POLYPHENOL OXIDASE INHIBITOR(S) FROM GERMAN COCKROACH (*BLATTELLA GERMANICA*) EXTRACT

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ABSTRACT

Enzymatic browning causes millions of dollars in losses yearly to the food industry by discoloration of fruits and vegetables. Food and Drug Administration's 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 the extract of German cockroach, *Blattella germanica*. Crude cockroach extract inhibited apple PPO activity 60–70% and potato PPO 15–25%. Inhibition occurred rapidly in the reaction mixture and was dependent on the pH of the system. The inhibitor(s) appear(s) to be large based on ultrafiltration studies and loss of inhibition with proteases.

PRACTICAL APPLICATIONS

An extract from German cockroach appears effective in inhibiting browning on apples and potatoes. Successful identification of inhibitor(s) of polyphenol oxidase (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. Identifying and understanding the mechanism of inhibition on fruit and vegetable PPOs could lead to inexpensive alternatives to prevent browning. Eventually a commercial spray or dip using a purified form of the inhibitor may be developed as an inexpensive treatment to preserve fruits and vegetables.

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 or browning (Sapers 1993).

Enzymatic browning commonly occurs in some fruit and vegetable crops after harvest during handling, processing and storage (Jang *et al.* 2002). Enzymatic browning is considered an undesirable reaction in such 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 *et al.* 1995; Weemaes *et al.* 1997).

Historically, sulfites have been used to effectively slow and/or prevent browning. In fresh fruits and vegetables, sulfites prevent PPO from working properly by preventing brown pigment formation (Sayaverdra-Soto and Montgomery 1986; Sapers 1993). However, a small percentage of the U.S. population is suspected to be sensitive to sulfites. Food and Drug Administration's banning of sulfites in 1986 has

created a large field of research in search of natural, effective and economic inhibitors of enzymatic browning (Yoruk and Marshall 2003).

Polyphenol oxidase is found in insects as well as plants (Sugumaran 1998). Insect PPO is considered to play a key role in important physiological processes such as cuticular tanning and sclerotization, as well as in wound healing and defense reactions against foreign pathogens (Tsukamoto *et al.* 1992; Sugumaran and Nellaiappan 2000; Wang *et al.* 2004). It seems possible that natural inhibitors within insects regulate these physiological processes and could serve to prevent or minimize browning of fruits and vegetables. Previous literature has demonstrated endogenous inhibitors in insects such as houseflies (Tsukamoto *et al.* 1992; Daquinag *et al.* 1995, 1999). Sugumaran and Nellaiappan (2000) isolated a heat-labile, high molecular weight (380,000 Da) glycoprotein inhibitor of PPO from the larval cuticle of tobacco horn worm, *Manduca sexta*. In addition, Yoruk *et al.* (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 nominal molecular weight limit (NMWL) membrane.

The hypothesis is that inhibitors regulating PPO's role in insect development could be useful as browning inhibitors for fruits and vegetables. Thus, the objectives of this study were to extract PPO inhibitor(s) from German cockroach and to demonstrate inhibition of PPO activity using these inhibitor(s) on potato and apple PPOs. Characterization of crude inhibitor(s) included temperature stability, pH extraction profile, incubation, dialysis, ultrafiltration, treatment with proteases and kinetic evaluation of the crude extract.

MATERIALS AND METHODS

PPO Activity and Inhibition

German Cockroach Samples. Samples of *Blattella germanica* (L.) were provided by the United States Department. of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology (Gainesville, FL). The male cockroaches were reared at 26C, 55% relative humidity, and a 12:12 light : dark photoperiod as described by Koehler and Patterson (1986). Newly molted adult males were collected using featherweight forceps. Adult males are typically used instead of females to minimize impact on the laboratory colonies.

Preparation of Inhibitor Extract. PPO inhibitor was extracted from German cockroach following a procedure adapted from Valles and Yu (1995). Cockroaches ($n = 10$–15) were anaesthetized with carbon dioxide and male samples were separated and put on ice. The cockroaches were decapi-

tated, and the bodies were put in a 10-mL tube with 0.1 M sodium phosphate buffer, pH 6.5. A Delta Machinery 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 (Beckman Instruments Inc., Fullerton, CA) at $10,000 \times g$ for 15 min at 4C. The supernatant was filtered through glass wool and then centrifuged again for 1 h at $105,000 \times g$. The supernatant was filtered through glass wool once again and stored at −20C 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. Apple PPO was extracted from an apple acetone powder made using whole Red Delicious apples acquired from a local grocery store. A Russet potato acetone powder was prepared from whole Russet potatoes acquired from a local grocery store. Washed apples and potatoes were cut up to small pieces (200 g of each), which were homogenized in a pre-chilled blender (Waring Products Inc., Torrington, CT) with acetone (−20C) 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 (−20C). The resulting white acetone powder was vacuum-dried in a FoodSaver Vac 350 (Tilia Inc., San Francisco, CA) at room temperature (25C) and stored in commercial vacuum bags (Tilia Inc.) at −20C until needed for PPO extraction.

The modified procedures of Murata *et al.* (1995) and Yoruk *et al.* (2003) were used for PPO extraction. One gram of acetone powder was added 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 at 4C for 20 min while stirring with a magnetic stirrer. The suspension was centrifuged in a Beckman Coulter Optima™ L-100 XP (Beckman Instruments Inc.) for 30 min at $12,000 \times g$ and 4C, and the resulting supernatant was filtered through glass wool. Finally, the filtered supernatant was stored in microcentrifuge tubes at −20C.

Assay of PPO Activity and Inhibition. PPO inhibition was quantified by measuring the PPO activity of apple and potato with and without added inhibitor extract. A standard assay consisted of 2.45 mL of either 0.1 M sodium acetate-acetic acid, pH 4.0–5.5 or 0.1 M sodium phosphate, pH 6.0–7.0 buffer, 0.3 mL of 0.5 M catechol substrate (Sigma Chemical Co., St. Louis, MO), 0.2 mL of test extract or control buffer depending on pH and 0.05 mL of the enzyme extract (apple or potato). PPO activity was measured by spectrophotometric determination of the greatest initial reaction rate at 420 nm and 25C with a Beckman Model DU 640

Ultraviolet-Visible spectrophotometer (Beckman Instruments Inc.). The pH of the reaction mixture was checked after each assay to confirm pH values were maintained for the control and test systems.

Polyphenol oxidase activity of apple and potato 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 or 0.1 M sodium phosphate) varied from pH 4.0 to 6.5 depending on the enzyme source. One unit of enzyme activity was defined as an increase in absorbance of 0.001 per minute at 25C. 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 (inhibitor) system.

Characterization of Crude Inhibitor(s) from German Cockroach

The pH Optimum of Plant PPO. The pH-activity profile for the oxidation of catechol by apple and potato 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 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 apple 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 PPO or catechol substrate, and incubated at room temperature (25C) for 0, 1, 5, 15 and 30 min. Reactions were initiated by either adding PPO (to inhibitor/substrate incubation) or substrate (to inhibitor/PPO incubation) and initial reaction rates were calculated at 420 nm and 25C.

Effect of Heating and Freezing and Thawing on Inhibitor(s). Aliquots of 2 mL of the inhibitor preparation were incubated in boiling water, 100C, for 5, 15, 30 and 60 min. The precipitate was separated by centrifugation for 5 min at 10,000 × g at room temperature. The clear supernatants were collected in clean microcentrifuge tubes. PPO inhibition was determined at 25C as described above.

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

The pH Extraction Profile. The inhibitor extract was prepared 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 −20C 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-, 2,000- and 25,000-Da molecular weight cutoff dialysis membranes (Spectrum Laboratory Products, Inc., New Brunswick, NJ) with constant stirring for 24 h at 4C against two and three changes of 0.1 M sodium phosphate buffer, pH 6.5, or distilled water. The dialysates were kept at 4C until they were tested for PPO inhibition.

Ultrafiltration. Ultrafree-4 centrifugal filter units (Millipore Corporation, Bedford, MA) with the NMWL membranes of 10,000, 50,000 and 100,000 were used. Filter units were rinsed with distilled water to remove any trace amount of glycerin. Aliquots of 1.5 mL inhibitor sample were placed onto the filter units and concentrated by centrifugation at 4C and 7,500 × g for 35 min. Filtrates were collected and kept at 4C. The sample was reconstituted to 2 mL by adding 0.1 M sodium phosphate buffer, pH 6.5, and centrifuged again. Concentrated inhibitor sample on the membrane surface, retentate, was reconstituted 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 0.01 g of trypsin (Sigma Chemical Co.) in 10 mL of a pH 7.2 0.1 M 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 by adding 0.3 mL of control buffer (without trypsin) to 5.7 mL of cockroach inhibitor solution. Then TRIS (0.2 M) was added to the control and test solutions to reach the optimum pH of 7.6 for trypsin as stated by the manufacturer. The solutions (PPO control [no inhibitor], pH control [inhibitor in buffer at optimum pH for trypsin], protease treatment, inhibitor control [inhibitor]) were incubated in a water bath at room temperature (25C) for 1 h. 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.) in 10 mL of pH 6.5 0.1 M sodium phosphate buffer. Then, 0.15 mL of the papain solution was added to 2.85 mL of inhibitor extract. The pH control solution was also made by

adding 0.15 mL of control buffer (without papain) to 2.85 mL of inhibitor extract. Because papain has an optimum pH range of 6.0–7.0, no further pH adjustment was necessary. The solutions (PPO control [no inhibitor], pH control [inhibitor in buffer at optimum pH for papain], protease treatment, inhibitor control [inhibitor]) were incubated in a water bath at room temperature (25C) for 1 h. 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, $^{1}/_{2}$X and $^{1}/_{4}$X) for five different fixed concentrations of catechol (1, 0.5, 0.25, 0.125 and 0.0625 M) at pH 5.5.

RESULTS AND DISCUSSION

PPO Activity and Inhibition

Optimization of pH Conditions for PPO Activity and Inhibition.
The pH activity profile for oxidation of catechol by potato PPO for the pH range of 4.0–6.5 was determined (Fig. 1). 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 and 5.0 and the second between 6.0 and 6.5 (Sanchez-Ferrer *et al.* 1993) while this was not apparent for this potato extract.

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. The amount of inhibition by cockroach extract on potato PPO varied with changing pH (Fig. 1). 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. The pH of the system was confirmed with a control containing no inhibitor. The pH curve (Fig. 1) is
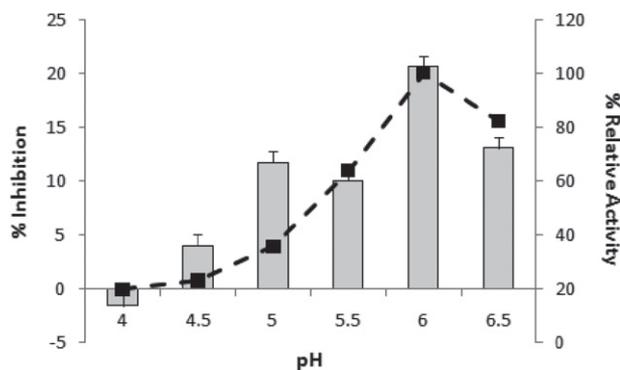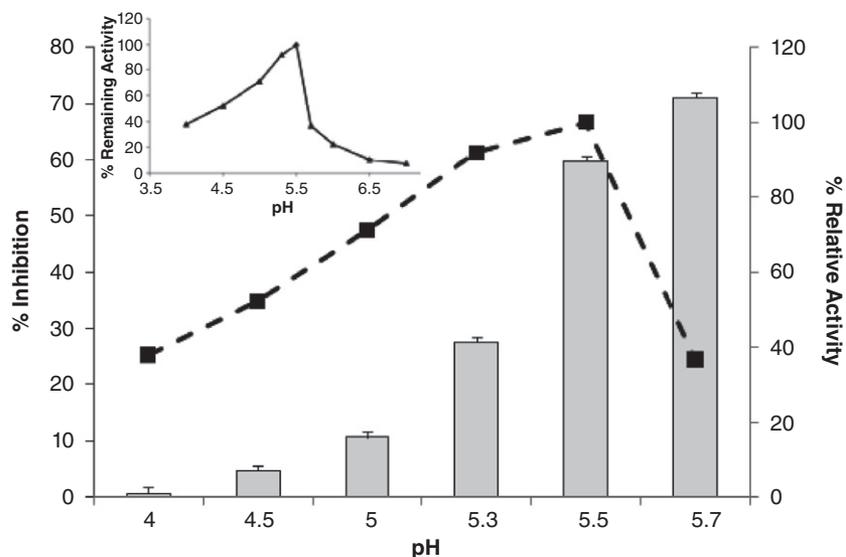

FIG. 1. OPTIMUM PH FOR POLYPHENOL OXIDASE (PPO) FROM POTATO AND ITS PH OPTIMUM FOR INHIBITION BY COCKROACH EXTRACT
This was determined by measuring the PPO activity of potato mixed with inhibitor(s) in a standard reaction mixture described in *Material and Methods*. Bars indicate % inhibition while the dashed line indicates % relative PPO activity without inhibitor(s). Each data point represents mean ± standard deviation of two experiments each with three replicates.

very similar to the pH optima performed in independent experiments (data not shown).

The pH activity profile for oxidation of catechol by apple PPO was also determined (Fig. 2). 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 as pH increased (Fig. 2, insert). The pH of the system was confirmed with a control containing no inhibitor. The pH curve (Fig. 2) is very similar to the pH optima performed in independent experiments (Fig. 2, insert). 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 to 5.5 (Janovitz-Klapp *et al.* 1989; Zhou *et al.* 1993; Yoruk and Marshall 2003). Stelzig *et al.* (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 *et al.* 1972; Janovitz-Klapp *et al.* 1989; Zhou *et al.* 1993; Whitaker 1994; Yoruk and Marshall 2003).
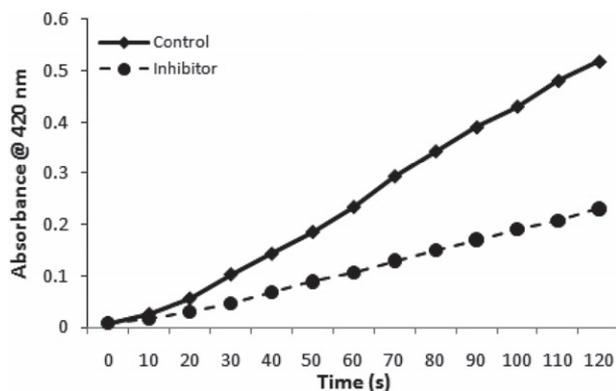
The ability of cockroach extract to inhibit apple PPO was demonstrated spectrophotometrically by mixing the inhibitor extract with an active extract of apple PPO at the determined pH optimum of 5.5. A comparison of absorbance at 420 nm between the control and test assays for 120 s was plotted (Fig. 3). 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

FIG. 2. OPTIMUM PH FOR APPLE POLYPHENOL OXIDASE (PPO) INHIBITION BY COCKROACH EXTRACT

This was determined by measuring the PPO activity of apple mixed with inhibitor(s) in a standard reaction mixture described in *Materials and Methods*. Bars indicate % inhibition while the dashed line indicated % relative PPO activity without inhibitor(s). Each data point represents mean ± standard deviation of two experiments each with three replicates. Insert is the pH optimum of apple PPO from a separate extract for comparison. Activities are expressed as % relative activity to the maximal activity determined at pH 5.5. Each data point represents mean ± standard deviation of two experiments each with three replicates and is representative of over 10 trials.

inhibitor(s) assay conditions demonstrated the extreme importance of reaction pH on inhibition activity by the cockroach extract (Fig. 2). 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 be attributed to the decline in PPO activity as a result of changing pH (Fig. 2, insert).

Inhibition of PPO by insect tissues has been reported previously. Tsukamoto *et al.* (1992) isolated inhibitors from the pupal extracts of houseflies and demonstrated inhibition on housefly PPO. Sugumaran and Nellaiappan (2000) isolated an endogenous inhibitor in the larval cuticle of the tobacco horn worm (*M. sexta*). This PPO inhibitor from *M. sexta* inhibited the activity of both insect and plant PPOs and laccase. Yoruk *et al.* (2003) isolated an inhibitor(s) from the common housefly (*M. domestica*). The isolated inhibitor(s) was found to be fairly heat-stable and low-molecular weight. The inhibitor(s) from *M. 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 *et al.* 1992; Sugumaran and Nellaiappan 2000).

Characterization of Crude Inhibitor(s) from German Cockroach

Characterization of the inhibitor from German cockroach was evaluated primarily using apple PPO. Apple PPO showed greater activity than potato PPO and it also showed the greatest inhibition with the inhibitor over 60% compared to 20%, respectively.

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 (Fig. 4). When the time of incubation increased, the rate of reaction at

FIG. 3. INHIBITION OF APPLE POLYPHENOL OXIDASE (PPO) ACTIVITY BY INHIBITOR(S) FROM GERMAN COCKROACH
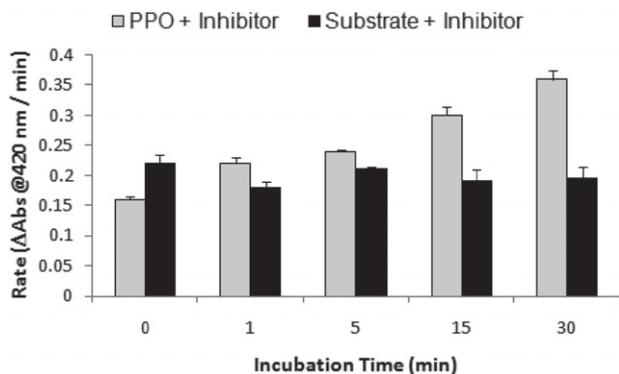
Solid line indicates results for PPO alone, while the dashed line indicates PPO with inhibitor(s). Inhibition activity was observed in the 3 mL standard reaction mixture described in *Materials and Methods*. The graph is representative of over 100 trials with cockroach extract on PPO activity of potato and apple.

FIG. 4. INCUBATION TIME STUDY
Rate of reaction at 420 nm was determined after incubation of inhibitor/polyphenol oxidase (PPO) compared to incubation of inhibitor/substrate over a 30-min time period. Each data point represents mean ± standard deviation of three replicates.

420 nm also increased, which corresponds to a decrease in inhibition. However, when inhibitor and substrate were incubated over the same 30-min time period, there was very little effect on inhibition. The rate at 420 nm remained fairly constant at each incubation time after slightly decreasing from 0 to 1 min. Therefore, PPO inhibition studies were carried out initiating the reaction with PPO as opposed to substrate. It also suggests that the inhibitor(s) may be causing apple PPO inhibition by affecting its substrate catechol.

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 100C 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 100C for 10 min. However, Tsukamoto *et al.* (1992) found their inhibitor(s) from housefly to be quite stable to heating, retaining 60% of their activity when heated to 80C for 1 h. Similarly, Yoruk *et al.* (2003) found their housefly inhibitor(s) to also be relatively heat stable, losing only 26% of its inhibitory activity on apple PPO upon boiling at 100C up to 1 h. This is a crude way to characterize the inhibitor(s) as a number of enzymes may act on a common complex substrate (Whitaker 1994). It should be noted that both of these inhibitors were considered to be either peptides (Tsukamoto *et al.* 1992) or small proteins (Yoruk *et al.* 2003).

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 *et al.* (2003) with housefly, which lost only

about 8% of its inhibitor activity to repeated freezing and thawing. Inhibitor activity of cockroach extract stored at −20C 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 (Fig. 5). The pH had a dramatic effect on the inhibitor(s). It was most active at an extraction 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 reported to have a dramatic effect on the inhibitor from tobacco horn worm (Sugumaran and Nellaiappan 2000). This inhibitor was most stable around a neutral pH, losing its total activity upon exposure to pH 10 for 10 min. Yoruk *et al.* (2003) reported the inhibitor from common housefly 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 *et al.* (1992) observed the inhibitor(s) in housefly 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.

Dialysis of Inhibitor(s). Dialysis of inhibitor extracts was performed using 500-, 2,000- and 25,000-Da molecular weight cutoff dialysis membranes with water and buffer (Fig. 6). Due to the changes pH has on the inhibitor(s) from cockroach extract, the extracts that went through dialysis with distilled water lost more than 40% inhibition compared to the control. When dialysis was performed using the control
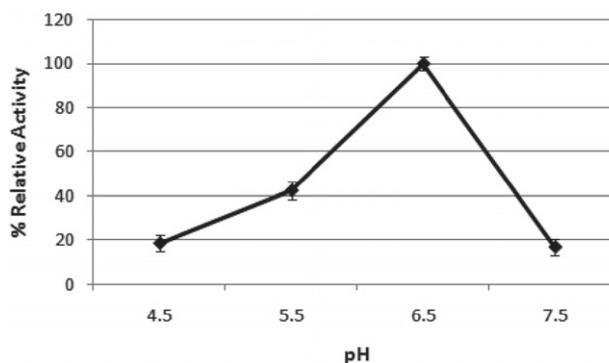
FIG. 5. THE PH EXTRACTION PROFILE OF INHIBITOR(S)
Inhibitor was extracted at pH values of 4.5, 5.5, 6.5 and 7.5 with 0.1 M sodium phosphate or sodium acetate buffer. Each data point represents mean ± standard deviation of two experiments each with two replicates.
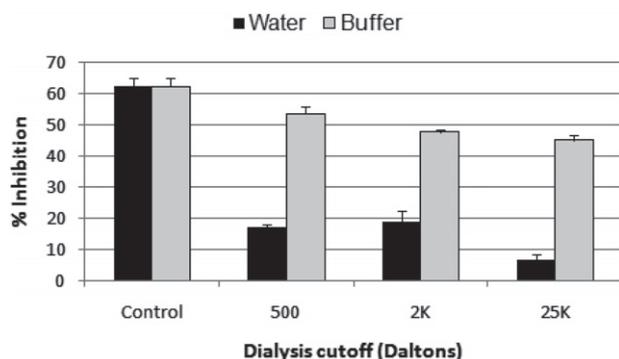
FIG. 6. PERCENT INHIBITION AFTER DIALYSIS WITH WATER OR CONTROL BUFFER

Each buffer data point represents mean ± standard deviation of two experiments each with three replicates. Each water data point represents mean ± standard deviation of three replicates.

buffer, a much smaller loss in percent inhibition was observed. The 500-Da molecular weight cutoff membrane lost only 8% inhibition compared to control. There was a slight decreasing trend as the molecular weight membrane got larger, with the 25,000-Da membrane losing about 17% compared to the control. Since it appeared the unknown inhibitor(s) may in fact be larger than 25,000-Da, ultrafiltration studies were performed to better approximate the molecular weight.

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 *et al.* 2006). Inhibitor preparation was also characterized by ultrafiltration studies (Table 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 indi-

TABLE 1. ULTRAFILTRATION OF THE INHIBITOR(S) EXTRACTS FROM GERMAN COCKROACH

| NMWL	Relative % inhibitor activity	
	Filtrate	Retentate
Control	0	100
10,000	4.6 ± 1.0	98.0 ± 1.5
50,000	4.6 ± 1.8	98.4 ± 0.8
100,000	4.0 ± 2.4	99.5 ± 2.4

Ultrafiltration was performed using centrifugal filter units with specified nominal molecular weight limit (NMWL) membranes. Each data point represents mean ± standard deviation of two experiments each with two replicates.

cated that the unknown inhibitor(s) from German cockroach is larger than 100,000 NMWL as more than 98% inhibitor activity remained in the retentate at all three size ultrafiltration units. Ultrafiltration units with 10,000, 50,000 and 100,000 NMWL retained 98.4, 98.0 and 99.5% activity, 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 380,000 on SDS-PAGE gels. The size results are much larger than those found by Tsukamoto *et al.* (1992) based on Sephadex G-25 gel filtration information, which showed the inhibitor to range in size from 3,000 to 3,500. Yoruk *et al.* (2003) reported ultrafiltration results on the inhibitor(s) from the common housefly to retain 83, 67 and 51% of the inhibitor activity on 10,000, 30,000 and 50,000 NMWL membranes. This would indicate a smaller inhibitor than the results from this study, although the crude mixture used in the cockroach and common housefly for ultrafiltration gives only an estimate of inhibitor size.

Treatment of Inhibitor(s) with Trypsin. Trypsin is a pancreatic serine protease and it is commonly used commercially and experimentally for hydrolyzing proteins. However, trypsin's high pH optimum of 8.0 is well beyond the optimal conditions for the inhibitor (pH 6.5) and PPO (pH 5.5–6.0), and thus, papain was also compared as its pH optimum is around 6.0–7.0. This was done to demonstrate that the inhibitor was a protein. Treatment of inhibitor(s) with trypsin was ineffective due to the instability of the inhibitor to changes in pH. By increasing the pH of the inhibitor above 6.5 and back down again, the inhibition was lost in both pH control and trypsin samples (Fig. 7 [protease treatment]). Therefore, it is 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.

Treatment of Inhibitor(s) with Papain. Papain is a sulf-hydryl protease from *Carica* papaya latex. Treatment of inhibitor(s) with papain proved effective in decreasing the inhibition in the papain sample (Fig. 7 [protease treatment]) but not the pH control 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 (Fig. 7 [protease treatment]). The PPO control had an average rate of 0.33 compared to an average of 0.31 for the protease treatment with papain. The pH control for papain had an average rate of 0.16 compared to an average rate of 0.15 for the inhibitor control. This shows that pH did not
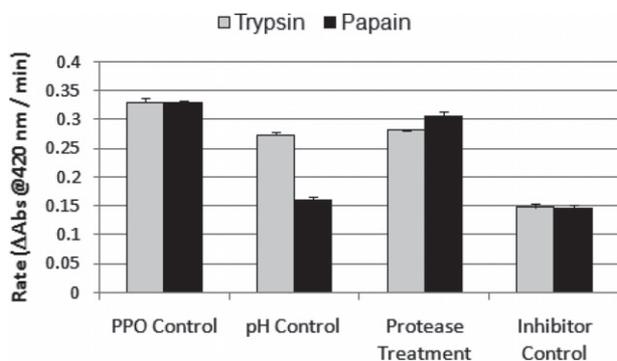
FIG. 7. TREATMENT OF INHIBITOR(S) WITH TRYPSIN AND PAPAIN
Treatments represent a polyphenol oxidase (PPO) control (no inhibitor or protease), pH control (inhibitor and buffers), protease treatment (inhibitor and trypsin or papain) and an inhibitor control (inhibitor), and assayed according to the *Materials and Methods* section. Each data point represents mean ± standard deviation of two experiments each with three replicates.

influence the rate of the reaction as the rates were similar for pH control and inhibitor control. When compared to the PPO control, inhibition was approximately 55% which was similar to the optimum for apple PPO of 60% found earlier. Also, the protease treatment with papain showed that inhibition was absent suggesting that the protease hydrolyzed the inhibitor(s). This along with the results from temperature stability and ultrafiltration suggests that the inhibitor(s) is a protein. It should be noted that electrophoresis or protein assay was not performed on these samples.
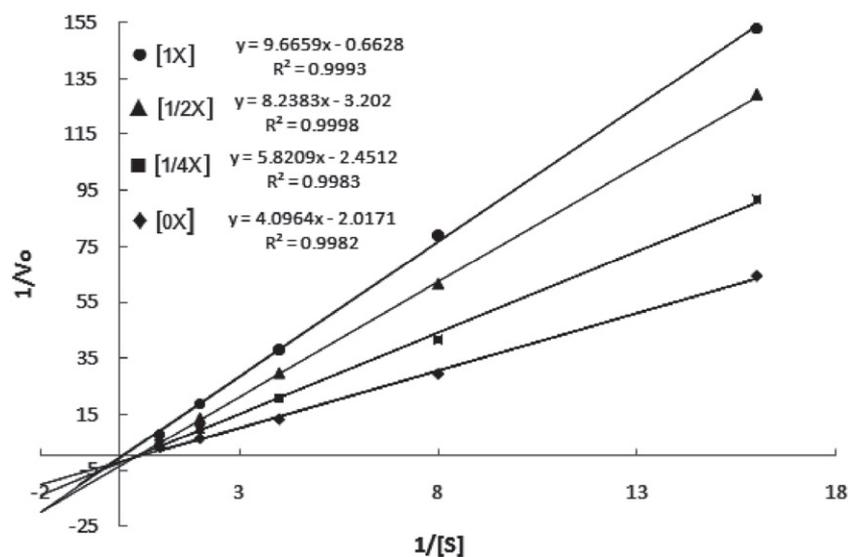
Inhibitor Kinetics. Inhibition by German cockroach extract on apple PPO was characterized using the Lineweaver–Burk method. Results indicated noncompetitive inhibition, as both the vertical intercept and slope terms of the equation are affected (Fig. 8). The lines intercept at a point left of the *y*-axis and below the *x*-axis. Noncompetitive 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 noncompetitive using a Lineweaver–Burk plot. In contrast, Son *et al.* (2000) found the inhibitory mode of oxalic acid on mushroom PPO to be of a competitive type. The crude nature of the inhibitor and partial purification of the PPOs make accurate interpretation of the type of inhibition difficult; although, the data do support inhibitor activity.

CONCLUSION

An inhibitor from cockroach was extracted and partially characterized against potato and apple PPO. The inhibitor showed greatest inhibition when extracted from cockroach at pH 6.5. The inhibitor worked optimally at pH 6 for potato PPO and pH 5.5 for apple PPO showing inhibition of 20% and 58%, respectively. The inhibitor caused greater inhibition when incubated with the substrate catechol compared to the enzyme. Dialysis and ultrafiltration studies suggest the inhibitor is quite large being retained on a 100,000 NMWL ultrafiltration membrane. This along with temperature stability and protease data suggests that the inhibitor is a protein and not a peptide. Because insects contain PPO for their growth and development, insects may present an opportunity to control PPO browning in fruits and vegetables by providing a source of unique PPO inhibitors.

FIG. 8. INHIBITOR KINETICS: PLOT OF SUBSTRATE-VELOCITY DATA ACCORDING TO LINEWEAVER–BURK METHOD
Inhibition by cockroach extract on apple polyphenol oxidase was determined in the presence of three different concentrations of inhibitor solution (1X, $\frac{1}{2}$X, $\frac{1}{4}$X) for five different fixed concentrations of catechol (1, 0.5, 0.25, 0.125 and 0.0625 M).

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