Apple Polyphenol Oxidase Inhibitor(s) from Common Housefly (Musca Domestica L.)

R. Yoruk, J.A. Hogsett, R.S. Rolle, and M.R. Marshall

ABSTRACT: A new natural apple polyphenol oxidase (PPO) inhibitor(s) from housefly (Musca domestica L.) was discovered. Crude inhibitor(s) isolated by buffer extraction, heat treatment, and dialysis from housefly pupae inhibited the activity of apple PPO up to 90% at pH values above 5.0. Inhibition was strictly pH-dependent. The inhibitor(s) was further characterized by employing heat, freezing and thawing, irradiation, pH adjustment, and ultrafiltration studies. The potential PPO inhibitor(s) was stable to heating at 100 °C for 1 h, repeated freezing and thawing, and irradiation. The inhibitor(s) was most stable at pH around 5.0 and least stable at alkaline pH. The PPO inhibition profile of housefly during metamorphosis also was evaluated.

Keywords: apple, PPO, housefly, pupae, natural inhibitor(s)

Introduction

Enzymatic browning, mainly associated with polyphenol oxidase (PPO; EC 1.10.3.1), causes millions of dollars in damage yearly to fruits, vegetables, and seafood (crustaceans) (Whitaker and Lee 1995; Kim and others 2000). PPO catalyzes the hydroxylation of monophenols (monophenolase activity) and the oxidation of o-diphenols into o-quinones (diphenolase activity) with oxygen as the primary oxidant (Whitaker 1994; Lerch 1985; Yoruk and Marshall 2003). The highly reactive quinones usually undergo further nonenzymatic condensation reactions leading to the generation of unpleasant dark brown melanins (Mathew and Papia 1971; Sapers 1993). Quality of many fruit and vegetable crops susceptible to this biochemical reaction diminishes rapidly due to unpleasant discoloration and off-flavor generation (Vamos-Vigayoz 1981; Zawistowski and others 1991; Yoruk and Marshall 2003).

In most cases, catalytic action of PPO is a limiting factor in the handling and technological processing of these crops because peeled, sliced, bruised, or diseased tissues rapidly undergo adverse enzymatic browning. Severe browning of plant products arises from stress conditions due to subcellular decompartmentalization and oxygen penetration leading to PPO-substrate contact (Vamos-Vigayoz 1981; Zawistowski and others 1991; Yoruk and Marshall 2003). Many proposed methods for controlling PPO are not applicable to foodstuffs because of problems related to food safety, regulations, and the cost associated with prevention (Mcevily and others 1992; Sapers 1993). One group of preservatives, sulfites and related substances, are very effective in preventing browning; however, they can cause harm to individuals who are asthmatic. Therefore, their use has been limited on fresh fruits and vegetables (Sapers 1993).

An active field of research is to find effective, natural, and inexpensive alternatives to prevent adverse browning in apple and apple products, a global fruit particularly prone to enzymatic browning. Housefly (Musca domestica L.), a ubiquitous insect, may be a cheap answer to this problem. In addition to its general occurrence in plants, PPO is found in insects (Brunet 1980; Sugumaran 1998). During insect development, PPO is believed to be responsible for various physiological roles. It is involved in sclerotization (hardening) and tanning of the insect cuticle (Sugumaran 1998) and in defense reactions, wound healing, and disease resistance (Gillespie and others 1997; Sugumaran and Nellaiappan 2000). It stands to reason that natural inhibitors within the housefly regulate this development, and previous literature has indicated endogenous inhibitors to housefly PPO (Tsukamoto and others 1992; Daquinag and others 1995, 1999). Additionally, Sugumaran and Nellaiappan (2000) have isolated a high-molecular weight (380000) glycoprotein inhibitor of PPO from the larval cuticle of the tobacco horn worm, Manduca sexta, and proposed that the presence of endogenous PPO inhibitor(s) would ensure the direct control of PPO activity in insects. The potent PPO inhibitors isolated from the pupae of housefly on housefly PPO 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; Daquinag and others 1995). There is little, if any, documentation on the potential applicability of these inhibitors to food systems.

The objectives of this study were to provide evidence for the existence of apple PPO inhibitors in common housefly and to demonstrate inhibition of apple PPO activity using inhibitor(s) isolated from housefly pupae by buffer extraction, heat treatment, and dialysis. PPO inhibition profile of the housefly during its growth and development was also evaluated.

Materials and Methods

Housefly pupae

Pupae of Musca domestica (L.) were provided by the U.S. Dept. of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology (Gainesville, Fla., U.S.A.). Larvae of the housefly were reared on the Gainesville Housefly Diet (Hogsett 1992). The diets seeded with housefly eggs were held at 26.7 °C and 60% relative humidity (RH). After 6 d, pupae were separated from the culture medium by flotation. They were further washed in distilled water, and dried on a paper towel. Care was taken to be sure that pupae samples were free of the culture medium.

Preparation of inhibitor extract

Pupae obtained 2 d after pupation (middle stage pupae) were powdered in a Waring blender after freezing in liquid nitrogen and stored at −20 °C until used for inhibitor extraction. The powder, homogenized in 4-fold (wt/vol) 0.1 M sodium acetate-acetic acid, pH 4.0 buffer, was filtered through 8 layers.
of cheesecloth and then incubated in a water bath at 70 °C for 30 min (Tsukamoto and others 1992). The resulting extract was centrifuged at 4 °C and 30000 × g for 30 min to remove coagulated materials. The supernatant, dialyzed in a 2000-Da molecular-weight cut-off (MWCO) dialysis membrane (Spectrum Laboratory Products, Inc., New Brunswick, N.J., U.S.A.) with constant stirring for 25 h against 3 changes of distilled water, was tested for PPO inhibition and properties of inhibitor(s). Dialysates were kept at 4 °C.

In the experiments designed to study the inhibitor activity of housefly during growth and development, samples from the following stages were collected: 3rd-instar larvae, 5 different pupal stages obtained through pupation, and adults. Housefly samples were stored at −20 °C until needed. They were directly used for extraction. Crude extracts prepared from samples (10 g) at each stage of the development by buffer extraction and heat treatment, as described above, were tested for PPO inhibition activity. The experiments were repeated twice each with 3 replicates.

Preparation of apple PPO

Apple PPO was extracted from apple acetone powder. Acetone powder was prepared from Red Delicious apples obtained from a local grocery store (Yemenicoglu and others 1997). Small pieces of peeled apple pulp (200 g) were homogenized in a prechilled Waring blender with acetone (−20 °C, 400 mL) for 1 min, and then filtered through a Buchner funnel using Whatman No 1 filter paper. The residue was reextracted 3 times with 200 mL of cold acetone. The resulting white powder was vacuum-dried at room temperature (22 °C) and stored in commercial vacuum bags at −20 °C until used for PPO extraction.

The modified procedures of Murata and others (1995) and Yemenicoglu and others (1997) were used for PPO extraction. The acetone powder (1 g) was incubated in 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, Calif., U.S.A.) for 20 min while stirring with a magnetic stirrer at 4 °C. The suspension was centrifuged for 30 min at 12000 × g, and the resulting supernatant, filtered through glass wool, was tested for PPO activity and properties of inhibitor(s). PPO activity of apple acetone extract kept in the freezer (−20 °C) remained stable for 1 wk with no visible intrinsic photodegradation.

Assay of PPO activity and inhibition

PPO inhibition was quantified by measuring PPO activity of apple with and without added inhibitor extract. For a standard assay, 0.2 mL of test extract or control buffer was mixed with 2.45 mL of 0.1 M sodium acetate-acetic buffer, pH 5.5 buffer as the main buffer, 0.3 mL of 0.5 M catechol (Sigma Chemical Co., St. Louis, Mo., U.S.A.) 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. The pH of the reaction mixture was checked after each assay to confirm similar pH values for control and test systems.

PPO activity of apple also was assayed by mixing it with an inhibitor extract in a standard reaction mixture where the pH of the main buffer (0.1 M 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 on the reaction was expressed as percent inhibition (%), calculated using the formula (100[(A - B)/A]), where A and B were enzyme activities in the control and test systems, respectively. Specific activity of inhibition for housefly stages was expressed as unit (U) inhibition per mg protein, where 1 U of inhibitor activity was defined as 10% inhibition of apple PPO.

The pH optimum of apple 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 0.1 mL of the enzyme solution, 0.3 mL of 0.5 M catechol and 2.6 mL of various buffer solutions. The reaction was initiated with the addition of the substrate prepared in distilled water. PPO activity was estimated as described above.

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 15, 30, and 60 min. The precipitate was separated by centrifugation for 5 min at 10000 × g and room temperature (22 °C). The clear supernatants were collected in clean test tubes and used for PPO inhibitory activity determination at 25 °C as described above. Repetted freezing and thawing (5 times) on crude inhibitor preparation also was determined. In the last freezing step, the samples were thawed and assayed after storing them overnight at −20 °C.

The pH stability of inhibitor(s)

A crude inhibitor extract (prior to dialysis) adjusted to pH values from 5.0 to 11.0 with HCl or NaOH was incubated for 5 h at the adjusted pH at refrigeration temperature (4 °C). Volume differences between samples were adjusted with distilled water and inhibitor samples were dialyzed using a 2000-Da MWCO membrane for 30 h against 3 changes of distilled water. Dialysates were centrifuged at room temperature for 5 min. Supernatants were tested for PPO inhibition as described above.

Irradiation

Because housefly pupae are routinely irradiated for preservation purposes, the pupae were exposed to 50 kR of γ-irradiation in a cesium-137 irradiator at the USDA laboratory in Gainesville, Fla., according to Morgan and others (1966). The irradiated pupae, subjected to buffer extraction and heat treatment as described above, were used as crude inhibitor extract. PPO activity of apple mixed with inhibitor extract in a standard reaction mixture was measured spectrophotometrically as described above.

Ultrafiltration

Ultrafree-4 centrifugal filter units (Spectrum Laboratory Products, Inc.) with the nominal molecular weight limit (NMWL) membranes of 10000, 30000, 50000, and 100000 were used. After rinsing with distilled water to remove a trace amount of glycerin, aliquots of 2 mL of the inhibitor sample were placed onto the filter units and concentrated by centrifugation at 4 °C and 7500 × g for 35 min. Filtrates were collected and kept at 4 °C. The sample was reconstituted back to 2 mL by adding distilled water and centrifuged again. Two wash cycles were performed. Concentrated inhibitor sample on the membrane surface (retentate) was reconstituted back to 2 mL by adding distilled water and assayed for PPO inhibitory activity as described above. Control inhibitor samples in centrifuge tubes also were subject to centrifugation with test samples together.

Protein determination

Protein was determined according to the method of Bradford using bovine serum albumin (Sigma Chemical Co.) as a standard (Bradford 1976).

Results and Discussion

Optimization of pH conditions for PPO activity and inhibition

The pH activity profile for oxidation of catechol by apple PPO is shown in Figure 1. PPO showed maximum activity at pH around 5.5. About 68% activity, relative to that determined at pH 5.5, was detected at pH 5.0, but there was barely any activity at or above pH 6.0. The acidic pH optimum for
apple PPO activity by 80%. Inhibition was rapid, occurring in the reaction mixture, and did not require incubation. Further studies of PPO inhibitor(s) assay conditions revealed the importance of reaction pH on inhibition activity by the pupal extract (Figure 3). Inhibition by the fly extract on apple PPO markedly differed as a function of pH. There was barely any inhibition at or below pH 5.0. Above pH 5.0, inhibition showed an increasing trend with increasing pH values tested. Greatest inhibition (90%) of PPO was observed at pH 5.7. However, this increased inhibition may occur as a result of the decline in PPO activity without inhibitor(s) as a function of pH (Figures 1 and 3). It should also be noted that under the optimal pH conditions (pH 5.5) for PPO activity, the housefly inhibitor(s) demonstrated only a 10% reduction in inhibition when compared with pH 5.7.

Tsukamoto and others (1992) isolated inhibitors from the pupal extracts of houseflies and demonstrated inhibition on housefly PPO. The presence of endogenous inhibitor in the larval cuticle of another insect, the tobacco horn worm (Manduca sexta), was reported by Sugumaran and Nellaiappan (2000). The specific endogenous PPO inhibitor of Manduca sexta inhibited the activity of not only insect PPOs but also plant PPOs and laccase. In addition to several other regulatory biochemical mechanisms of endogenous PPO activity in insects (Sugumaran and Nellaiappan 2000), it is believed that PPO activity could also be regulated by intrinsic inhibitors during development (Tsukamoto and others 1992; Daquinag and others 1995, 1999; Sugumaran and Nellaiappa 2000).

Inhibitory activity of the housefly during metamorphosis

Figure 4 illustrates the developmental stages of housefly (Musca domestica L.). The housefly life cycle varies greatly in length depending on environmental factors, such as temperature, moisture level, and the pH of the larval medium (West 1951). The following times were observed through the developmental stages of the laboratory housefly: eggs hatched in 24 h, and the periods for the 3 larval stages or instars and the pupal stage averaged 3 and 5 d, respectively.

PPO inhibition activity in houseflies during its growth and development is shown in Figure 5. The percent inhibition per 0.2 mL of inhibitor extracts obtained from larvae through adult stages was not much different, especially at pupal stages. The late pupal (P5), larval, and adult stages showed slightly lower percent inhibition. However,
specific inhibitor(s) activity (U inhibition/mg protein) showed an influence in inhibition throughout the developmental stages, where highest inhibition was observed in the larvae stage. Based on specific activity of inhibitor(s), apple PPO inhibition was relatively lower with the inhibitor(s) in early pupae stages (P1 and P2) than middle-stage and late-stage pupae (Figure 5). These are contradictory to the findings of Tsukamoto and others (1992) who observed significant variation in the inhibitor activity of the developmental stages (per gram body weight) on housefly PPO, and housefly PPO inhibition was prominent with the inhibitors in aged pupae and little with those in larvae and adults.

In this investigation, none of the housefly extracts exhibited PPO activity under the standard assay conditions containing catechol as substrate. This observation is consistent with the results that Tsukamoto and others (1992) obtained for the inhibitors on housefly PPO with Dopa as substrate.

Characterization of crude inhibitor(s) from housefly pupae

Effect of inhibitor concentration. Crude inhibitor(s) preparation was initially analyzed for its concentration dependency on PPO activity. Serial dilution of the inhibitor preparation demonstrated a decrease in percent inhibition with increasing dilution factor. The overall dilution profile gave an almost logarithmic relationship between inhibitor(s) concentration and activity. However, a linear relationship \( (r = 0.99) \) was observed upon dilution of the inhibitor between 1/2 and 1/16. About 40% of the inhibitor activity was observed upon dilution by 1/4. Inhibitor activity calculated on the basis of the protein amount found in the standard reaction mixture is shown in Figure 6. In this case, about 40% of the activity was observed at 13 \( \mu \)g protein in the 3-mL standard reaction mixture. Higher protein concentration \( (\geq 25 \mu g) \) in the standard reaction mixture did not cause a substantial increase in inhibition, indicating maximum inhibition was achieved.

Temperature stability. The effect of heat treatment on the activity of crude inhibitor(s) is shown in Table 1. The unknown inhibitor(s) was relatively stable to heat, losing about 26% of its inhibitory activity on apple PPO upon boiling at 100 °C up to 1 h. When relative inhibition was calculated based on specific activity of inhibitor(s) (U inhibition/mg protein), inhibitor(s) retained about 88% of original activity after 1 h of heating. In this respect, inhibitor(s) was similar to those isolated by Tsukamoto and others (1992) who noted that the inhibitors in housefly pupae for housefly PPO are quite stable to heating at 80 °C for 1 h as they retained greater than 60% of their activity. However, the inhibitor isolated from tobacco horn worm was a heat-labile glycoprotein, totally inactivated by heating at 100 °C for 10 min (Sugumaran and Nellaippan 2000).

Further investigations of the inhibitor(s) from housefly pupae demonstrated that

<table>
<thead>
<tr>
<th>Table 1—Heating effect on inhibitor activity</th>
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<tbody>
<tr>
<td>Heating period (min at 100 °C)</td>
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<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>15</td>
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<tr>
<td>30</td>
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<td>60</td>
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*One unit (U) of inhibitor activity was defined as 10% inhibition of apple PPO.

Figure 4—Life cycle of housefly. Housefly development through the stages of egg, larva, pupa, and adult. The larva molts twice (1st-instar larva not shown) during development.

Figure 5—Inhibitory effect of housefly on apple PPO during the developmental stages. Crude extracts prepared from 3rd instar larvae (L3), young pupae just formed (P1), pupae samples obtained on day 2 (P2), 3 (P3), 4 (P4), and 5 (P5) after pupation, and adults (A) were tested for inhibitory effect on apple PPO in a standard reaction mixture. % inhibition (---); specific inhibitor activity (-----). One unit (U) of inhibitor activity was defined as 10% inhibition of apple PPO. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.
Table 2—Ultrafiltration

<table>
<thead>
<tr>
<th>NMWL</th>
<th>Retentate</th>
<th>Filtrate</th>
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<tbody>
<tr>
<td>2000</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3000</td>
<td>83.2 ± 3.1</td>
<td>19.8 ± 4.2</td>
</tr>
<tr>
<td>5000</td>
<td>66.5 ± 4.0</td>
<td>35.2 ± 5.6</td>
</tr>
<tr>
<td>10000</td>
<td>50.9 ± 6.9</td>
<td>53.7 ± 7.6</td>
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<tr>
<td></td>
<td>9.7 ± 8.0</td>
<td>86.5 ± 11.9</td>
</tr>
</tbody>
</table>

Ultrafiltration was performed using centrifugal filter units with specified nominal molecular weight limit (NMWL) membranes.

Dialyzed inhibitor preparation was used as starting material. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates.

the inhibitor(s) also was stable after repeated freezing and thawing, losing only about 8% of its inhibitor activity. This result also indicated that inhibitor(s) are quite stable to freezing temperatures. Inhibitor activity of pupal extract kept at a refrigerated temperature (4 °C) remained stable over a period of a week.

The pH stability. Inhibitor(s) exposed to a range of pH values from 3.0 to 11.0 for 5 h was compared for its inhibitory activity on apple PPO. The pH stability curve is shown in Figure 7. pH had a dramatic effect on the inhibitor(s). It was most stable at acidic pH values, specifically pH 5.0, and least stable at alkaline pH values. The pH stability of the inhibitor(s) increased from 3.0 to 5.0 and showed a significant decreasing trend above pH 5.0. The observed inhibition upon exposure to pH 7.0, 9.0, and 11.0 were about 57%, 36%, and 38% when compared with those at pH 5.0, respectively (Figure 7). However, exposure to pH 3.0 caused only 14% loss in inhibition. Sugumar and Nellaiappan (2000) also reported the dramatic effect of pH on the inhibitor from tobacco hornworm. However, in their case, the inhibitor was most stable at neutral pH values, losing its total activity upon exposure to pH 10 for 10 min. The findings in our investigation were contradictory to those reported by Tsukamoto and others (1992) who observed that the inhibitor(s) in housefly pupae are quite stable over a wide range of pH (4.0 to 10.0). However, the extent of stability obtained for these particular studies was not presented in the publication.

γ irradiation. Housefly pupae exposed to γ irradiation based on a technique using 50 kr γ radiation (Morgan and others 1986) were tested for PPO inhibitory activity to determine whether irradiation influenced inhibition. Based on the results obtained from the investigation, exposing the pupae to 50 kr γ irradiation did not cause any change in PPO inhibition effect of the pupal extract. This irradiation technique developed by Morgan and others (1986) was routinely used at the mass-rearing laboratory of the USDA in Gainesville, Fla., to supplement the daily production of housefly pupae for maintenance of the parasitoid colonies. The irradiation radiation was shown to prolong the 2- and 3-day-old housefly pupal period from only 24 h to up to 8 wk of storage at around 44 °C (Morgan and others 1986). Studies on Caribbean fruit fly larvae exposed to γ rays revealed reductions in brain growth (Nation and others 1995). Additionally, radiation treatment caused a stress condition in mango fruit, leading to a gross distortion of normal biochemical patterns during ripening and senescence (Frylinck and others 1987).

Ultrafiltration. Inhibitor preparation was further evaluated by ultrafiltration studies, and the results are shown in Table 2. Because the inhibitor preparation used in these studies was subjected to dialysis in a 2000-NMWL dialysis membrane after heat treatment as described in Materials and Methods, relative inhibitor activity in the filtrated samples were compared with that in the dialysate (Table 2). Ultrafiltration studies were carried out to determine the approximate molecular weight of unknown inhibitor(s) required for future dialysis information during purification and to determine whether it was similar to the peptides referred to by Tsukamoto and others (1992).

The overall results showed that there was a decreasing trend in inhibitor activity measured in the retentate with increasing NMWL of the membranes (Table 2). In all cases, the inhibitor activity lost in the retentate was recovered in the filtrate. Ultrafiltration units with 10000, 30000, and 50000 NMWL membranes would retain about 83%, 67%, and 51% of the inhibitor activity, respectively. However, only 10% inhibition was retained for the 100000 NMWL membranes. It seems that the unknown inhibitor(s) could be larger than 3000 to 3500 found by Tsukamoto and others (1992) based on Sephade G-25 gel filtration information. The primary structure of one of the inhibitors was determined and found to have a cysteine motif commonly found in snail and spider toxins (Daqunag and others 1995, 1999). However, the endogenous glycoprotein inhibitor from tobacco hornworm, Manduca sexta, was found to have a molecular weight of 380000 on SDS-PAGE gels (Sugumar and Nellaiappan 2000). In our investigation, considering the system is a crude mixture, ultrafiltration gave only an estimate of the inhibitor(s) size in housefly pupae and may reflect multiple inhibitors in the extract. Purification studies in the future will address this issue.

Conclusions

It is evident that housefly pupae contain inhibitor(s) of apple PPO. Successful identification of the unknown inhibitor(s) may be of value to the apple industry. In light of the fact that these results represent the 1st attempt at identifying inhibitor(s) of housefly on PPO from higher plants, further studies will be needed to determine how this inhibitor(s) works on other plant PPOs, and the mechanism by which it works on PPOs.
With additional characterization and study, the inhibitor(s) may have the potential to become a low-cost alternative to the limited number of methods currently available for controlling enzymatic browning in other fruits and vegetables. Purification and characterization of the inhibitor(s) is ongoing.

References


