (1992):

- Reducing agents (ascorbic acid and analogues, sulfites)

- Chelating agents (ethylenediaminetetraacetate [EDTA], sodium diethyldithiocarbamate [DIECA], sodium azide)
- Complexing agents (cyclodextrins, chitosan)
- Acidulants (ascorbic acid, citric acid, malic acid, phosphoric acid)
- Enzyme inhibitors (substrate analogs, halides)
- Enzyme treatments (proteases, *o*-methyltransferase)

Reducing Agents

Reducing agents are widely used in the food industry to inhibit enzymatic browning. They act to prevent formation of melanin by preventing the accumulation of *o*-quinones, or by forming stable colorless products (Eskin and others 1971; Nicolas and others 1994; Kim and others 2000). The action of a reducing agent on the enzymatic browning can be seen in Figure 2-2.

Sulfites

Sulfites are inorganic salts that have antioxidant and preservative properties. Many compounds capable of producing sulfite called sulfiting agents, have been used as food additives since antiquity to help prevent enzymatic and nonenzymatic browning; control growth of microorganisms; act as bleaching agents, antioxidants, or reducing agents; and carry out various other technical functions (Taylor and others 1986; Sapers 1993; Girelli and others 2004). Examples of sulfiting agents include sulfur dioxide, sodium sulfate, sodium and potassium bisulfites and metabisulfites. Specifically, sulfites are used on fruits and vegetable to prevent unpleasant browning, applied to shrimp and lobster to prevent melanosis, or "black spot", used in wines to discourage bacterial growth, as a conditioner in dough and to bleach certain food starches and cherries.

In fresh fruits and vegetables, sulfites prevent PPO from working properly by preventing brown pigment formation (Sayavedra-Soto and Montgomery 1986; Sapers 1993). Sulfites are thought to inhibit browning by acting as a reducing agent that combines with the ortho-quinones and converts them back to colorless diphenols. This prevents the nonenzymatic condensation of *o*-quinones to complex brown polymers (Figure 2-2) (Weaver 1974).

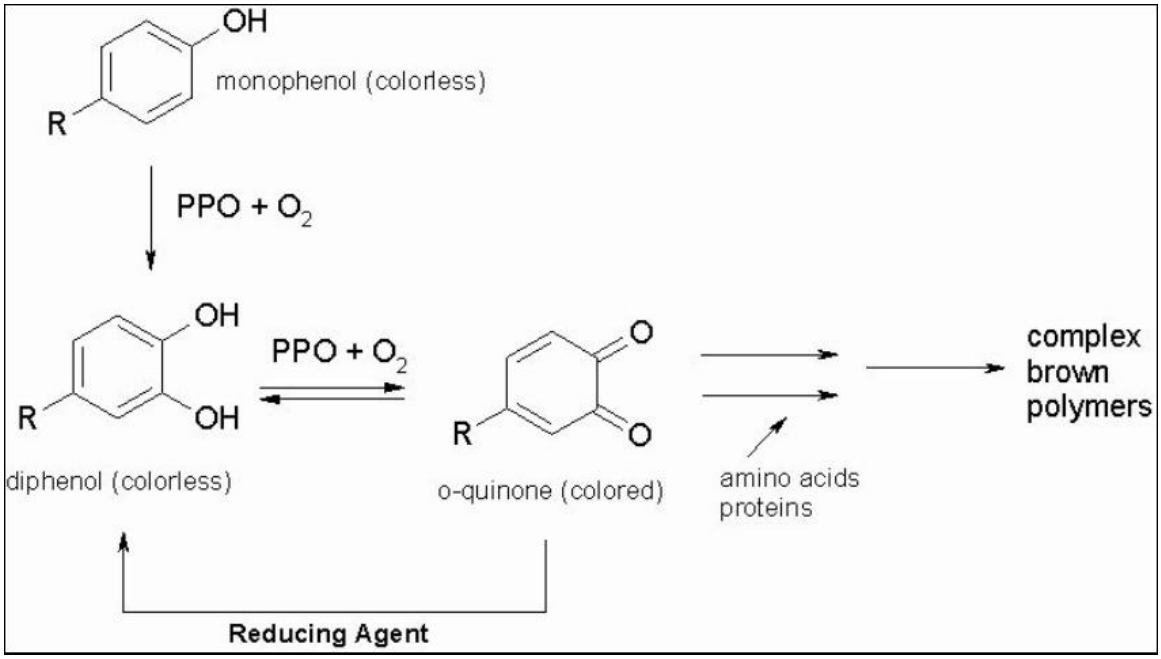

Figure 2-2. Inhibition of enzymatic browning using a reducing agent. (Adapted from Walker, J.R.L. 1977. Enzymatic Browning in foods: Its chemistry and control. Food Technol. NZ, 12: 19-25)

A small percentage of the U.S. population is suspected to be sensitive to sulfites. Sensitivity to sulfites can develop at any time during a human lifespan, with some initial reactions not showing up until a person has reached their forties or fifties. The manifestations of sulfite sensitivity include a large array of dermatological, pulmonary, gastrointestinal, and cardiovascular symptoms. Adverse reactions to sulfites in nonasthmatics are extremely rare, but asthmatics that are steroid-dependent or have a great degree of airway hyperreactivity may be at an increased risk of having a reaction to a sulfite containing food (Lester 1995). Varying degrees

of bronchospasm, angiodema, urticaria, nausea, abdominal cramping, and diarrhea are commonly reported (Knodel 1997).

Sulfites have such a long history of use that when the Federal Food, Drug, and Cosmetic Act was amended in 1958 to regulate preservatives and other food additives, FDA classified sulfites as generally recognized as safe (GRAS) (Papazian 1996). However, in response to a 1982 FDA proposal to affirm the GRAS status of sulfating agents, FDA began to receive reports of adverse health reactions to sulfites (Papazian 1996; Sapers 1993). FDA contracted the Federation of American Societies for Experimental Biology (FASEB) to examine the link between sulfites and reported health claims.

The FASEB submitted its final report to FDA in 1985, concluding that sulfites are safe for most people, but could pose a hazard of unpredictable severity to asthmatics and others who are sensitive to them. Based on this report, FDA took the following regulatory actions in 1986: sulfites were prohibited to maintain color and crispness of fresh fruits and vegetables, such as in salad bars or fresh produce. Companies were required to list sulfites or chemical components that give rise to sulfites with at least 10 part per million (ppm) or higher, and any sulfiting agents that had a technical or functional effect in the food regardless of the amount present (FDA 1988a; Papazian 1996).

Currently, sulfiting agents are not considered GRAS for use in meats, foods recognized as a major source of vitamin B-1, or "fruits or vegetables intended to be served raw to consumers or to be presented to consumers as fresh" (FDA 1988b). Sulfites have been found to destroy thiamin and have therefore been banned for use in foods that are a major source of vitamin B-1.

Ascorbic acid

Ascorbic acid (vitamin C), which is acidic in nature, is known as the best alternative reducing agent to sulfites. It forms neutral salts with bases, and is highly water soluble (Marshall

and others 2000). Ascorbic acid is commonly used as an antibrowning agent in the manufacturing of fruit juices, purees, frozen sliced fruits, and canned fruits and vegetables (Yoruk and Marshall 2003). Ascorbic acid retards the formation of melanins by directly reducing *o*-quinones back to corresponding reactants, *o*-diphenols, before the quinones can undergo additional non-enzymatic polymerization to form dark brown melanins, (Figure 2-2). Unfortunately, ascorbic acid is irreversibly oxidized to dehydroascorbic acid during the reduction process, which allows browning to occur upon its depletion, limiting it's usefulness as an inhibitor (Sapers 1993).

There is an interest in investigation of using ascorbic acid in combination with other inhibitors for better control of enzymatic browning. Eskin and others (1971) and Sapers (1993) found ascorbic acid in combination with citric acid to be a more effective inhibitor than ascorbic acid alone. This increased inhibition is likely due to the increased stability of ascorbic acid in an acidic environment as well as inhibition of the acidic environment on the catalytic activity of the enzyme (Yoruk and Marshall 2003). Citric acid can also function as a PPO inhibitor through its chelating action (Eskin and others 1971); and ascorbic acid through the site-directed specificity toward histidine residues on the PPO protein (Osuga and others 1994).

Chelating Agents

Chelating agents are another group of enzymatic browning inhibitors. Copper is critical for PPO activity because the enzyme is a copper metalloprotein (Lerch 1995; Van Gelder and others 1997). The active site of PPO is comprised of two copper atoms through which the enzyme interacts with its phenolic substrates and oxygen (Lerch 1995; Van Gelder and others 1997). Chelating agents have the ability to react with metals through chelation, making copper at the active site unavailable, thereby inhibiting PPO (McEvily and others 1992). Examples of agents serving as chelators for copper include EDTA, phosphates, citric acid,

diethyldithiocarbamate (DIECA), and sodium azide (Eskin and others 1971; McEvily and others 1992; Yoruk and Marshall 2003).

Acidulants

Acidulants are another group of inhibitors used by the food industry to prevent enzymatic browning because they are often part of the natural food composition. Acidulants, such as ascorbic, citric, malic, and phosphoric acids, are applied to maintain the pH well below that required for optimum catalytic activity an enzyme. These acids can shift the food pH to 3 or lower, and slow the action of PPO (Richardson and Hyslop 1985; Eskin 1990; Osuga and others 1994). PPO is typically more active in a pH range from 4.0-8.0, and the activity drops steadily in acidic environments (Yoruk and Marshall 2003). Citric acid is one of the most widely used acidulants in the food industry.

Under conditions of extreme acidic pH, resulting inhibition can be attributed to protonation of catalytic groups essential for catalysis, conformational changes in the active site of the enzyme, irreversible denaturation of the protein, and/or reduction in the stability of the substrate as a function of pH (Whitaker 1994). In addition, acidity also diminishes the strong binding of the enzyme to its active site copper, which increases the ability of citric acid to react with copper through chelation (Osuga and others 1994).

Physical Treatments

Physical treatments are another option in controlling enzymatic browning of food products. These include heating, freezing, refrigeration, dehydration, irradiation, and high pressure treatments (Ashie and others 1996; Kim and others 2000; Yoruk and Marshall 2003). Although effective, these physical treatments can also produce undesirable side effects, such as subcellular decompartmentalization, which leads to enzyme-substrate contact and deterioration in texture (Macheix and others 1990). Irradiation can result in tissue darkening and decreased

vitamin content, while freezing can lead also lead to changes in texture and freshness. A proper combination of pressure and temperature can be used to enhance enzyme activation in certain foods (Marshall and others 2000). Additional methods for preventing enzymatic browning that are being investigated include controlled atmosphere packaging, and edible films and coatings that act to prevent oxygen penetration (Ahvenainen 1996).

Natural Inhibitors

The use of browning inhibitors by the food industry is limited due to factors such as off-flavors/odors, food safety, economic feasibility, and effectiveness of inhibition (Eskin 1990; McEvily and others 1992; Sapers 1993; Park 1999; Chen and others 2000; Yoruk and Marshall 2003). With sulfites banned by the FDA in 1996, there is an even greater research interest in finding new effective, natural, and safe antibrowning agent(s) to prevent browning of fruits and vegetables. Substantial investigations are performed on natural antibrowning agents, such as amino acids (Kahn 1985). Research has been published involving natural inhibitors such as glucose (Tan and Harris 1995) honey (Osmianski and Lee 1990; Chen and others 2000), and pineapple juice (Lozano-de-Gonzalex and others 1993). These natural PPO inhibitors are not being commercially utilized on food at this time.

There is research being conducted on inhibition of PPO by naturally occurring oxalic acid. Oxalic acid in both soluble and insoluble forms is a natural component of many higher plants. Oxalic acid was suggested to have some selective, fuctional significance or adaptation during normal plant development (Ilarslan and others 1997). Despite not being an approved food additive, oxalic acid is a common component of a large variety of plant foods such as asparagus, broccoli, Brussel sprouts, carrot, garlic, lettuce, onion, parsley, pea, potato, purslane, spinach, tomato, and turnip (USDA 1984). Research by Sato (1980) demonstrated oxalic acid was a naturally occurring inhibitor of PPO in spinach, while Ferrar and Walker (1996) reported that

oxalic acid produced by the phytopathogen *Sclerotinia sclerotiorum* inhibited PPO activity in infected apples and bean pods.

German Cockroach, *Blattella germanica*

The German cockroach (*Blattella germanica*) is about 5/8-inch in length and brown in color with two dark longitudinal streaks that run the length of the pronotum. The male is light brown and somewhat boat-shaped and slender, while the female is slightly darker in color with a broader and rounded posterior (Suiter and Koehler 2000). The German cockroach has three life stages typical of insects with incomplete metamorphosis: the egg, nymph, and adult (Valles 1996). The German cockroach produces more eggs per capsule than other pest cockroach species, and the life cycle can be completed in about 3 months (Koehler and Castner 1997; Suiter and Koehler 2000). They tend to breed continuously, with many overlapping generations present at any one time (Valles 2000).

Although the German cockroach is a primitive insect, it is well adapted to the human environment (Lin and others 2002). It is found worldwide in association with humans (Valles 1996). In the United States, it is the principle domestic household cockroach species (Bhat and others 2003). It is prevalent in and around homes, apartments, supermarkets, food processing plants, and restaurants (Suiter and Koehler 2000). The major limiting factor for the survival of German cockroaches appears to be cold temperatures, which prevent them from being able to colonize. Other limiting factors include availability of food, water, and harborage (Valles 1996).

The German cockroach is considered an aesthetic pest, therefore, the action threshold for it is dependent on the tolerance of the people living in the infested dwelling (Valles 1996). The most successful means of control is use of baits (stations or gel form). When the level of infestation is high, use of an insecticide mixed with an insect growth regulator to act as a "clean-out" may be necessary (Suiter and Koehler 2000).

Objectives

Since insects have a natural PPO mechanism, which appears to be regulated during their growth and development, we hypothesize that this regulation is due to inhibitors in the insect. Thus, the objective of this research was to provide evidence for the existence of plant PPO inhibitor(s) from German cockroach, *Blattella germanica*. Past research supports findings of an inhibitor(s) in insects that influences the enzyme activity of polyphenol oxidase. If such an inhibitor(s) is present in German cockroach, it may also be effective in inhibiting the browning in fruits and vegetables caused by PPO. Specific objectives included testing different plant PPOs to determine effectiveness with the extracts, and characterizing the extract as an inhibitor to determine the effect of temperature, pH, and incubation time on it. In addition, the approximate size of the inhibitor(s) was determined.

CHAPTER 3
MATERIALS AND METHODS

German Cockroach Samples

Samples of *Blattella germanica* (L.) were provided by the U.S. Dept. of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology (Gainesville, FL). The cockroaches were reared at 26°C, 55% relative humidity, and with a 12:12 light:dark photoperiod as described by Koehler and Patterson (1986). Newly molted adult males were collected using featherweight forceps.

Preparation of Inhibitor Extract

PPO inhibitor was extracted from German cockroach following a procedure adapted from Valles and Yu (1995). Cockroaches were anaesthetizied with carbon dioxide and male samples were separated and put on ice. The cockroaches were decapitated, and the bodies were put in a 10 mL tube with 0.1 M sodium phosphate buffer, pH 6.5. A Delta Model 11-990 12 inch drill press (Pittsburgh, PA) equipped with a Thomas Scientific Potter-Elvehjem Teflon pestle (Swedesboro, NJ) was used to homogenize the cockroaches. Next, the homogenate was filtered through a double layer of cheese cloth and centrifuged in a Beckman Model L8-M Ultracentrifuge (Fullerton, CA) at 10,000 × *g* for 15 minutes at 4°C. The supernatant was filtered through glass wool and then centrifuged again for 1 hour at 105,000 × *g*. The supernatant was filtered through glass wool once again and stored at -20°C in microcentrifuge tubes until used. The supernatant is the soluble fraction and contains the cytosome. The resulting pellet containing the microsomes was discarded.

Preparation of Plant PPO

In preliminary studies, mushroom and banana were tested for PPO activity and inhibition. Fresh Crimini mushrooms and bananas were acquired from a local grocery store and prepared

into an acetone powder. Washed mushrooms and peeled bananas were cut up to small pieces (200g of each), which were homogenized in a pre-chilled blender (Waring Products Inc., Torrington, CT) with acetone (-20°C) for 1 min, and then filtered through Whatman No. 1 filter paper. The residue was re-extracted three times with 200 ml of cold acetone (-20°C). The resulting white acetone powder was vacuum-dried in a FoodSaver Vac 350 (Tilia Inc., San Francisco, CA) at room temperature (25°C) and stored in commercial vacuum bags (Tilia Inc., San Franciso, CA) at -20°C until needed for PPO extraction.

Russet potatoes were selected as a source of potato PPO because this variety has shown high enzyme activity in past research confirmed by visual tests conducted in our lab. Previous research indicated higher PPO activity in the Russet variety potato (Hsu and others 1988). A potato acetone powder was prepared from Russet potatoes acquired from a local grocery store. The potatoes were washed and sliced into small pieces and then prepared into an acetone powder as described above.

Red Delicious variety of apple was selected as a source of apple PPO because past research indicated it to have the highest enzyme activity among the apple cultivars (Janovitz-Klapp and others 1989). Apple PPO was extracted from an apple acetone powder. Red Delicious apples, which were acquired from a local grocery store, were washed, peeled, and cut into small pieces and then prepared into an acetone powder as described above.

PPO extraction was accomplished by adding 1 gram of acetone powder to 50 mL of 0.1 M KH_2PO_4/Na_2HPO_4, pH 7.2 buffer containing 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) and incubated for 20 min while stirring with a magnetic stirrer at 4°C. The suspension was centrifuged in a Beckman Model DU 640 Ultraviolet-Visible spectrophotometer (Beckman Instruments Inc., Fullterton, CA) for 30 min at 12,000 × g, and the resulting

supernatant was filtered through glass wool. Finally, the filtered supernatant was stored in microcentrifuge tubes at -20°C.

Assay of PPO Activity and Inhibition

PPO inhibition was quantified by measuring the PPO activity of apple with and without the added inhibitor extract. A standard assay consisted of 2.45 mL of 0.1 M sodium acetate-acetic acid, pH 5.5 buffer, 0.3 mL of 0.5M catechol substrate (Sigma Chemical Co., St. Louis, MO), 0.2 mL of test extract or control buffer and 0.05 mL of the enzyme extract. PPO activity was measured by spectrophotometric determination of initial reaction rates at 420 nm and 25°C with a Beckman Model DU 640 Ultraviolet-Visible spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The pH of the reaction mixture was checked after each assay to confirm pH values were maintained for the control and test systems.

PPO activity of apple was also assayed by mixing it with an inhibitor extract in a standard reaction mixture where the pH of the main buffer (0.1 M sodium acetate-acetic acid) varied from pH 4.0 to 5.7. One unit of enzyme activity was defined as an increase in absorbance of 0.001 per min at 25°C. The degree of inhibition was expressed as percent inhibition (I), calculated using the formula [100(A-B)/A], where A represents enzyme activity of the control system and B represents enzyme activity of the test system.

The pH Optimum of Plant PPO

The pH-activity profile for the oxidation of catechol by PPO was determined using 0.1 M sodium acetate, pH 4.0 to 5.5, and 0.1 M sodium phosphate, pH 6.0 to 7.0. The reaction mixture contained 0.05 mL of the enzyme solution, 0.3 mL of 0.5 M catechol and 2.65 mL of various buffer solutions. The reaction was initiated with the addition of the enzyme solution. PPO activity was determined as described above.

Effect of Timed Incubation on Inhibitor(s)

Initiation of the assay described above was done in two different ways to compare the differences between incubating the inhibitor(s) with the PPO or substrate for varying periods of time. The main reaction buffer (sodium acetate-acetic acid, pH 5.5) and inhibitor were placed in the cuvette with either the PPO or the catechol substrate and incubated at room temperature (25°C) for 0, 1, 5, 15, and 30 min. Reactions were initiated with the component not used for incubation and initial reaction rates were calculated at 420nm and 25°C.

Effect of Heating and Freezing and Thawing on Inhibitor(s)

Aliquots of 2 mL of the inhibitor preparation were incubated in boiling water, 100°C, for 5, 15, 30, and 60 min. The precipitate was separated by centrifugation for 5 min at 10000 × *g* at room temperature. The clear supernatants were collected in clean microcentrifuge tubes. PPO inhibition was determined at 25°C as described above.

Aliquots of 2 mL for the inhibitor preparation were exposed to repeated freezing and thawing and storage (3X). In the last step, the samples were thawed after storage at -20°C. Aliquots were also stored at -20°C for up to 6 months

The pH Extraction Profile

The inhibitor extract was extracted according to the methods described above but at pH values ranging from 4.5-7.5 with 0.1 M sodium phosphate or sodium acetate buffer. The samples were stored at -20°C in microcentrifuge tubes until they were used to compare PPO inhibition as described above.

Dialysis of Inhibitor(s)

Dialysis of inhibitor extracts was performed using 500, 2000, and 25000-Da molecular-weight cut off (MWCO) dialysis membranes (Spectrum Laboratory Products, Inc., New

Brunswick, NJ) with constant stirring for 24 hours against 2 and 3 changes of 0.1 M sodium phosphate buffer, pH 6.5, and distilled water. The dialysates were kept at 4°C until they were tested for PPO inhibition.

Ultrafiltration

Ultrafree-4 centrifugal filter units (Millipore Corporation, Bedford, MA) with the nominal molecular weight limit (NMWL) membranes of 10000, 50000, and 100000 were used. Filter units were rinsed with distilled water to remove a trace amount of glycerin. Aliquots of 1.5 mL of inhibitor sample were placed onto the filter units and concentrated by centrifugation at 4°C and 7,500 × *g* for 35 min. Filtrates were collected and kept at 4°C. The sample was reconstituted back to 2 mL by adding 0.1 M sodium phosphate buffer, pH 6.5, and centrifuged again. Concentrated inhibitor sample on the membrane surface, rententate, was reconstituted back to 2 mL by adding distilled water and assayed for PPO inhibitor activity as described above. Control inhibitor samples in centrifuge tubes also were subjected to centrifugation with test samples.

Treatment of Inhibitor(s) with Trypsin

A trypsin solution was created by dissolving 0.01g of trypsin (Sigma Chemical Co., St. Louis, MO) in 10 mL of a pH 7.2 sodium phosphate buffer. Next, 0.3 mL of the trypsin solution was added to 5.7 mL of crude cockroach inhibitor extract. A control solution was also made up by adding 0.3 mL of control buffer to 5.7 mL of cockroach inhibitor solution. 0.2 M TRIS was added to the control and test solutions to reach the optimum pH for trypsin of 7.6 as stated by the manufacturer. The solutions were incubated in a water bath at room temperature (25°C) for 1 hour. Finally the solutions were titrated back to pH 5.5 using 0.2 M acetic acid and assayed for inhibitor activity.

Treatment of Inhibitor(s) with Papain

A papain solution was made by dissolving 0.15 g of papain (Sigma Chemical Co., St. Louis, MO) in 10 mL of pH 6.5 sodium phosphate. Then, 0.15 mL of the papain solution was added to 2.85 mL of inhibitor extract. A control solution was also made by adding 0.15 mL of control buffer to 2.85 mL of inhibitor extract. Since papain has an optimum pH range of 6-7, no further pH adjustment was necessary. The solutions were incubated in a water bath at room temperature (25°C) for 1 hour. Finally, the solutions were assayed for inhibitor activity.

Inhibition Kinetics

Plots based on experimental data are often complicated by substrate inhibition or activation; therefore it is best to use methods of plotting v_0 versus (A_0) data to display them in linear form (Whitaker 1994). The Lineweaver-Burk method, first described in 1934, is the method most frequently used.

A plot of substrate-velocity data was made according to the Lineweaver-Burk method. Inhibition by cockroach extract on apple PPO was determined in the presence of three different concentrations of inhibitor solution (1x, ½X, and ¼X) for five different fixed concentrations of catechol (1, 0.5, 0.25, 0.125, and 0.0625M) at pH 5.5.

CHAPTER 4
RESULTS AND DISCUSSION

Optimization of pH Conditions for PPO Activity and Inhibition

The pH activity profile for oxidation of catechol by mushroom and banana PPO were both determined in preliminary studies (Figures 4-1 and 4-2 respectively). Maximum activity for mushroom PPO for the range of pH 4.5-6.5 was found at around pH 6.5, although activity at pH 6.0 was similar to 6.5, especially given the standard deviation. At pH 5.5, only about 72% activity was found relative to that at pH 6.5. Minimal activity was detected at pH 4.5, only 37% relative to that at pH 6.5. In comparison, Gouzi and Benmansour (2007) found two pH optima for mushroom PPO, at 5.3 and 7.0. Meanwhile Colak and others (2007) found a pH optimum of 7.0 for three different wild mushroom species.

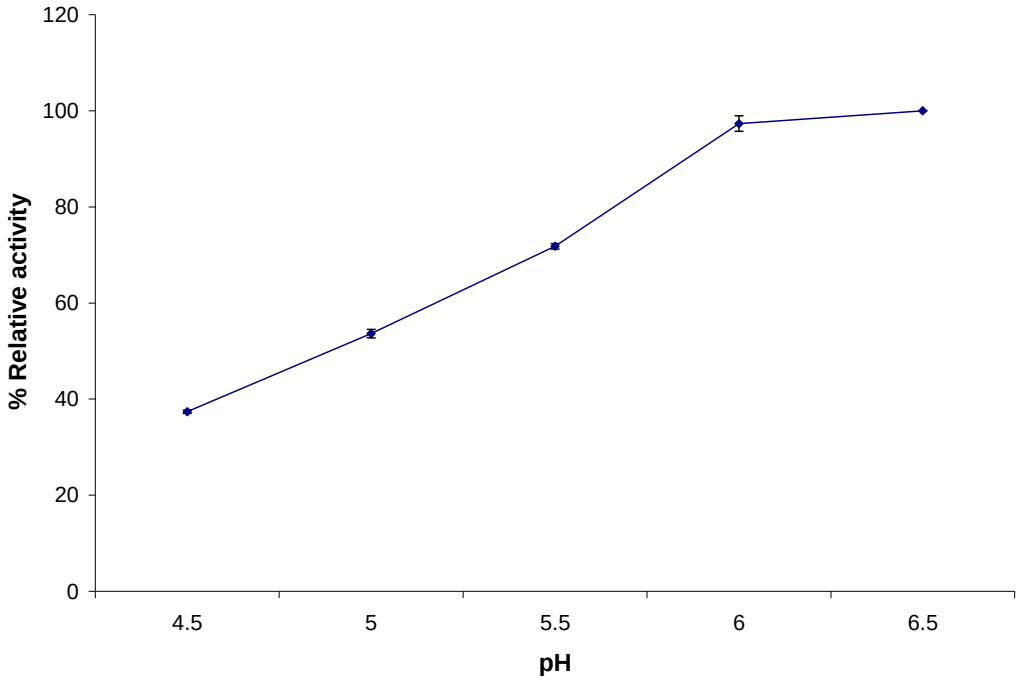

Figure 4-1. The pH optimum of mushroom PPO. Activities are expressed as % relative activity to the maximal activity determined at pH 6.5. Each data point represents mean ±standard deviation of 2 experiments each with 3 replicates.

Maximum activity for banana PPO for the range of pH 4.0-6.5 was also found at pH 6.5. Activity between pH 4-5 ranged from a minimum of 38% at pH 4 up to around 50% at pH 5. However, at pH 5.5 the activity jumped to around 73%. At pH 6, about 80% activity was observed relative to that at pH 6.5. Yang and others (2000) also found a pH optimim of 6.5 for banana pulp, while Palmer (1963) found a pH optimum of 7.0 for banana when catalyzing the oxidation of dopamine. Although some inhibition studies were carried out using these plants, there was not enough evidence of significant inhibition to continue more comprehensive studies.

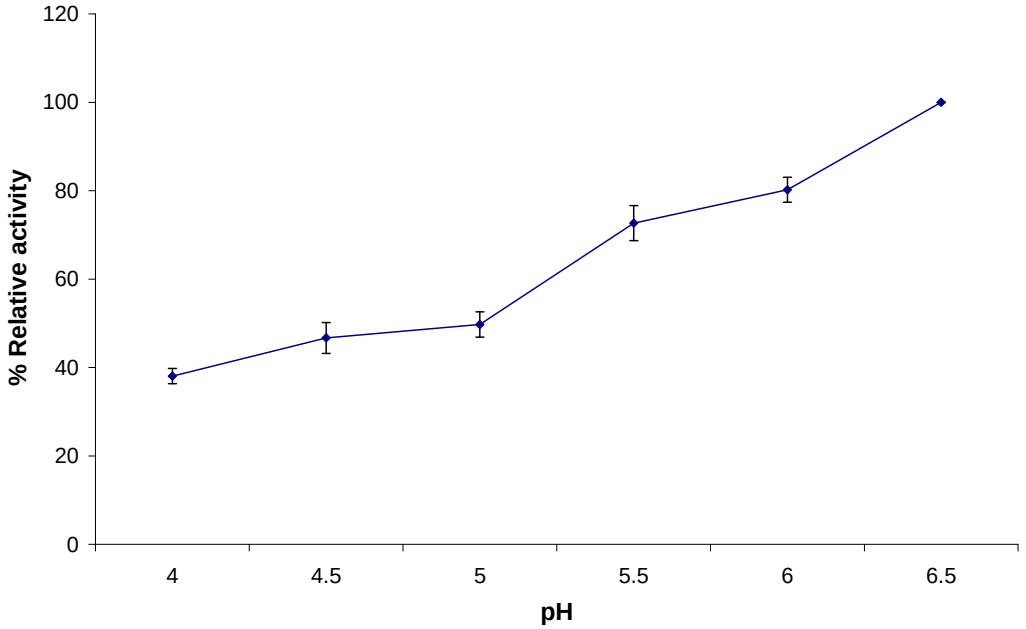

Figure 4-2. The pH optimum of banana PPO. Activities are expressed as % relative activity to the maximal activity determined at pH 6.5. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

The pH activity profile for oxidation of catechol by potato PPO for the pH range of 4.0-6.5 was determined (Figure 4-3). Maximum activity for potato PPO was found around pH 6.0, while the minimum activity was found at pH 4.0. A steady increasing trend occurred from pH

4.0 up to the maximum at 6.0. At pH 6.5, activity dropped down to about 82% relative to that at 6.0. Potato PPO has been reported to have two pH optima, the first between 4.5-5.0 and the second between 6.0-6.5 (Sanchez-Ferrer and others 1993a).

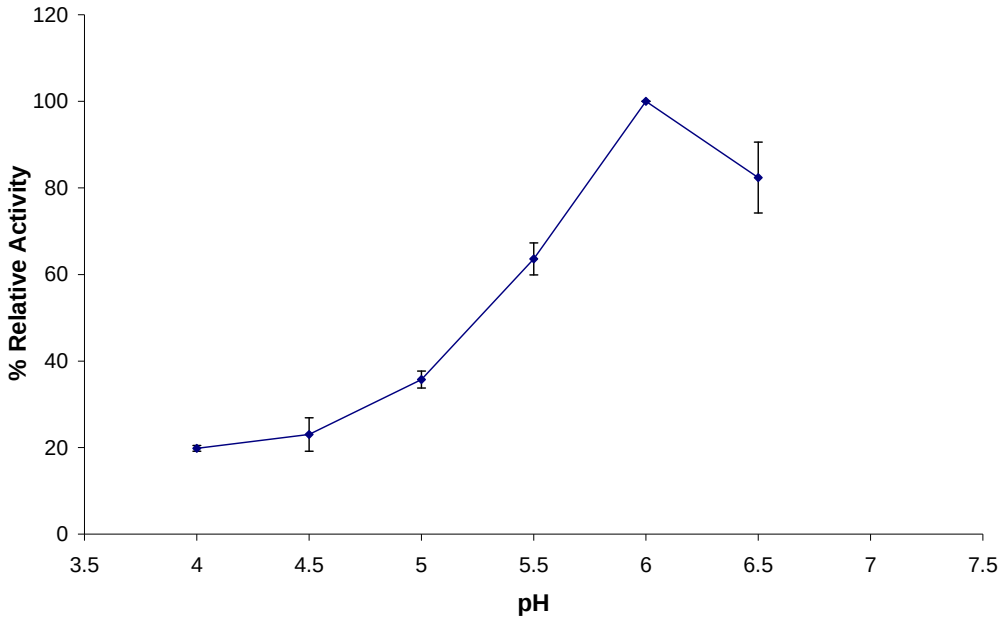

Figure 4-3. The pH optimum of potato PPO. Activities are expressed as % relative activity to the maximal activity determined at pH 6.0. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

The ability of the cockroach extract to inhibit potato PPO was demonstrated spectrophotometrically by mixing the inhibitor extract with an active extract of potato PPO at the determined pH optimum of 6.0. The crude inhibitor preparation reduced potato PPO activity by 20%. The inhibition was fairly rapid, and occurred during the assay analysis. The amount of inhibition by the cockroach extract on potato PPO varied with changing pH (Figure 4-4). Inhibition showed an increasing trend from pH 4.0, although the inhibition at pH 5.0 was slightly

higher than that seen at pH 5.5. At a pH of 6.5, only 13% inhibition was seen, compared to the 20% seen at the pH optimum of 6.0.

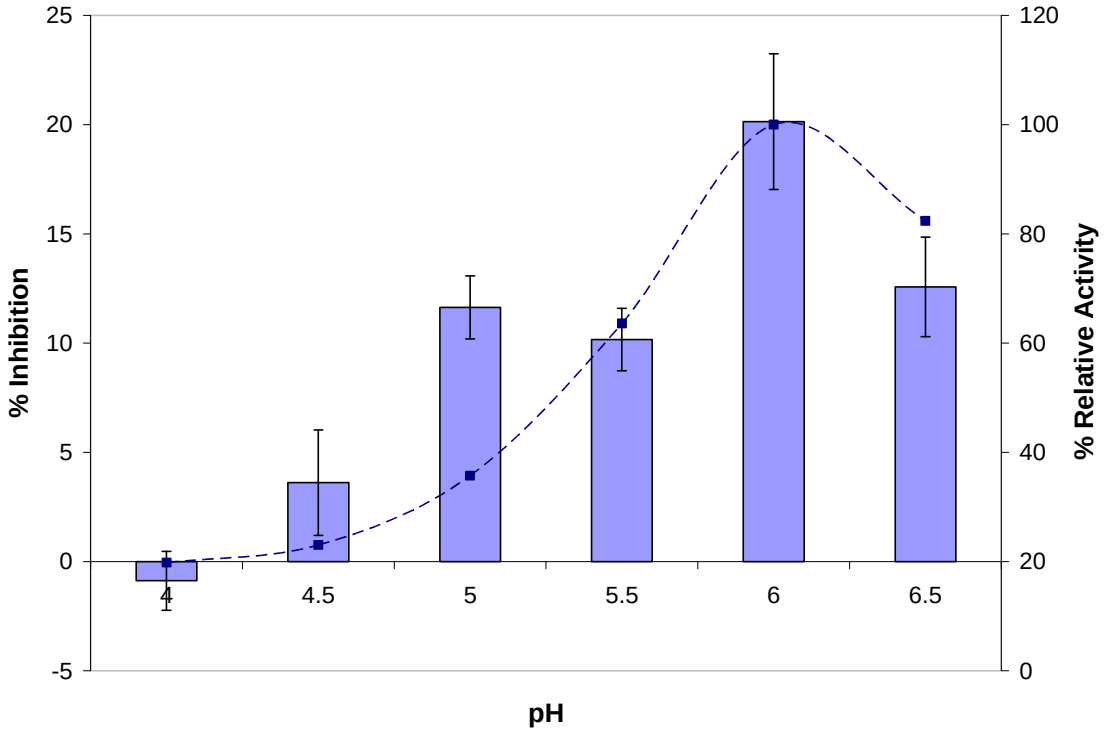

Figure 4-4. Optimum pH for PPO inhibition by cockroach extract. This was determined by measuring the PPO activity of potato mixed with the inhibitor(s) in a standard reaction mixture where the pH of the main buffer (0.1 M acetate-acetic acid) varied from 4.0 to 6.5. Bars indicate %inhibition while the dashed like indicated %relative PPO activity without inhibitor(s). Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

The pH activity profile for oxidation of catechol by apple PPO was also determined (Figure 4-5). Maximum activity for apple PPO for the range of pH 4.0-7.0 was found around pH 5.5, with a steady decreasing trend above that. At pH 5.0, around 71% activity was found relative to that at pH 5.5. Minimal activity was detected at pH 6.0 or higher. The pH optimum of apple PPO is commonly found to be acidic, with other researchers finding the pH optimum in the range from 4.5-5.5 (Janovitz-Klapp and others 1989; Zhou and others 1993; Yoruk and

Marshall 2003). Stelzig and others (1972) reported the PPO of apple peel to have two pH optima, 4.2 and 7.0. For all plant PPOs, several factors can affect the optimum pH value, such as the method of extraction, temperature, nature of the phenolic substrate, and type of buffer system used in determination of maximum activity (Stelzig and others 1972; Janovitz-Klapp and others 1989; Zhou and others 1993; Whitaker 1994; Yoruk and Marshall 2003).

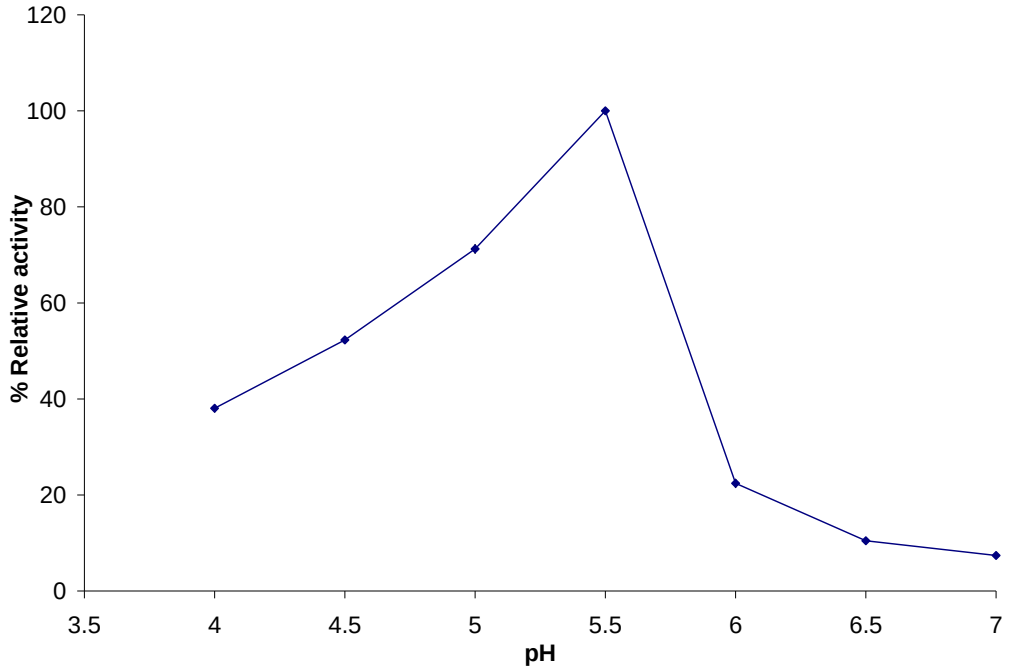

Figure 4-5. The pH optimum of apple PPO. Activities are expressed as % relative activity to the maximal activity determined at pH 5.5. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

The ability of the cockroach extract to inhibit apple PPO was demonstrated spectrophotometrically by mixing the inhibitor extract with an active extract of apple PPO at the determined pH optimum of 5.5. A comparison of absorbance at 420 nm between the control and test assays for 120 seconds was plotted (Figure 4-6). The crude inhibitor preparation reduced apple PPO activity by 60%. As with potato PPO, the inhibition was fairly rapid, occurring in the

assay mixture, and did not require a long incubation. In depth studies of PPO inhibitor(s) assay conditions demonstrated the extreme importance of reaction pH on inhibition activity by the cockroach extract (Figure 4-7). The amount of inhibition by the cockroach extract on apple PPO varied with changing pH. Inhibition showed an increasing trend from pH 4.0 and higher with greatest inhibition of 72% seen at pH 5.7. At the pH optimum of 5.5, 60% inhibition was observed. However, below pH 5.0 very low (levels < 5%) inhibition was observed. It should also be noted that at pH levels above 5.5 the increased amount of inhibition may in fact be attributed to the decline in PPO activity on the activity profile as a result of changing pH.

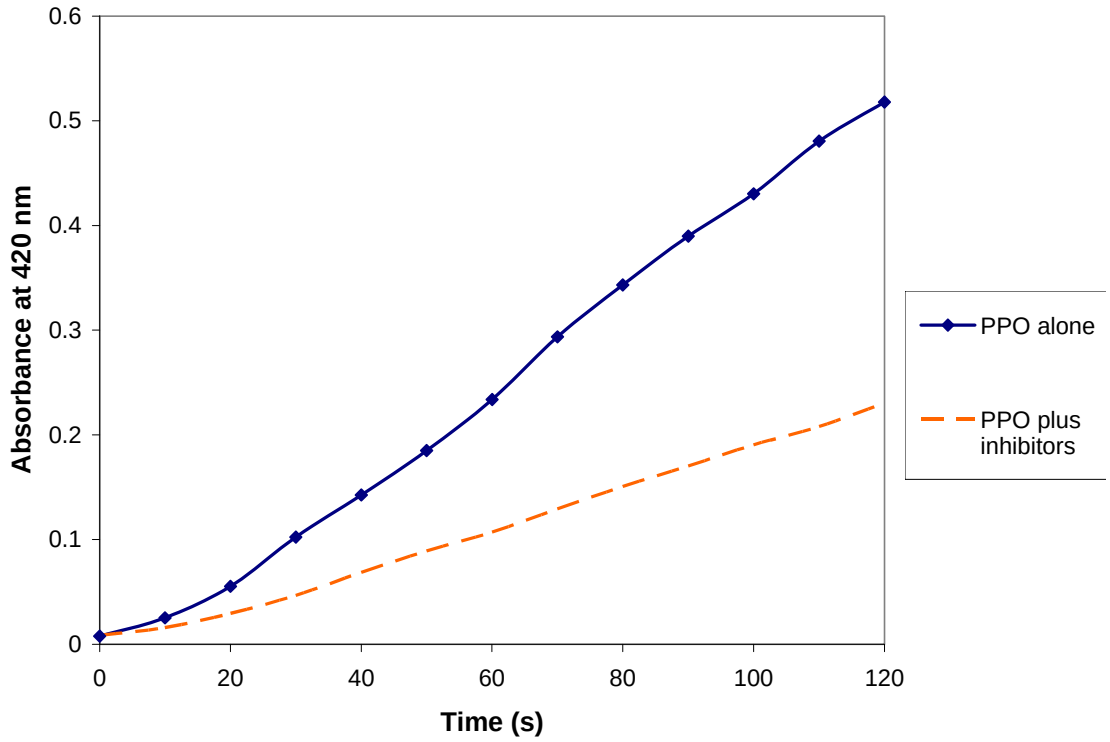

Figure 4-6. Inhibition of apple PPO activity by inhibitor(s) from German cockroach. Connected line indicates results of PPO alone, while the dashed line indicated PPO with inhibitor(s). Inhibition activity was observed in the 3 mL standard reaction mixture.

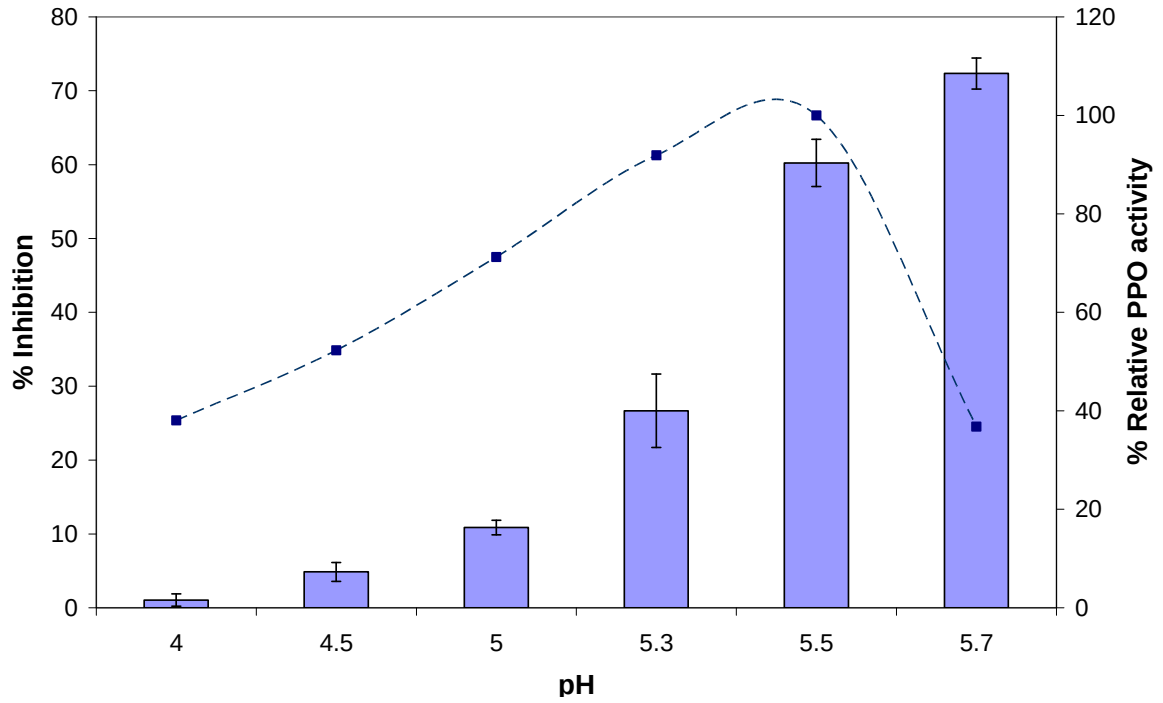

Figure 4-7. Optimum pH for apple PPO inhibition by cockroach extract. This was determined by measuring the PPO activity of apple mixed with the inhibitor(s) in a standard reaction mixture where the pH of the main buffer (0.1 M acetate-acetic acid) varied from 4.0 to 5.7. Bars indicate %inhibition while the dashed like indicated %relative PPO activity without inhibitor(s). Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

Inhibition of PPO by insect tissues has been reported previously. Tsukamoto and others (1992) isolated inhibitors from the pupal extracts of houseflies and demonstrated inhibition on house fly PPO. Sugumaran and Nellaiappan (2000) isolated an endogenous inhibitor in the larval cuticle of the tobacco horn worm (*Manduca sexta*). This PPO inhibitor from *Manduca sexta* inhibited the activity of both insect and plant PPOs and lacase. Yoruk and others (2003) isolated an inhibitor(s) from the common house fly (*Musca domestica*). The isolated inhibitor(s) was found to be fairly heat-stable and low-molecular weight. The inhibitor(s) from *Musca domestica* inhibited the activity of apple PPO up to 90% at pH values above 5.0. PPO is thought to play a role in several regulatory biochemical mechanisms of endogenous PPO activity in

insects (Sugumaran and Nellaiappan 2000). Insect PPO activity may also be regulated by intrinsic inhibitors during development (Tsukamoto and others 1992; Sugumaran and Nellaiappan 2000).

Characterization of Crude Inhibitor(s) From German Cockroach

Effect of Timed Incubation on the Inhibitor(s)

Incubation had a large effect on the unknown inhibitor(s) when the inhibitor and PPO were left to sit up to 30 min (Figure 4-8). When the time of incubation increased, rate of reaction at 420 nm also increased, which corresponds to a decrease in inhibition. However, incubation had minimal effect when inhibitor and substrate were incubated over a length of 30 min. The rate at 420 nm remained fairly constant at each time after slightly decreasing from 0 to 1 min. Therefore, PPO inhibition studies were carried out initiating the reaction with the PPO as opposed to the substrate.

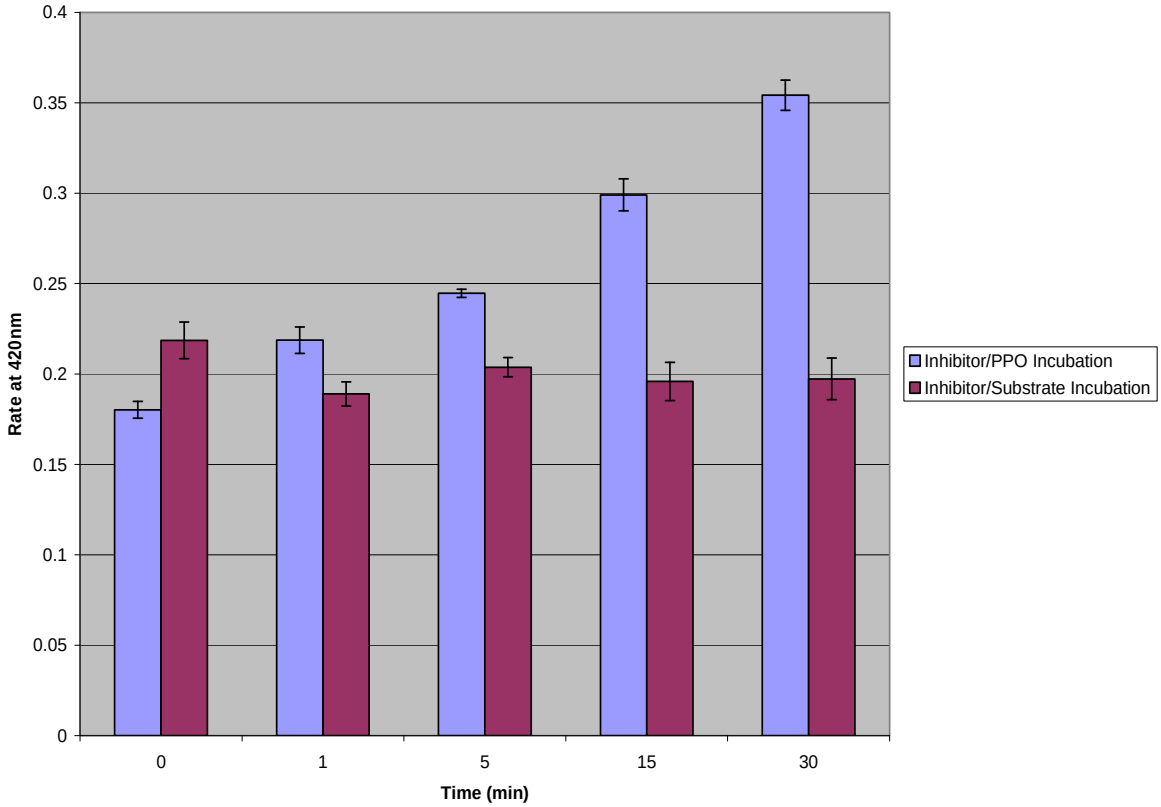

Figure 4-8. Incubation time study. Rate of reaction at 420 nm was determined after incubation of inhibitor/PPO compared to incubation of inhibitor/substrate over a time of 30 min. Each data point represents mean ± standard deviation of 3 replicates.

Temperature Stability

Temperature stability was studied in order to measure the effect of temperature on inactivation of inhibitor(s). The unknown inhibitor(s) from German cockroach extract used in this study was not very heat stable. It was completely inactivated by heating at 100°C for as little as 5 min. This is similar to results found by Sugumaran and Nellaiappan (2000) with tobacco horn worm, which was a heat-labile glycoprotein, inactivated by heating at 100°C for 10 min (Sugumaran and Nellaiappan 2000). However, Tsukamoto and others (1992) found their inhibitor(s) from housefly to be quite stable to heating, retaining 60% of their activity when heated to 80°C for 1 hour. Similarly, Yoruk and others (2003) found their house fly inhibitor(s) to also be relatively heat stable, losing only 26% of its inhibitory activity on apple PPO upon

boiling at 100°C up to an hour. This is a crude way to characterize the inhibitor(s) since a number of enzymes may act on a common complex substrate (Whitaker 1994).

The inhibitor(s) from German cockroach was found to be stable to repeated freezing and thawing cycles, losing only about 9% of its inhibitor activity. This is similar to results found by Yoruk and others (2003) with house fly, which lost only about 8% of its inhibitor activity to repeated freezing and thawing. Inhibitor activity of cockroach extract stored at -20°C was found to remain stable up to 6 months.

The pH Extraction Profile

Inhibitor(s) extracted at a range of pH values from 4.5-7.5 was compared for its inhibitory activity on apple PPO to determine the pH extraction profile (Figure 4-9). The pH had a dramatic effect on the inhibitor(s). It was most stable at a pH of 6.5. At a pH of 5.5 only about 40% relative inhibition was observed compared to pH 6.5. At pH levels of 4.5 and 7.5 only about 20% relative inhibition was observed when compared to those at pH 6.5.

Similarly, pH was also reported to have a dramatic effect on the inhibitor from the tobacco horn worm (Sugumaran and Nellaiappan 2000). This inhibitor from the tobacco horn worm was also most stable around a neutral pH, losing its total activity upon exposure to pH 10 for 10 min. Yoruk and others (2003) also reported the inhibitor from common house fly to be unstable to changes in pH.

In contrast to the German cockroach and tobacco horn worm, the housefly inhibitor(s) was most stable at acidic pH values and least stable at alkaline values. However, Tsukamoto and others (1992) observed the inhibitor(s) in house fly pupae was quite stable over a wide pH range

from 4.0 to 10.0. The extent of stability obtained in these studies was not presented.

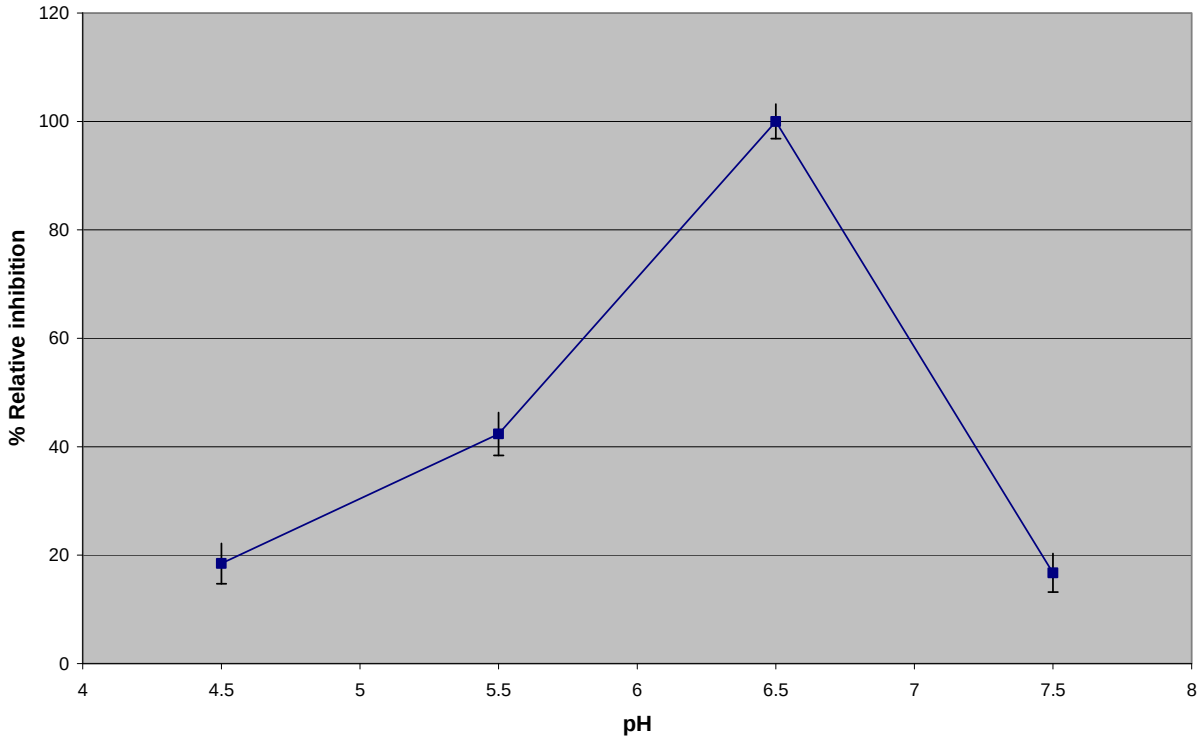

Figure 4-9. The pH extraction profile of inhibitor(s). Inhibitor was extracted at pH values of 4.5, 5.5, 6.5, and 7.5 with 0.1 M sodium phosphate or sodium acetate buffer. Each data point represents mean ± standard deviation of 2 experiments each with 2 replicates.

Dialysis of Inhibitor(s)

Dialysis of inhibitor extracts was performed using 500, 2000, and 25000-Da molecular-weight cut off (MWCO) dialysis membranes with water and buffer (Figure 4-10). Due to the changes pH have on the inhibitor(s) from coackroach extract, the extracts that went through dialysis with distilled water lost more than 40% inhibition compared to that of the control. When dialysis was performed using the control buffer, a much smaller loss in %inhibition was observed. The 500-Da molecular weight cut off membrane lost only 8% inhibition compared to that of control. There was a slight decreasing trend as the molecular weight membrane got

larger, with the 25000-Da membrane losing about 17% compared to that of the control. Since it appeared the unknown inhibitor(s) may in fact be larger than 25000-Da, ultrafiltration studies were performed to better approximate the molecular weight.

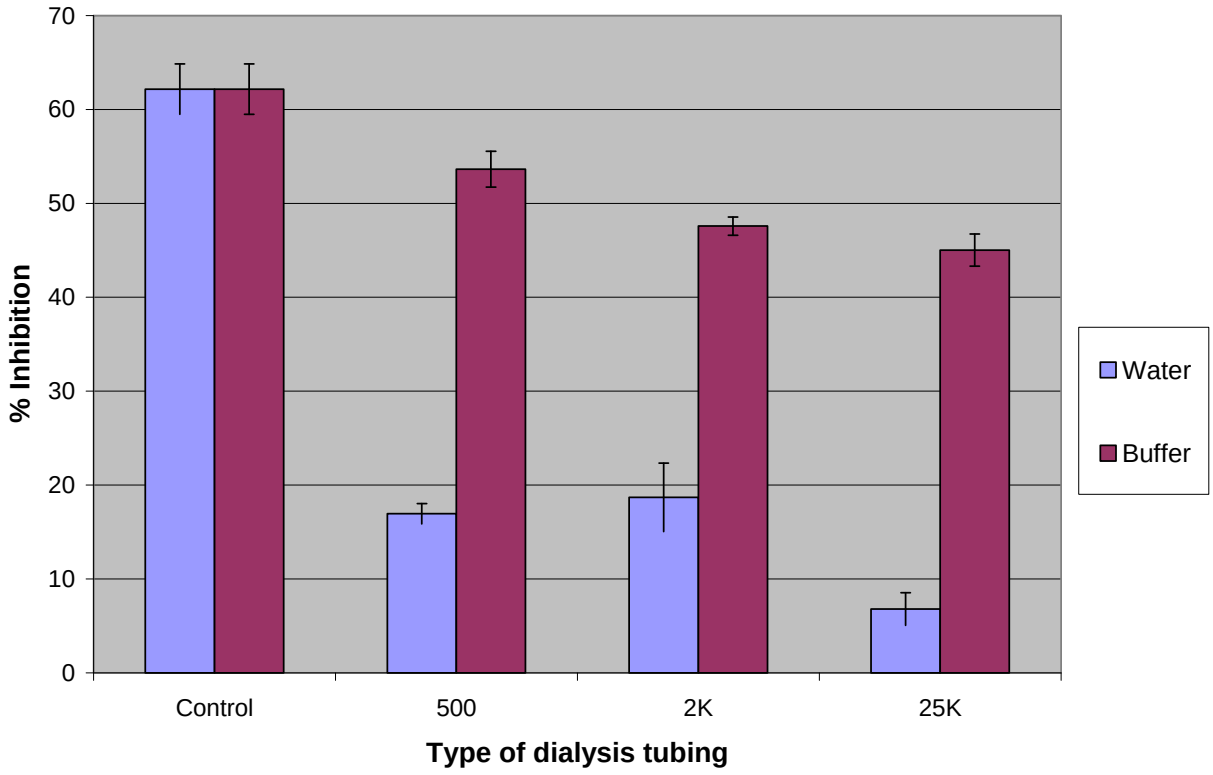

Figure 4-10. Percent inhibition after dialysis with water or control buffer. Each buffer data point represents mean ± standard deviation of 2 experiments each with 3 replicates. Each water data point represents mean ± standard deviation of 3 replicates.

Ultrafiltration

Ultrafiltration is a type of membrane filtration in which hydrostatic pressure forces liquid against a semipermeable membrane to separate solids and solutes of high molecular weight from water and low molecular weight solutes which pass through the membrane (Matella and others, 2006). Inhibitor preparation was also characterized by ultrafiltration studies (Table 4-1). Ultrafiltration studies were conducted to determine the approximate molecular weight of the unknown inhibitor(s) required for future purification and to compare with sizes from previous

studies. The results indicated that the unknown inhibitor(s) from German cockroach is larger than 100000 NMWL as more than 98% inhibitor activity remained in the retentate at all 3 size ultrafiltration units. Ultrafiltration units with 10000, 50000, and 100000 NMWL retained 98.0, 98.7, and 99.5% respectively.

These results may be similar to those found by Sugumaran and Nellaiappan (2000), from the inhibitor in the tobacco horn worm. They reported their endogenous glycoprotein to have a molecular weight of 380000 on SDS-PAGE gels. The size results are much larger than those found by Tsukamoto and others (1992) based on Sephadex G-25 gel filtration information, which showed the inhibitor to range in size from 3000 to 3500. Yoruk and others (2003) reported ultrafiltration results on the inhibitor(s) from the common house fly to retain 83, 67, and 51% of the inhibitor activity on 10000, 30000, and 50000 NMWL membranes. This would indicate a smaller inhibitor than the results from this study, although the crude mixture used in the cockroach and common house fly for ultrafiltration gives only an estimate of inhibitor size.

Table 4-1. Ultrafiltration

	Relative % Inhibitor Activity	
NMWL	Filtrate	Retentate
Control	0	100
10000	4.6 ± 1.0	98.0 ± 1.5
50000	5.4± 1.8	98.7 ± 0.8
100000	4.0 ± 2.4	99.5 ± 2.4

Ultrafiltration was performed using centrifugal filter units with specified nominal molecular weights limit (NMWL) membranes. Each data point represents mean ± standard deviation of an experiment with 3 replicates.

Treatment of Inhibitor(s) with Trypsin

Trypsin is a pancreatic serine protease. Trypsin has a pH optimum of 8. Treatment of inhibitor(s) with trypsin was ineffective due to the instability of the inhibitor to changes in pH. By raising the pH of the inhibitor above 6.5 and back down again, the inhibition was lost in both control and test trypsin samples (Figure 4-11). Therefore, it's inconclusive whether there was an

effect on the inhibitor(s) by trypsin, or if it was just the pH change that caused the loss of inhibition being shown in both.

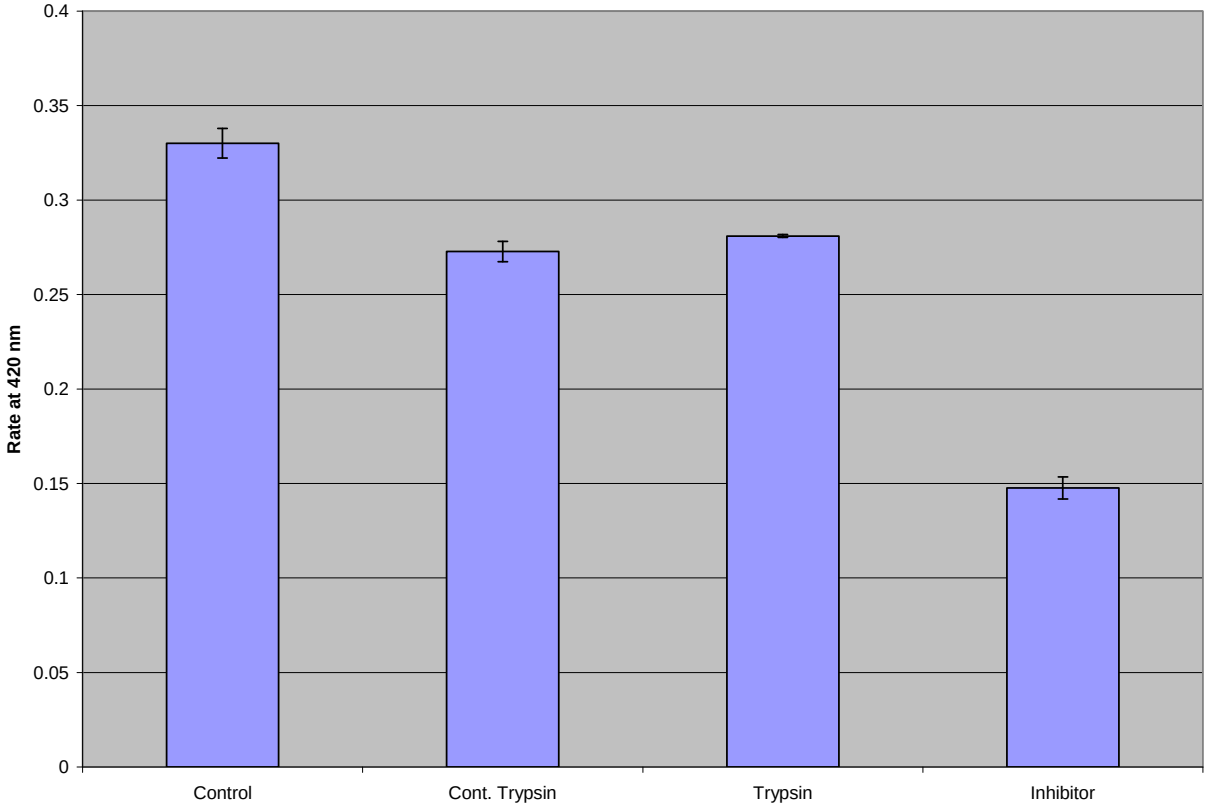

Figure 4-11. Treatment of inhibitor(s) with trypsin. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

Treatment of Inhibitor(s) with Papain

Papain is a sulfhydryl protease from *Carica* papaya latex. Treatment of inhibitor(s) with papain proved effective in decreasing the inhibition in the test sample but not the control papain sample. The German cockroach inhibitor was too sensitive to pH changes required for treatment with trypsin to determine its effect on it. However, papain has an optimum pH of 6.0-7.0, which is similar to the German cockroach inhibitor, and the results indicated that cysteine protease may be effective in disrupting the inhibition (Figure 4-12). The control had an average rate of 0.3284 compared to an average of 0.3058 for the test sample with papain. The control papain sample

had an average rate of 0.1598 compared to an average rate of 0.1467 for regular inhibitor, showing only a small decrease on the level of inhibition.

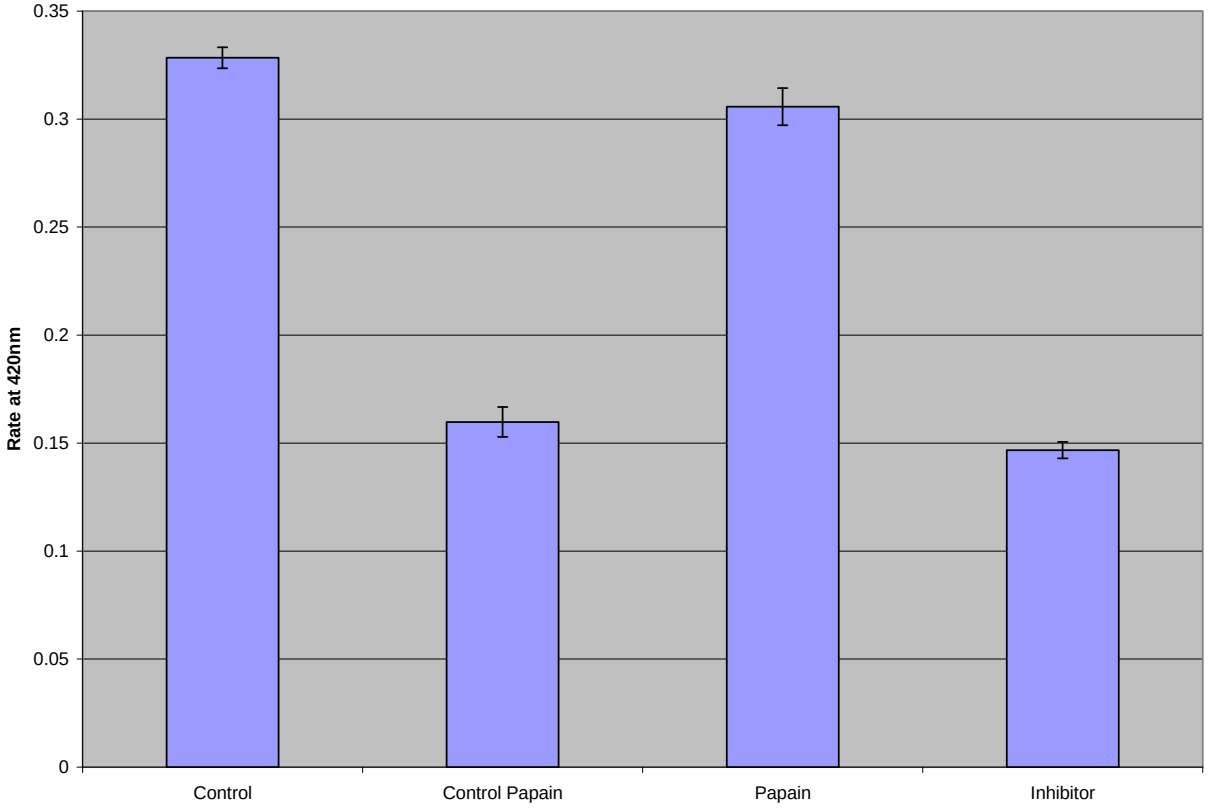

Figure 4-12. Treatment of inhibitor(s) with papain. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

Inhibitor Kinetics

Inhibition by German cockroach extract on apple PPO was characterized using the Lineweaver-Burk method. Results indicated non-competitive inhibition, as both the vertical intercept and slope terms of the equation are affected. (Figure 4-13). The lines intercept at a point left of the y-axis and below the x-axis. Non-competitive inhibition is when a reversible inhibitor can bind to the enzyme at a site that is distinct from the active site.

Inhibitor kinetics results from different inhibitor sources vary. Oszmianski and Lee (1990) found the inhibitory effect of honey on apple PPO to also be non-competitive using a

Lineweaver-Burk plot. In contrast, Son and others (2000) found the inhibitory mode of oxalic acid on mushroom PPO to be of a competitive type.

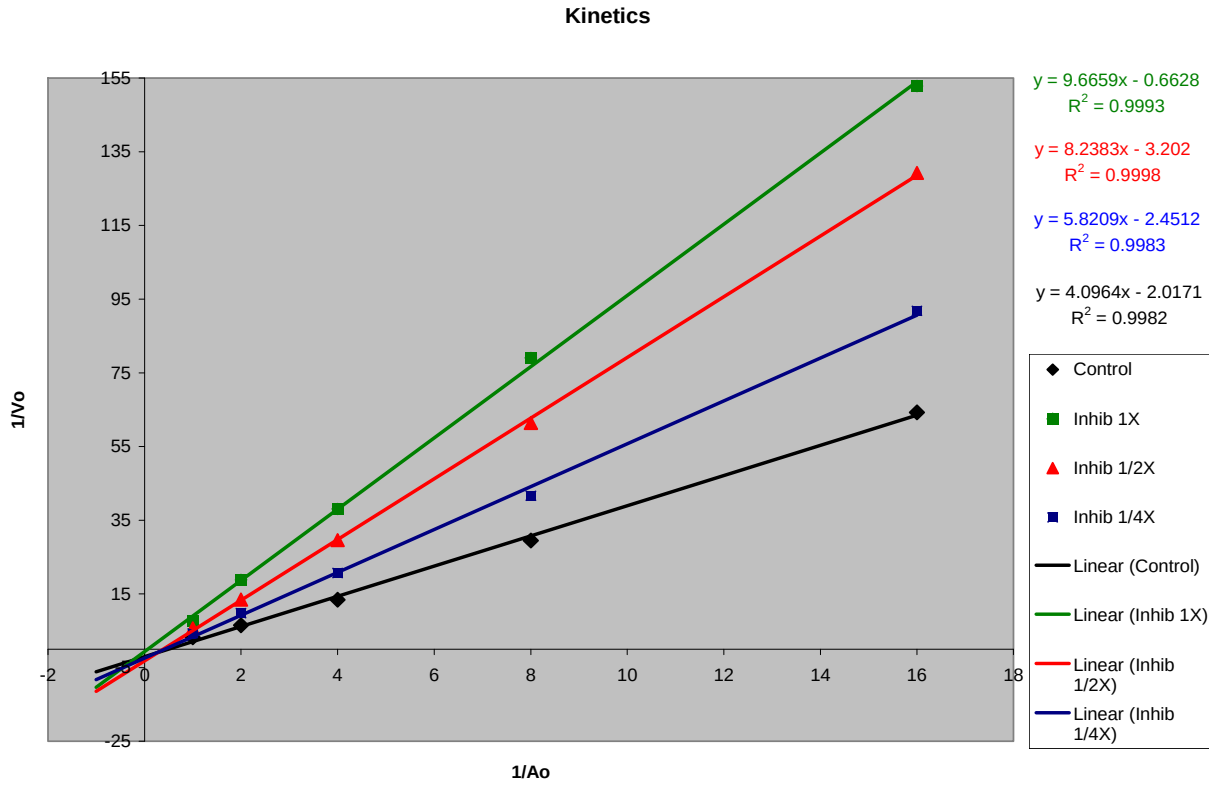

Figure 4-13. Inhibitor kinetics: Plot of the substrate-velocity data according to Lineweaver Burk method. Inhibition by cockroach extract on apple PPO was determined in the presence of three different concentrations of inhibitor solution (1X, ½X, ¼X) for five different fixed concentrations of catechol (1, 0.5, 0.25, 0.125, and 0.0625M).

CHAPTER 5
CONCLUSIONS

A new type of inhibitor(s) was discovered from German cockroach (*Blattella germanica*). Although minimal inhibition was found on mushroom and banana, the crude inhibitor preparation reduced apple PPO activity up to 70% and potato PPO up to 25% at a reaction pH optimal for the enzyme activity. The potential PPO inhibitor(s) was characterized using temperature stability, extraction pH adjustment, incubation time, treatment with protease, dialysis, ultrafiltration studies, and enzyme inhibitor kinetics.

The inhibitor(s) from German cockroach was found to be heat labile, becoming inactivated when heated to 100°C for 5 min. However, the inhibitor was stable to freeze-thaw cycles and refrigerated storage. It was also discovered that the inhibitor(s) activity was influenced by changes in extraction pH, being most stable at pH 6.5 and dropping in % inhibition drastically when the pH moved in either direction. Incubation time was found to affect the inhibitor(s) when the inhibitor and PPO were allowed to incubate together for 30 min, however when the inhibitor and substrate were incubated, there was no effect on inhibition.

Treatment with trypsin was inconclusive because of the sensitivity of the inhibitor(s) to changes in pH, which conflicts with the pH activity range of trypsin. Therefore, treatment of the inhibitor(s) with papain was attempted since it has a pH optimum that is similar to the unknown inhibitor(s). Results showed a loss of inhibition, which may indicate the presence of a protein.

Sensitivity to changes in pH also proved to be a problem when performing dialysis with distilled water, but the inhibitor retained most of its inhibition when a sodium phosphate control buffer was used in place of water. Ultrafiltration studies were performed to better estimate the molecular weight of the unknown inhibitor(s). Results indicated that the inhibitor(s) may be larger than 100000 NMWL, as more than 90% inhibition was retained compared to the control.

Kinetic studies were performed using a Lineweaver-Burk plot, and results indicated a non-competitive inhibition of the cockroach inhibitor(s) on apple PPO.

In order to study the amino acid composition, molecular weight, tertiary structure, substrate specificity, or kinetic parameters of the inhibitor(s), it must be completely separated from all other substances. These results are the first attempt at identifying an inhibitor(s) from the German cockroach on PPO from higher plants; therefore further research is needed to completely purify the unknown inhibitor(s). Further studies may help clarify the mechanism by which this inhibitor(s) works on other plant PPOs, especially those from apple and potato.

APPENDIX A
RAW DATA

Table A-1. The pH optimum of potato PPO

pH	1	2	3	4	5	6	Average	St Dev	% Rel. Act.
4	0.02931	0.03086	0.02936	0.02901	0.02892	0.02774	0.0292	0.001004	19.8121
4.5	0.02947	0.03067	0.03297	0.03711	0.03875	0.03463	0.033933	0.003613	23.0236
5	0.05075	0.05215	0.05093	0.05567	0.05361	0.05277	0.052647	0.001837	35.7205
5.5	0.09095	0.09355	0.08853	0.09765	0.09535	0.09648	0.093752	0.003474	63.6100
6	0.15604	0.15607	0.17072	0.12762	0.14098	0.13288	0.147385	0.016352	100.0000
6.5	0.11579	0.13151	0.12991	0.11979	0.11909	0.1125	0.121432	0.007658	82.3908

Table A-2. Optimum pH for potato PPO inhibition

pH	c	I	% inhibition	Average	St Dev
4	0.02901	0.02957	-1.93037		
	0.02874	0.02871	0.104384		
	0.02783	0.02782	0.035932		
	0.02856	0.02874	-0.63025		
	0.02892	0.02981	-3.07746		
	0.02774	0.02768	0.216294	-0.88024	1.341958
4.5	0.03875	0.0386	0.387097		
	0.03723	0.03616	2.874026		
	0.03476	0.03351	3.596087		
	0.03746	0.03627	3.176722		
	0.03711	0.0342	7.841552		
	0.03463	0.03331	3.811724	3.614535	2.410945
5	0.05567	0.04855	12.78965		
	0.05503	0.04866	11.5755		
	0.05434	0.04758	12.44019		
	0.05385	0.048094	10.68895		
	0.05361	0.04662	13.03861		
	0.05277	0.04787	9.285579	11.63642	1.440139
5.5	0.09765	0.08936	8.489503		
	0.09638	0.08495	11.85931		
	0.09726	0.08832	9.191857		
	0.09673	0.08714	9.914194		
	0.09535	0.08396	11.94546		
	0.09648	0.08723	9.587479	10.16463	1.427687
6	0.15604	0.11852	24.04512		
	0.15315	0.12147	20.6856		
	0.14976	0.12032	19.65812		
	0.15589	0.12676	18.68625		
	0.14098	0.11956	15.19364		
	0.15607	0.12086	22.56039	20.13819	3.105412
6.5	0.11979	0.10619	11.3532		
	0.11934	0.10345	13.3149		
	0.11385	0.10057	11.66447		
	0.11857	0.10511	11.35194		
	0.11909	0.09897	16.89479		
	0.1125	0.10027	10.87111	12.57507	2.277233

Table A-3. The pH optimum of apple PPO

pH	4	4.5	5	5.3	5.5	5.7	6	6.5	7
	0.22845	0.30943	0.423	0.53897	0.58159	0.21057	0.13228	0.06163	0.04653
	0.22633	0.30893	0.41107	0.53826	0.59414	0.21842	0.13394	0.06286	0.0401
	0.22462	0.30631	0.41896	0.54243	0.59251	0.21765	0.12858	0.06005	0.04166
	0.21856	0.30539	0.42292	0.52614	0.58625	0.21534	0.12987	0.06129	0.04456
	0.21965	0.30169	0.41533	0.54262	0.57525	0.21498	0.12398	0.06067	0.04243
	0.21853	0.30455	0.41155	0.53926	0.58306	0.21549	0.13845	0.06138	0.04521
Average	0.22269	0.30605	0.417138333	0.537947	0.585467	0.215408	0.131183	0.061313	0.043415
St Dev	0.00433	0.00288	0.005337218	0.00607	0.00708	0.002746	0.00494	0.000949	0.00242
% R.A.	38.03632	52.27454	71.24886131	91.8834	100	36.79259	22.40663	10.47256	7.415452
% Rel St Dev	0.739627	0.491972	0.911617752	1.036733	0	0.469008	0.843697	0.162171	0.413301

Table A-4. Inhibition of apple PPO activity by inhibitor(s) from German cockroach

	Control	Inhibitor
0	0.0078	0.0085
10	0.0252	0.0159
20	0.0554	0.0296
30	0.1024	0.0466
40	0.1427	0.0687
50	0.1851	0.0891
60	0.2338	0.1071
70	0.2937	0.1293
80	0.3432	0.1506
90	0.3899	0.1702
100	0.4303	0.1905
110	0.4806	0.208
120	0.5179	0.2305

Table A-5. Optimum pH for apple PPO inhibition

% inhibition at varying pH					
pH	c	I	% inhibition	Average	St Dev
4	0.13576	0.13657	-0.59664113		
	0.13478	0.13298	1.33550972		
	0.13268	0.13047	1.665661743		
	0.12945	0.12774	1.320973349		
	0.13239	0.1308	1.200997054		
	0.12826	0.12648	1.387806019	1.052384	0.82247
4.5	0.19705	0.18423	6.505962954		
	0.19688	0.18421	6.435392117		
	0.19345	0.18456	4.595502714		
	0.18078	0.17367	3.932957186		
	0.1809	0.17373	3.963515755		
	0.18011	0.17341	3.71994892	4.85888	1.282483
5	0.32254	0.28648	11.18000868		
	0.32304	0.28699	11.15960872		
	0.32277	0.28546	11.55931468		
	0.32234	0.28913	10.30278588		
	0.32166	0.29217	9.168065659		
	0.32249	0.2843	11.84222767	10.86867	0.981699
5.3	0.36795	0.2428	34.01277347		
	0.34745	0.26266	24.4035113		
	0.34731	0.23772	31.55394316		
	0.35744	0.2818	21.16159355		
	0.34983	0.26329	24.73772975		
	0.34586	0.2624	24.13115133	26.66678	4.968462
5.5	0.33058	0.11455	65.34878093		
	0.32784	0.12506	61.85334309		
	0.32213	0.13136	59.22143234		
	0.32957	0.12888	60.89449889		
	0.28904	0.1236	57.23775256		
	0.28611	0.12366	56.77886128	60.22244	3.198734
5.7	0.08827	0.0238	73.03727201		
	0.08832	0.02392	72.91666667		
	0.08735	0.02678	69.34172868		
	0.08769	0.0215	75.48181092		
	0.08752	0.02546	70.9095064		
	0.08947	0.02479	72.29238851	72.3299	2.084577

Table A-6. The pH extraction profile of inhibitor

4.5	C	I	% inhibition	% relative
	0.33963	0.29675	12.62550423	
	0.33755	0.28186	16.49829655	
	0.32779	0.2931	10.58299521	
	0.38387	0.34396	10.3967489	
	0.38385	0.34773	9.409925752	
	0.37544	0.35598	5.183251651	
Average	0.358022	0.319897	10.78278705	18.45855
St Dev			3.728032792	

5.5	C	I	% inhibition	% relative
	0.32017	0.24251	24.25586407	
	0.32803	0.25192	23.20214615	
	0.33137	0.24075	27.34707427	
	0.3877	0.2684	30.77121486	
	0.38776	0.29589	23.6924902	
	0.38447	0.31067	19.19525581	
Average	0.356583	0.268357	24.74400756	42.35812
St Dev			3.939876636	

6.5	C	I	% inhibition	% relative
	0.31495	0.13021	58.65692967	
	0.31595	0.13619	56.89507834	
	0.30746	0.14458	52.97599688	
	0.32784	0.12506	61.85334309	
	0.32213	0.13136	59.22143234	
	0.32957	0.12888	60.89449889	
Average	0.31965	0.132713	58.4162132	100
St Dev			3.180310962	

7.5	C	I	% inhibition	% relative
	0.07942	0.0706	11.10551498	
	0.07611	0.06621	13.00748916	
	0.0767	0.06943	9.478487614	
	0.07192	0.06914	3.865406007	
	0.06458	0.05947	7.91266646	
	0.07055	0.06118	13.28136074	
Average	0.073213	0.066005	9.77515416	16.73363
St Dev			3.549459924	

Table A-7. Dialysis

Dialysis	C	I	% Inhibition	Average	St Dev
Control	0.22959	0.09308	59.45816455		
	0.22526	0.08511	62.2169937		
	0.23955	0.08426	64.82571488	62.16696	2.684125
500	0.32677	0.26842	17.85659638		
	0.32031	0.26981	15.76597671		
	0.32747	0.27102	17.23822029	16.9536	1.073979
2K	0.32947	0.27958	15.14250159		
	0.34214	0.2654	22.42941486		
	0.32647	0.26601	18.51931265	18.69708	3.646708
25K	0.25895	0.24113	6.881637382		
	0.24773	0.22669	8.493117507		
	0.24659	0.23419	5.028589967	6.801115	1.733667
500 buffer	0.33536	0.14751	56.01443225		
	0.32008	0.14988	53.17420645		
	0.31817	0.15383	51.65163277		
	0.32716	0.14406	55.96649957		
	0.31128	0.14922	52.06245181		
	0.31804	0.14943	53.01534398	53.64743	1.90261
2K buffer	0.26329	0.13455	48.89665388		
	0.26252	0.13684	47.87444766		
	0.26281	0.14214	45.91530003		
	0.26879	0.14059	47.69522676		
	0.26523	0.13995	47.23447574		
	0.26169	0.1365	47.8390462	47.57586	0.978894
25K buffer	0.28562	0.16231	43.17274701		
	0.29943	0.16671	44.32421601		
	0.28842	0.16377	43.21822342		
	0.2984	0.15772	47.14477212		
	0.298	0.15886	46.69127517		
	0.29031	0.15805	45.55819641	45.01824	1.716992

Table A-8. Treatment of inhibitor(s) with trypsin and papain

	Trypsin					Papain			
	Control	Cont. Trypsin	Trypsin	Inhibitor		Control	Control Papain	Papain	Inhibitor
	0.33737	0.26589	0.28143	0.15159		0.32645	0.15235	0.30193	0.14985
	0.33511	0.27924	0.27972	0.1555		0.32707	0.17134	0.29145	0.15139
	0.3316	0.27495	0.28148	0.13843		0.33143	0.15782	0.31456	0.14523
	0.32706	0.27594	0.28032	0.14863		0.32843	0.16238	0.30543	0.14679
	0.33338	0.26673	0.28156	0.14513		0.33567	0.15356	0.31402	0.14673
	0.31583	0.27391	0.28133	0.1464		0.32145	0.16143	0.30754	0.14045
Average	0.330058333	0.2727767	0.28097333	0.147613333	Average	0.328416667	0.159813333	0.30582167	0.14674
St Dev	0.007795197	0.005325	0.00076607	0.005848414	St Dev	0.004814705	0.006940379	0.00858332	0.003825

Table A-9. Ultrafiltration

Ultrafiltration			% Inhib	Avg					
Control	0.29496	0.11734	60.21833	61.35971					
	0.29018	0.11344	60.90702						
	0.29393	0.10889	62.95376						
	Control	**Filtrate**	**Retentate**	**% Inhib F**	**% Inhib R**	**Avg. F**	**Avg R.**	**Std Dev F**	**Std Dev R**
10,000	0.25625	0.25128	0.10604	1.939512	58.61854	2.821443	60.15248	1.047909	1.511957
	0.25423	0.24776	0.10119	2.54494	60.19746				
	0.24649	0.23668	0.09455	3.979877	61.64145				
50,000	0.29496	0.28594	0.11596	3.058042	60.68619	3.341065	60.58759	1.82771	0.800619
	0.29018	0.28533	0.11682	1.671376	59.74223				
	0.29393	0.27837	0.11365	5.293777	61.33433				
100,000	0.25625	0.24633	0.09523	3.87122	62.83707	2.429494	61.07837	2.354919	2.378966
	0.25423	0.24481	0.09654	3.705306	62.02651				
	0.24649	0.2472	0.10261	-0.28804	58.37154				

NMWL	Filtrate	St Dev +/-	Retentate	Std Dev +/-
Control	0		100	
10000	4.6	1.0	98.0	1.5
50000	5.4	1.8	98.7	0.8
100000	4.0	2.4	99.5	2.4

Table A-10. Inhibitor kinetics

	Ao	Vo rep 1	Vo rep 2	1/Ao	1/Vo rep 1	1/Vo rep 2	1/Vo Avg		Inhib Conc	Slope
Control	1	0.31212	0.30897	1	3.2038959	3.2365602	3.220228		1	9.6659
	0.5	0.15186	0.15591	2	6.5850125	6.4139568	6.499485		0.5	8.2383
	0.25	0.07609	0.07308	4	13.142331	13.683634	13.41298		0.25	5.8209
	0.125	0.03464	0.03323	8	28.86836	30.093289	29.48082		0	4.0964
	0.0625	0.01558	0.01553	16	64.184852	64.3915	64.28818			
Inhib 1X	1	0.13398	0.13132	1	7.4638006	7.6149863	7.539393			
	0.5	0.05341	0.05292	2	18.723086	18.896447	18.80977			
	0.25	0.02535	0.02725	4	39.447732	36.697248	38.07249			
	0.125	0.01216	0.01318	8	82.236842	75.872534	79.05469			
	0.0625	0.006656	0.006432	16	150.23136	155.47264	152.852			
Inhib 1/2X	1	0.20159	0.15756	1	4.9605635	6.3467885	5.653676			
	0.5	0.08604	0.06521	2	11.622501	15.335071	13.47879			
	0.25	0.03976	0.02936	4	25.150905	34.059946	29.60543			
	0.125	0.01984	0.01381	8	50.403226	72.411296	61.40726			
	0.0625	0.008594	0.007037	16	116.35754	142.10399	129.2308			
Inhib 1/4X	1	0.27142	0.20427	1	3.6843269	4.8954815	4.289904			
	0.5	0.1325	0.08331	2	7.5471698	12.003361	9.775265			
	0.25	0.06678	0.03793	4	14.974543	26.364355	20.66945			
	0.125	0.03355	0.01872	8	29.806259	53.418803	41.61253			
	0.0625	0.01539	0.008424	16	64.977258	118.71409	91.84567			

LIST OF REFERENCES

Ahvenainen, R. 1996. New approaches in improving the shelf life of minimally processed fruits and vegetables. Trends Food Sci. Technol. 7: 179-200.

Andersen, S.O., Peter, M.G., and Roepstorff, P. 1996. Cuticular sclerotization in insects. Biochem. Physiol. 113: 689-705.

Ashie, I.N.A., Simpson, B.K. and Smith, J.P. 1996. Mechanisms for controlling enzymatic reactions in foods. Crit. Rev. Food Sci. Nutr. 36: 1-30.

Becker, D.K., Dugdale, B., Smith, M.K., Harding, R.M., and Dale, J.L. 2000. Genetic transformation of Cavendish banana (*Musa* spp. group) cv 'Grand Nain' via microprojectile bombardment. Plant Cell Rep. 19: 229–34.

Bhat, R.K., Page, K., Tan, A., and Hershenson, M.B. 2003. German cockroach extract increases bronchial epithelial cell interleukin-8 expression. Clin Exp Allergy 33: 35-42.

Brunet, P.C.J. 1980. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. Insect Biochem. 10: 467-500.

Cary, J.W., Lax, A.R. and Flurkey, W.H. 1992. Cloning and characterization of cDNAs coding for *Vicia faba* polyphenol oxidase. Plant Mol. Biol. 20: 245-253.

Chase, M.R., Raina, K., Bruno, J., and Sugumaran, M. 2000. Purification, characterization, and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. Insect Biochem. Mol. Biol. 30: 953-967.

Chen, J.S., Preston, J.F., Wei, C., Hooshar, P., Gleeson, R.A. and Marshall, M.R. 1992. Structural comparison of crustacean, potato, and mushroom polyphenol oxidases. J. Agric. Food Chem. 40: 1326-1330.

Chen, L., Mehta, A., Berenbaum, M., Arthur, R.Z. and Engeseth, N.J. 2000. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. J. Agric. Food Chem. 48: 4997-5000.

Cho, Y.K. and Ahn, H.K. 1999. Purification and characterization of polyphenol oxidase from potato: I. Purifcation and properties. J. Food Biochemistry 23: 577-592.

Colak, A., Sahin, E., Yildirim, M., and Sesli, E. 2007. Polyphenol oxidase potentials of three wild mushrooms species harvest from Lişer High Plateau, Trabzon. Food Chemistry. 103(4): 1426-1433.

Daquinag, A.C., Nakamura, S., Takao, T., Shimonishi, Y., Tsukamoto, T. 1995. Primary structure of a potent endogenous Dopa-containing inhibitor of phenol oxidase from *Musca domestica*. Proc. Natl. Acad. Sci. U.S.A. 92: 2964-2968.

Daquinag, A.C., Sato, T., Koda, H., Takao, T., Fukuda, M., Shimonishi, Y., Tsukamoto, T. 1999. A novel endogenous inhibitor of phenoloxidase from *Musca domestica* has a cysteine motif commonly found in snail and spider toxins. Biochem. 38: 2179-2188.

Eskin, N.A.M. 1990. Biochemistry of food spoilage: Enzymatic browning. In *Biochemistry of Foods*, (N.A.M. Eskin, ed.) pp. 401-432, Academy Press, NewYork.

Eskin, N.A.M., Henderson, H.M. and Townsend, R.J. 1971. Browning reactions in foods. In *Biochemistry of Foods*, (N.A.M. Eskin, H.M. Henderson and R.J. Townsend, eds.) pp. 69-83, Academic Press, New York.

Feng, C., and Fu, W. 2004. Tissue distribution and purification of prophenoloxidase in larvae of asian corn borer, *Ostrinia furnacalis* Guenee (Lepidoptera: Pyralidae). Acta Bio.Biophysica Sinica. 36(5): 360-364.

Ferrar, P.H. and Walker, J.R.L. 1996. Inhibition of diphenol oxidases: A comparative study. J. Food Biochemistry. 20: 15-30.

FDA. 1988a. Sulfiting agents in standardized foods: Labeling requirements. Food and Drug Admin., Fed Reg. 53:51062-51084.

FDA. 1988b. Sulfiting agents: Affirmation of GRAS status. Food and Drug Admin., Fed Reg. 53:51065-51084.

Fraignier, M., Marques, L., Fleuriet, A. and Macheix, J. 1995. Biochemical and immunochemical characteristics of polyphenol oxidase from different fruits of *Prunus*. J. Agric. Food Chem. 43: 2375-2380.

Gandía-Herrero, F., Escribano, J. and García-Carmona, F. 2005. Betaxanthins as substrates for tyrosinase: An approach to the role of tyrosinase in the biosynthetic pathway of betalains. Plant Physiol. 138: 421-432.

Gillespie, J.P., Kanost, M.R., Trenczek, T. 1997. Biological mediators of insect immunity. Ann. Rev. Entomol. 42: 611-643.

Girelli, Anna M., Mattei, Enrico, Messina, Antonella, and Tarola, Anna M. 2004. Inhibition of polyphenol oxidases activity by various dipeptides. J. of Agric. And Food Chem. 52: 2741-2745.

Gouzi, H. and Benmansour, A. 2007. Partial purification and characterization of polyphenol oxidase extracted from *Agaricus bisporus* (J.E.Lange) Imbach," International Journal of Chemical Reactor Engineering. Vol. 5: A76.

Hara, T., Miyoshi, T., Funatsu, M. 1993. Comparative studies on laral and pupal phenoloxidases of the housefly *Musca domestica*. Compar. Biochem. and Physiol. 106B: 287-292.

Harel, E., Mayer, A.M. and Shain, Y. 1965. Purification and multiplicity of catechol oxidase from apple chloroplasts. Phytochemistry 4: 783-790.

Hopkins, T.L. and Kramer, K.J. 1992. Insect cuticle sclerotization. Annu. Rev. Entomol. 37: 273-302.

Hsu, A.F., Thomas, C.E. and Brauer, D.1988. Evaluation of several methods for estimation of the total activity of potato polyphenol oxidase. J. Food Sci. 53(6): 1743-1745.

Hunt, M.D., Eannetta, N.T., Haifeng, Y., Newman, ,S.M. and Steffens, J.C. 1993. cDNA cloning and expression of potato polyphenol oxidase. Plant Mol. Biol. 21: 59-68.

Ilarslan, H., R.G. Palmer, J. Imsande, and H.T. Horner. 1997. Quantitative determination of calcium oxalate and oxalate in developing seeds of soybean (Leguminosae). Am. J. Bot. 84: 1042–1046.

Jang, Mi Soon, Sanada, Aiko, Ushio, Hideki, Tanaka, Munehiko, and Ohshima, Toshiaki. 2002. Inhibitory effects of "Enokitake" mushroom extracts on polyphenol oxidase and prevention of apple browning. Tokyo University of Fisheries, Department of Food Sci. and Tech. Elsevier Science Ltd.

Janovitz-Klapp, A., Richard, F. and Nicolas, J. 1989. Polyphenoloxidase from apple, partial purification and some properties. Phytochemistry 28: 2903-2907.

Kahn, V. 1985. Effect of proteins, proteins hydrolyzates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana. J. Food Sci. 50: 111-115.

Kim, J., Marshall, M.R., Wie, C. 2000. Polyphenoloxidase. In *Seafood enzymes utilization and influence on postharvest seafood quality* (Haard, N.F., Simpson, B.K., ed.) New York: Marcel Dekker. pp. 271-315.

Knodel, L.C. 1997. Current issues in drug toxicity: Potential health hazards of sulfites. Toxic Subst. Mech. 16(3):309-311.

Koehler, P.G. and Castner, J.L. 1997. The German cockroach. Department of Entomology and Nematology, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. SP-147.

Koehler, P.G. and Patterson, R.S. 1986. A comparison of insecticide susceptibility in seven nonresistant strains of the German cockroach, *Blattella germanica* (Dictyoptera: Blattellidae), J. Med. Entomol. 23: 298.

Koussevitzky, S., Ne'eman, E., Sommer, A., Steffens, J.C. and Harel, E. 1998. Purification and properties of a novel chloroplast stromal peptidase. J. Biol. Chem. 272:: 27064-27069.

Kowalski, S.P., Bamberg, J., Tingey, W.M., and Steffens, J.C. 1990. Insect resistance in the wild potato *Solanum berthaultii*: inheritance of glandular trichrome polyphenol oxidase. J Hered. 81: 475-478.

Lax, A.R., Vaughn, K.C. and Templeton, G.E. 1984. Nuclear inheritance of polyphenol oxidase in Nicotiana. J. Heredity 75: 285-287.

Lax, A.R. and Vaughn, K.C. 1991. Colocalization of polyphenol oxidase and photosystem II proteins. Plant Physiol. 96: 26-31.

Le Bourvellec, C., Le Quere, J., Sanoner, P., Drilleau, J., and Guyot, S. 2004. Inhibition of apple polyphenol oxidase by procyanidins and polyphenol oxidation products. J. Agric Food Sci. 52: 122-130.

Lee, C.Y., Smith, N.L., and Pennesi, A.P. 1983. Polyphenoloxidase from DeChaunac grapes. J Sci. Food Agric. 34: 987-991.

Lee, M., Kim, Y., Kim, N., Kim, G., Kim, S., Bang, K. and Park, I. 2001. Prevention of browning in potato with a heat-treated onion extract. Biosci. Biotechnol. Biochem. 66(4): 856-858.

Lehninger, A.L., Nelson, D.L., and Cox, M.M. 1993. *Principles of Biochemistry*, Second Ed., pp. 198-229. Worth Publishers, New York.

Lerch, K. 1995. Tyrosinase: Molecular and active-site structure. In *Enzymatic Browning and Its Prevention*, (C.Y. Lee and J.R. Whitaker, eds.) pp. 64-80, ACS Symposium Series 600; American Chemical Society, Washington DC.

Lester, M.R. 1995. Sulfite sensitivity: Significance in human health. J. Am. Coll. Nutr. 14(3): 229-232.

Lin, G.G., Liou, R., and Lee, H. 2002. The period gene of the German cockroach and its novel linking power between vertebrate and invertebrate. Chronobiology International. 19(6): 1023-1040.

Lourenco, E.J., Neves, V.A. and Silva, M.A.D. 1992. Polyphenol oxidase from sweet potato: purification and properties. J. Agric. Food Chem. 40: 2369-2373.

Lozano-de-Gonzalex, P.G., Barrett, D.M., Wrolstad, R.E. and Durst, R.W. 1993. Enzymatic browning inhibited in fresh and dried apple rings by pineapple juice. J. Food Sci. 58: 399-404.

Macheix, J., Fleuriet, A. and Billot, J. 1990. Phenolic compounds in fruit processing. In *Fruit Phenolics*, (J. Macheix, A. Fleuriet and J. Billot, eds.) pp. 239-312, CRC Press, Boca Raton, Florida.

Maki, H., and Morohashi, Y. 2006. Development of polyphenol oxidase activity in the micropylar endosperm of tomato seeds. J. Plant Physiol. 163: 1-10

Marques, L., Fleuriet, A. and Macheix, J. 1995. Characterization of multiple forms of polyphenoloxidase from apple fruit. Plant Physiol. Biochem. 33: 193-200.

Marshall, M.R., Kim, J., and Wei, C. 2000. Enzymatic browning in fruits, vegetables, and seafood. Food and Agriculture Organization of the United Nations. http://www.fao.org/ag/Ags/agsi/ENZYMEFINAL/Enzymatic%20Browning.html#ACID

Martinez-Cayuela, M., Rodriguez-Vico, F., Faus, M.J. and Gil, A. 1989. Partial purification and intracellular localization of cherimoya (Annona cherimolia Mill.) polyphenol oxidase. J. Plant Phyisol. 133: 660-663.

Matella, N.J., Dolan, K.D., and Lee, Y.S. 2006. Comparison of galactooligosaccharide production in free-enzyme ultrafiltration and in immobilized-enzyme systems. J. Food Sci. 71(7): C363-C638.

Mayer, A.M., and Harel, E. 1979. Polyphenol oxidase in plants. Phytochemistry 18: 193-215.

Mcevily, A.J., Iyegar, R., Otwell, W.S. 1992. Inhibition of enzymatic browning in foods and beverages. Crit. Rev. Food Sci. Nutr. 32: 253-273.

Miller, Malinda. 2005. Commodity apple profile. Agricultural Marketing Resource Center, AgMRC. http://www.agrc.org/agmrc/commodity/fruits/apples/commodityapples.htmo0Last viewed 7/7/07.

Moussian, B., Schwarz, H., Bartoszewski, S., and Nusslein-Volhard, C. 2005. Involvement of chitin in exoskeleton morphogenesis in Drosophila melanogaster. J. Morphol. 264: 117-130.

Murata, M., Tsurutani, M., Tomita, M., Homma, S. and Kaneko, K. 1995. Relationship between apple ripening and browning: Changes in polyphenol content and polyphenol and polyphenol oxidase. J. Agric. Food Chem. 43: 1115-1121.

Murata, M., Tsurutani, M., Hagiwara, S. and Homma, S. 1997. Subcellular location of polyphenol oxidase in apples. Biosci. Biotech. Biochem. 61: 1495-1499.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M. and Aubert, S.Y. 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34: 109-157.

Osuga, D., Van Der Schaaf, A. and Whitaker, J.R. 1994. Control of polyphenol oxidase activity using a catalytic mechanism. In *Protein Structure-Function Relationships in Foods*, (R.Y. Yada, R.L. Jackman and J.L. Smith, eds.) pp. 62-88, Chapman and Hall, New York.

Oszmianski, J. and Lee, C.Y. 1990. Inhibition of polyphenol oxidase activity and browning by honey. J. Agric. Food Chem. 38: 1892-1895.

Palmer, J.K. 1963. Banana polyphenoloxidase: preparation and properties. Plant Physiol. 38(5): 508-513.

Papazian, R. 1996. Sulfites: Safe for most, dangerous for some. FDA Comsumer Magazine. 30(10).

Park, E.Y. and Luh, B.S. 1985. Polyphenol oxidase of kiwifruit. J. Food Sci. 50: 678-684.

Park, H.J. 1999. Development of advanced edible coatings for fruits. Trends Food Sci. Technol. 10: 254-260.

Peter, M.G. 2006. Review: Oxidative metabolism of tyrosine in insects. Institut fur organische chemie und biochemie der rheinischen. Friedrich-Wilhelms-Universitat.

Pryor, M.G.M. 1940. On the hardening of the ootheca of *Blatta orientalis*. Proc. Roy. Soc. 128: 378-393.

Richardson, T. & Hyslop, D.B. 1985. Enzymes. *In* O.R. Fennema, ed. *Food Chemistry*, p. 371-476. New York, Marcel Dekker.

Robinson, S.P. and Dry, I.B. 1992. Broad bean leaf polyphenol oxidase is a 60-kilodalton protein susceptible to proteolytic cleavage. Plant Physiol. 99: 317-323.

Sanchez-Ferrer, A., Levada, F. and Garcia-Carmona, F. 1993a. Substrate-dependent activation of latent potato leaf polyphenol oxidase by anionic surfactants. J. Agric. Food Chem. 41: 1583-1586.

Sanchez-Ferrer, A., Laveda, F. and Garcia-Carmona, F. 1993b. Partial Purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system. J Agric. Food Chem. 41: 1219-1224.

Sanchez-Ferrer, A., Francisco, L. And Garcia-Carmona, F. 1993c. Cresolase activity of potato tuber partially purified in a two-phase partition system. J. Agric. Food Chem. 41: 1225-1228.

Sanchez-Ferrer, A., Rodriguex-Lopez, J.N., Garcia-Canovas, F. and Garcia-Carmona, F. 1995. Tyrosinase: A comprehensive review of its mechanism. Biochim. Biophys. Acta. 1247: 1-11.

Sapers, G. M. 1993. Browning of foods: control by sulfites, antioxidants, and other means. Food Technol. 47(10): 75-84.

Sato, M. 1980. Inhibition by oxalates of spinach chloroplast phenolase in unfrozen and frozen states. Phytochemistry. 19: 1613-1617.

Sayaverdra-Soto, L.A., and Montgomery, M.W. 1986. Inhibition of polyphenoloxidase by sulfite. J. Food Sci. 51: 1531-1536.

Serradell, M.D.L.A., Rozenfeld, P.A., Martinez, G.A., Civello, P.M., Chaves, A.R. and Anon, M.C. 2000. Polyphenoloxidase activity from strawberry fruit (*Fragaria* × *ananassa*, Duch., cv Selva) characterization and partial purification. J. Sci. Food Agric. 80: 1421-1427.

Shahar, T., Hennig, N., Gutfinger, T., Hareven, D. and Lifschitz, E. 1992. The tomato 66.3 kD polyphenol oxidase gene: molecular identification and developmental expression. Plant Cell. 4: 135-147.

Sheptovitsky, Y.G. and Brudvig, G.W. 1996. Isolation and characterization of spinach photosystem II membrane-associated catalase and polyphenol oxidase. Biochemistry 35: 16255-16263.

Sherman, T.D., Gardeur T.L. and Lax, A.R. 1995. Implications of the phylogenetic distribution of polyphenol oxidase in plants. In *Enzymatic Browning and Its Prevention*, (C.Y. Lee and J.R. Whitaker, eds.) pp. 103-119, AACS Symposium Series 600; American Chemical Society, Washington, DC.

Sherman, T.D., Vaughn, K.C. and Duke, S.O. 1991. A limited survey of the phylogenetic distribution of polyphenol oxidase. Phytochemistry 30: 2499-2506.

Simpson, B.K., Marshall, M.R. and Otwell, W.S. 1988. Phenoloxidases from pink and white shrimp: Kinetic and other properties. J. Food Biochemistry 12: 205-217.

Solomon, E.I., Baldwin, M.J. and Lowery, M.D. 1992. Electronic structures of active sites in copper proteins. Contributions to reactivity. Chem. Rev. 92: 521-542.

Solomon, E.I., and Lowery, M.D. 1993. Electronic structure contributions to function in bioinorganic chemistry. Science 259: 1575-1581.

Sommer A., Ne'eman, E.., Steffebsm J.C., Mayer, A.M. and Harel, E. 1994. Import, targeting and processing of a plant polyphenol oxidase. Plant Physiol. 105: 1301-1311.

Son, S.M., Moon, K.D., and Lee, C.Y. 2000. Kinetic study of oxalic acid inhibition on enzymatic browning. J. Agric. Food Chem. 48: 2071-20-74.

Stelzig, D.A., Akhtar, S. and Ribeiro, S. 1972. Catechol oxidase of red delicious apple peel. Phytochemistry 11: 535-539.

Suderman, R.J., Dittmer, N.T., Kanost, M.R., and Kramer, K.J. 2006. Model reactions for insect cuticle sclerotization: Cross-linking of recombinant cuticular proteins upon their laccase-catalyzed oxidative conjugation with catechols. Insect Biochem. Mol. Biol. 36: 353-365.

Sugumaran, M., Giglio, L., Kundzicz, H., Saul, S., and Semensi, V. 1992. Studies on the enzymes involved in puparial cuticle sclerotization in drosophilia melanogaster. Arch. Insect Biochem. Physiol. 19: 271-283.

Sugumaran, M. 1998. Unified mechanism for sclerotization of insect cuticle. Adv. Insect Physiol. 27: 229-334.

Sugumaran, M and Nellaiappan K. 2000. Characterization of a phenoloxidase inhibitor from the cuticle of *Manduca Sexta*. Biochem Biophys Res Commun 268(2): 379-83.

Sugumaran, M. and Nelson, E. 1998. Model sclerotization studies. 4. Generation of n-acetylmethionyl catechol adducts during tyrosinase-catalyzed oxidation of catechols in the presence of n-acetylmethionine. Arch. Insect Biochem. Physiol. 38: 44-52.

Sugumaran, M. and Ricketts, D. 1995. Model sclerotization studies. 3. Cuticular enzyme catalyzed oxidation of peptidyl model tyrosinase and dopa derivatives. Arch. Insect Biochem. Physiol. 28: 17-32.

Suiter, D.R. and Koehler, P.G. 2000. German cockroach, *Blattella germanica*. Entomology and Nematology Department, Florida Coopereative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. ENY-238.

Tan, B.K. and Harris, N.D. 1995. Maillard reaction products inhibit apple polyphenoloxidase. Food Chem. 53: 267-273.

Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: Uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. Adv. Food Res. 30: 1-76.

Thygesen, P.W., Dry, I.B. and Robinson, S.P. 1995. Polyphenol oxidase in potato: A multigene family that exhibits differential expression patterns. Plant Physiol. 109: 525-531.

Tipton, K.F. and Dixon, H.B.F. 1983. Effects of pH on enzymes. In *Contemporary Enzyme Kinetics and Mechanism*, (D.L. Purich, ed.) pp. 97-148, Academic Press, New York.

Tolbert, N.E. 1973. Activation of polyphenol oxidase of chloroplasts. Plant Physiol. 51: 234-244.

Tsukamoto T, Ichimaru Y, Kanegae N, Watanabe K, Yamaura I, Katsura Y, Funatsu M. 1992. Identification and isolation of endogenous insect phenoloxidase inhibitors. Biochem Biophys Res Commun 184(1): 86-92.

United States Department of Agriculture. USDA. 1984. Composition of foods: vegetables and vegetable products. U.S. Dept. of Agriculture Handbook Number 8-11. Washington, D.C.; U.S. Dept. of Agriculture, Human Nutrition Information Service.

United States Department of Agriculture, USDA. 2005. World Apple Situation. http://www.fas.usda.gov/htp/horticulture/Apples/World%20Apple%20Situation%20MY%202004%202005.pdf. Last viewed 7/7/07.

United States Department of Agriculture, USDA. 2006**.** 2005 Potato Production and Disposition, National Agricultural Statistics Service. http://www.nass.usda.gov/Statistics_by_State. Last viewed 9/7/07.

Valero, E. and Garcia-Carmona, F. 1998. pH-dependent effect of sodium chloride on latent grape polyphenol oxidase. J. Agric. Food Chem. 46: 2447-2451.

Valles, S. 1996. German cockroach, *Blattella germanica* (Linnaeus) (Insecta: Blattodea: Blattellidae). Entomology and Nematology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. EENY-002.

Valles, S.M. and Yu, S.J. 1995. Tissue localization, induction, and developmental expression of cytochrome P450 monooxygenases in the German cockroach. Pestic. Biochem. Physiol. 54: 31-39.

Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sco. Nutr. 15: 49-127.

Van Gelder, C.W.G., Flurkey, W.H. and Wichers, H.J. 1997. Sequence and structural features of plant and fungal tyrosinases. Phytochemistry 45: 1309-1323.

Vaughn, K.C. and Duke, S.O. 1984a. Function of polyphenol oxidase in higher plants. Physiol. Plant. 60: 106-112.

Vaughn, K.C. and Duke, S.O. 1984b. Tentoxin stops the processing of polyphenol oxidasae into an active protein. Physiol. Plant. 60: 257-261.

Vaughn, K.C., Lax, A.R. and Duke, S.O. 1988. Polyphenol oxidase: The chloroplasat oxidase with no established function. Physiol. Plant. 72: 659-665.

Walker, J.R.L. 1977. Enzymatic browning in foods: Its chemistry and control. *Food Technol. NZ*, 12: 19-25.

Wang, Q., Chen, Q., Huang, X., Ke, L., Shi, Y., and Wang, J. 2004. Enzymatic characterization and functional groups of polyphenol oxidase from the pupae of blowfly (*Sarcophaga bullata*). Biochemistry (Moscow). Papers in Press.

Wang, Y. and Jiang, H. 2004. Purification and characterization of *Manduca sexta* serpin-6: a serine proteinase inhibitor that selectively inhibits prophenoloxidase-activating proteinase-3. Insect Biochem. Mol. Biol. 34: 387-395.

Weemaes, C., Rubens, S., De Cordt, S., Ludikhuyze, L., Van Den Broeck, I., Hendrickx, M., Heremans, K., and Tobback, P. 1997. Temperature sensitivity and pressure resistance of mushroom polyphenoloxidase. J. Food Sci. 62(2): 261-266.

Weaver, C.M. 1974. Factors influencing enzymatic browning of ripening bananas. Department of Foods and Nutrition. Oregon State University Master of Science Thesis.

Wesche-Ebeling, P., and Montgomery, M.W. 1990. Strawberry Polyphenoloxidase: It's Role in Anthocyanin Degradation. J. Food Sci. 55(3): 731–734.

Whitaker, J.R. 1994. *Principles of Enzymology for the Food Sciences*, Second Ed., pp. 271-556, Marcel Dekker, New York.

Whitaker, J.R. 1995. Polyphenol oxidase. In *Food Enzymes Structure and Mechanism*, (D.W.S. Wong, ed.) pp. 271-307, Chapman and Hall, New York.

Whitaker, J.R. 1996. Polyphenol Oxidase. In *Food Chemistry*, (O.R. Fennema, ed.) pp. 492-494, Marcel Dekker, New York.

Whitaker, J. R. and C. Y. Lee. 1995. Recent advances in enzymatic browning. In "Enzymatic Browning and It's Prevention" Ed. by Lee, C. Y. and Whitaker, J. R. American Chemical Society Symposium Series 600, 2-7, American Chemical Society, Washington, D. C.

Wilcox, D.E., Porras, A.G., Hwang, Y.T., Lerch, K., Winkler, M.E. and Solomon, E.I. 1985. Substrate analog binding to the coupled binuclear copper active site in tyrosinase. J. Am. Chem. Soc. 107: 4015-4027.

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

Yalovsky, S., Paulsen, H., Michaeli, D., Chitnis, P.R. and Nechushtai, R. 1992. Involvement of a chloroplast HSP70 heat shock protein in the integration of a protein (light-harvesting complex protein precursor) into the thylakoid membrane. Proc. Natl. Aca. Sci. USA 89: 5616-5619.

Yemenicioglu, A., Ozkan, M. and Cemeroglu, B. 1997. Heat inactivation kinetics of apple polyphenoloxidase and of its latent form. J. Food Sci. 62: 508-510.

Yoruk R., Hogsette J.A., Rolle R.S. and Marshall MR. 2003. Apple polyphenol oxidase inhibitor(s) from common housefly (*Musca Domestica* L.). J. Food Sci 68(6): 1942-47.

Yoruk, R, Marshall, M.R. 2003. Physiochemical properties and function of plant polyphenol oxidase.A review. J. Food Biochem. 27: 361-422.

Yoruk, R., Yoruk, S., Balaban, M.O. and Marshall, M.R. 2004. Machine vision analysis of antibrowning potency for oxalic acid: A comparative investigation on banana and apple. J. Food Sci. 69(6): 281-289.

Zhou, P., Smith, N.L. and Lee, C.Y. 1993. Potential purification and some properties of Monroe apple peel polyphenol oxidase. J. Agric. Food Chem. 41: 532-536.

Zufelato, M.S., Lourenco, A.P., Simoes, Z.L.P., Jorge, J.A., and Bitondi, M.G. 2004. Phenoloxidase activity in *Apis mellifera* honey bee pupae, and ecdysteroid-dependent expression of the prophenoloxidase mRNA. Insect Biochem. Mol. Biol. 34: 1257-1268.

BIOGRAPHICAL SKETCH

Paul Justin Kurt Grotheer was born in Springfield, Virginia outside Washington D.C. while his dad was working at the Pentagon. His dad's career in the Air Force led his family to move frequently while he was growing up, including stays in Tampa, Florida; Stuttgart, Germany; and Vicenza, Italy. When his dad finally retired from the military, Paul finished up high school in Tampa, Florida, before coming to the University of Florida. Paul earned a Bachelor's degree in Food Science and Human Nutrition specializing in Dietetics and also kept busy as a member of the Florida Drumline playing at home and away football games.

Paul then decided to pursue a Master's of Food Science, working with Dr. Marshall in the Food and Environmental Toxicology Lab. He is also completing a minor in Food and Resource Economics. Paul enjoys playing and watching sports, traveling, kayaking, and brewing beer in his spare time. He plans to pursue a career in food safety in the food industry upon completion of his degree.