High Performance Liquid Chromatography Assay for p-Nitroanisole o-Demethylation by Cytochrome P450 Enzymes in Musca domestica L.

AI Guo-Min, WANG Qing-Min, ZOU Dong-Yun, GAO Xi-Wu*, LI Fu-Gen
Department of Entomology, College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China

Abstract: A novel sensitive method was developed for measuring p-nitroanisole o-demethylation (PNOD) catalyzed by cytochrome P450 enzymes in crude homogenates of housefly, Musca domestica L., using high performance liquid chromatography (HPLC) with diode array detector. This method is based on the use of liquid-liquid extraction and HPLC with reversed-phase C18 column and gradient ultraviolet detection at 316 nm. The detection limit (S/N of 3/1) of p-nitrophenol (pNP) was 0.1 ng. The relative standard deviation (RSD) of peak area response of the HPLC analysis system was 1.0%. The average recoveries of 0.351, 1.755 and 8.755 μg of pNP added to incubation mixtures ranged from 92.3% to 87.6%, and the RSD of recoveries of three added levels ranged 2.64%–5.90%. The relationships between peak areas and concentration were linear in the range of 9.72–486 ng with good coefficient of determination (r² = 0.9995). This method is sensitive, precise, and reproducible and is also applicable to the assay of p-nitroanisole o-demethylases, which have low catalytic activities, present in other insects or organisms. With this method, the comparison of P450 o-demethylation activity of an imidacloprid-resistant strain with a susceptible strain of M. domestica (L.) was conducted to identify the roles of P450 enzymes involved in housefly resistance to imidacloprid. The results indicated that the resistance mechanism of housefly to imidacloprid was intensely assisted with elevated P450 activities.

Key Words: High performance liquid chromatography; o-Demethylation; Cytochrome P450; Musca domestica; Imidacloprid

1 Introduction

Cytochrome P450s (P450s or CYPs), namely mixed-function oxidases, exist in virtually all living organisms, from bacteria to protists, plants, fungi and animals[1]. Many P450s are involved in biosynthesis and biodegradation of endogenous substrates (steroid hormones, lipids, and so on), but much of their notoriety has been associated with the metabolism or detoxification of xenobiotic (pesticides, drugs, organic solvents, and so on)[2]. In insects, they play a significant role in the metabolism of the foreign compounds, resulting in many cases of insecticide resistance and botanical poison tolerance[3]. There are many different P450 enzymes, of which the catalytic competence is broad or narrow, overlapping with other P450 enzymes or not. Therefore, there are a large number of assays for P450 activity, and the activity based on the specific substrate is the sum of the contributions of all P450 enzymes that catalyze the metabolism of the substrate[2]. In the investigation of entomology toxicology, P450 activities associated with o-dealkylation, alkyl (aryl) hydroxylation and aldrin epoxidation are usually included to identify the roles of P450 enzymes involved in insect resistance to insecticides[3]. P450 activity of o-dealkylation is generally determined by measuring o-demethylation and/or o-deethylation activity. There are a number of different available assay systems for characterizing o-dealkylation by P450s. For assay of o-demethylation, p-nitroanisole (pNA) is used as substrate for colorimetric[4] or gas chromatography-electron-capture detector (GC-ECD) assay[5], and 7-methoxyresorufin[6] and 7-methoxycoumarin[7] are also used for fluorescent assay. For
2.1 Instruments and reagents

China). Phenylmethylsulfonyl fluoride (PMSF, 99.0% purity), Spectrometer (PGENERAL Instruments Co., Ltd, Beijing, Wilmington, DE). The protein concentration were conducted using ChemStation software (Agilent Technologies, MA, USA). Tris base and DL-dithiothreitol (DTT) were purchased from Promega Corporation (Madison, WI). Ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were purchased from Beijing Tongzheng Biological Company (Beijing, China). Analytical grade pNA, ethyl acetate, and n-hexane were purchased from Beijing Chemical Reagents Company (Beijing, China).

2.2 Chromatographic conditions

The HPLC separation was carried out using Agilent ZORBAX Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., 5 μm) with a guard column containing the same packing material (Agilent, USA). The mobile phases were solvents A (acetonitrile) and B (H2O, adjusted to pH 3.0 with acetic acid) with a flow rate of 1.0 ml min⁻¹. The gradient elution program (linear increase) was 0 min (22% A, 78% B), 10 min (50% A, 50% B), 14 min (50% A, 50% B), 17 min (100% A, 0% B), 20 min (100% A, 0% B), and from 23 min to 28 min (22% A, 78% B). The isocratic elution program was 33% A and 67% B (V/V). The temperature of column was set at 25 °C. The detection wavelength of DAD was 316 nm, and the injection volume was 20 μl. Under gradient elution, pNA substrate and metabolite pNP eluted at approximately 11.74 and 7.26 min, respectively.

2.3 Calibration procedures

A total of 0.0234 g of pNP was dissolved in 10-ml methanol as the standard stock solution. A series of standard working solutions were prepared by further dilution of the standard stock solution and were injected and run for calibration curve. The calibration curve was plotted subsequently for linear regression analysis of the peak area with the quantities (ng) of the injected analytes. The standard curve was used to determine recovery of pNP.

2.4 Insects

Housefly population, Musca domestica (L.), was collected around the Wrestling Museum in China Agricultural University East Campus (Beijing, China) in June 2007 and was maintained in the laboratory without being exposed to insecticides for 12 generations until selected by imidacloprid. An imidacloprid-resistant strain (CRS) was obtained by consecutive selection for 11 generations with increasing concentration of imidacloprid in the diet selecting second instar, and 85-fold resistance to imidacloprid was developed. The susceptible strain (CSS) was reared in the laboratory for many years without being exposed to insecticides. The insects were reared in the conditioned room maintained at (25 ± 1) °C, 60%–70% relative humidity, with a 14:10 (L:D) photoperiod, and supplied with water, sugar, and milk powder.

2.5 Enzyme preparation

Crude homogenates of the CRS and CSS were prepared.
using the method followed by Lee and Scott\textsuperscript{[15]} with some modification. Only abdomens of 4-day-old houseflies from each strain were homogenized in ice-cold homogenization buffer (0.1 M phosphate buffer, pH 7.5, containing 1.0 mM EDTA, 0.1 mM DTT, 1.0 mM PMSF, 1.0 mM PTU, and 15% (V/V) glycerol). The homogenate was centrifuged at 4 °C, 10000 g for 20 min. The supernatant was filtered through glass wool and collected to a clean ice-cold eppendorf tube and was used immediately for P450-dependent PNOD activity. The separate preparation of the enzyme sample from each strain was carried out thrice.

The determination of protein concentration was carried out by an improved method\textsuperscript{[16]} described previously using BSA as the standard.

### 2.6 pNA o-demethylation reaction

The PNOD activity was measured as described previously\textsuperscript{[4,15]} with some modifications. The o-demethylation reaction of pNA was carried out in a volume of 2 ml at 30 °C for 1 h in a water bath with occasional shaking. The incubation mixture consisted of 0.1 M Tris-HCl buffer (pH 8.2), 315 μM pNA, 7.5 mM MgCl\textsubscript{2}, 0.75 mg ml\textsuperscript{-1} BSA, and 1 mM NADPH. The reaction mixture was preincubated for 5 min, and then, the reactions were initiated by adding 0.5 ml of enzyme sample. After 1-h incubation, the metabolic reactions were terminated by adding 2 ml of ice-cold ethyl acetate:n-hexane (2:1, V/V) containing 1% phosphoric acid. Another total volume of 2 ml of ice-cold ethyl acetate:n-hexane (2:1, V/V) was added to extract the remaining metabolites twice. The organic fraction of three extracts was combined together and evaporated to dryness under a gentle nitrogen stream. The residue was redissolved in 200-μl methanol and placed on ice, and a 20 μl filtered solution was injected for HPLC analysis. The metabolic reactions at each substrate were performed in triplicate. Additionally, the control incubations (without enzyme samples) and the blank incubations (without substrates) were prepared to differentiate between metabolites originating from the enzyme samples and possible metabolites from the incubation procedure. The formation of pNP was measured according to the calibration curve, and the enzymatic activity data are presented as means (± S.E.) of three replicates expressed as pmol pNP/mg protein/min.

### 2.7 Application to compare PNOD activity by P450s between CRS and CSS

Using this developed HPLC assay for determination of PNOD by P450s, comparison of PNOD activity of an imidacloprid-resistant strain with a susceptible strain of *M. domestica* (L.) was conducted to identify the roles of P450 enzymes involved in housefly resistance to imidacloprid.
solvent acetonitrile after 6-min isocratic elution.

3.1.3 Limit of detection and calibration curve

A signal three times higher than the noise \((S/N \geq 3)\) was regarded as the detection limit, repeated thrice for confirmation. Accordingly the detection limits of \(p\)NP under gradient elution and isocratic elution were 0.1 and 0.04 ng, respectively. In general, organic compounds have higher sensitivity under gradient elution than under isocratic elution. This inconsistency might be interpreted from two aspects. First, a better baseline and shorter elution time were obtained under isocratic elution than under gradient elution. Second, because \(p\)NP is relative high polar and has low capacity factor in HPLC analysis, the polarity changes of mobile phases have no significant impact on its sensitivity. By considering the metabolite concentration levels of the metabolic reactions and the standard addition for the recovery studies, the calibration curves were developed in the range of 9.7–486 ng, although the quantification limit of \(p\)NP was 0.486 ng under gradient elution program. The regression equation was \(Y = 0.2640X - 3.1391\) with correlation coefficient of determination \((r^2)\) of 0.9995.

3.1.4 Precision

Six replicates of standard solution were analyzed using HPLC system for the precision. The relative standard deviation (RSD) of peak area response was 1.0%, showing the satisfactory reproducibility of the HPLC method.

3.1.5 Accuracy

The accuracy was evaluated by the recovery at the added levels of 0.351, 1.755 and 8.755 \(\mu\)g (five samples for each level). The metabolite \(p\)NP has relative high polarity and requires neutralization prior to extraction, and thus, polar organic solvent with acidic modifier is appropriate for extracting polar \(p\)NP. Because polar solvents such as ethyl acetate are dissolvable in water to some extent, the addition of nonpolar organic solvent is necessary. In this experiment, a mixed organic solvent of ethyl acetate and n-hexane \((2/1, V/V)\) with 1% phosphoric acid (85% purity) was used to stop the metabolic reactions and extract \(p\)NP. Average recoveries of 0.351, 1.755 and 8.755 \(\mu\)g of \(p\)NP added to incubation mixtures ranged from 92.3% to 87.6%. The RSD of recovery of three added levels was in the range of 2.64%–5.90% (Table 1). The results are considered satisfactory for trace analyses.

3.2 Comparison of PNOD activity by P450s between CRS and CSS

The PNOD catalyzed by P450 enzymes in the CRS and CSS was conducted simultaneously. The PNOD activity in the CRS was 3.34-fold of that in the CSS (Table 2), implicating that the resistance mechanism of \(M. domestica\) to imidacloprid was intensely assisted with elevated P450 activities. In 2004, imidacloprid was registered for housefly control in the United States[17]. The preapplication of piperonyl butoxide (PBO, inhibitor for P450s) suppressed the metabolism of imidacloprid in housefly[18], suggesting that P450s played a significant role in imidacloprid metabolism. In our research, P450s contributed greatly to the housefly resistance to imidacloprid, which can be confirmed by exploring the metabolic differences toward imidacloprid between the CRS and CSS.

4 Conclusions

Modern separation and analytical techniques, especially HPLC or HPLC-mass spectrometry techniques, have been popular and useful in the characterization of insecticides and model substrate metabolism in animals. However, in insects, just a few literatures concerning chromatographic assay with UV and/or mass spectrometric detection for characterizing model substrate metabolism are available, and the research on detoxifying enzyme activities related to direct insecticide metabolism based on chromatographic assay of metabolites is few. It can be predicted that more and more chromatographic assay with UV and/or mass spectrometric detection will be used in the investigation of entomology toxicology, characterizing detoxifying enzyme activities related to insecticides and model substrate metabolism to elucidate the metabolic resistance mechanisms of insect resistant to insecticides. The developed and validated HPLC assay in this study for measuring PNOD activity by P450s has three outstanding characterizations. First, the HPLC assay method is more sensitive than colorimetric assay and is also available for the determination of enzymes that have low catalytic activities, present in other organisms. Second, the HPLC assay method is more accurate than colorimetric and fluorescent assay because the possible interferences from by-products or enzyme matrices can be avoided via chromatographic separation. At last, only crude homogenates is needed and used as enzyme

### Table 1 Recovery of the product \(p\)NP \((n = 5)\)

<table>
<thead>
<tr>
<th>Amount added ((\mu)g)</th>
<th>Average recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.351</td>
<td>92.3</td>
<td>5.9</td>
</tr>
<tr>
<td>1.755</td>
<td>90.4</td>
<td>2.6</td>
</tr>
<tr>
<td>8.755</td>
<td>87.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

### Table 2 PNOD activity of CRS and CSS of \(Musca domestica\) (L.)

<table>
<thead>
<tr>
<th>Housefly strains</th>
<th>PNOD activity ((pmol\ pNP/mg\ protein/min))</th>
<th>Resistance ratio ((R/S))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRS</td>
<td>459.0 ± 2.6</td>
<td>3.34</td>
</tr>
<tr>
<td>CSS</td>
<td>137.5 ± 11.1</td>
<td></td>
</tr>
</tbody>
</table>
sample, and the classical preparation of microsomal fractions from insect has been omitted.

References
