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Continuous Spectrophotometric Assay for Plant Pectin Methyl Esterase

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A simple continuous assay for plant pectin methyl esterase (EC 3.1.1.11) has been developed. During the enzymatic reaction, the galacturonic acid methyl esters in pectin are hydrolyzed, producing acid. In the assay, the pH indicator bromothymol blue is protonated to produce a change in the absorbance at 620 nm. The absorbance change is spectrophotometrically monitored. The assay is calibrated with galacturonic acid. The sensitivity of the assay is 0.020 μmol of acid produced/min, and the precision is 12% (relative standard deviation). The assay requires only 2–3 min and 2 mL of substrate/trial. The measured activities are directly proportional to the amount of commercial pectin methyl esterase added, and the activity obeys saturation kinetics. In addition, the assay can be used to determine pectin methyl esterase in crude extracts of plant tissues. There is no interference from colored components or other activities in the extracts.

INTRODUCTION

Pectin degradation plays an important role in plant disease (Cooper, 1983), fruit ripening (Hobson et al., 1984), nutrition (Oppermann et al., 1973), and food product stability (McLellan et al., 1985). For example, ripening fruit softens because pectin and other cell wall carbohydrates are broken down enzymatically. Pectin, which is composed of α-1,4-linked galacturonic acid and galacturonic acid methyl ester, is degraded by a group of pectinases (Rexova-Benkova and Markovic, 1976). The galacturonic acid methyl esters are hydrolyzed by pectin methyl esterase (PME) (EC 3.1.1.11) (reaction 1).

$$\text{pectin–COOCH}_3 + \text{H}_2\text{O} \rightarrow \text{pectin–COO}^- + \text{H}^+ + \text{CH}_3\text{OH} \quad (1)$$

The existing assays for pectin methyl esterase are inconvenient or insensitive. Several methods have been described for determining the products of pectin hydrolysis. For example, the methanol produced (reaction 1) can be determined chromatographically (McFeeters and Armstrong, 1984) or colorimetrically (Wood and Siddiqui, 1971). The acid produced (reaction 1) can be determined by titration with a pH stat or a pH meter (Kertesz, 1937; Lee and Macmillan, 1968). The chromatographic method for methanol determination is very sensitive but is not convenient for routine enzyme determination. The colorimetric method for methanol and the titration methods require large volumes of reactants and are time consuming. In an alternative assay, hydrolysis of *p*-nitrophenyl acetate by the esterase is measured spectrophotometrically (Huggins and Lapides, 1947). This method is not useful for determining PME in crude plant extracts because it is neither specific nor sensitive.

We have developed a new continuous assay for PME that is convenient, sensitive, and specific. The new assay, like several qualitative assays previously described for PME (Rexova-Benkova and Markovic, 1976; Zimmerman, 1978), is based on the color change of a pH indicator during the PME-catalyzed reaction. As the ester bonds are hydrolyzed, acid groups are produced (reaction 1) and the

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the pH is lowered, causing the indicator dye to change color. The color change is continuously monitored spectrophotometrically, and the initial rate of the reaction is determined. The method is specific for pectin-degrading esterases because the natural substrate of PME is used. The new assay has been characterized with partially purified plant PME and has been used to determine PME in crude extracts of several plant tissues.

MATERIALS AND METHODS

Continuous Spectrophotometric Assay. The assay must always be started at the same pH to ensure reproducible color changes. However, buffers interfere with the measurement of acid production, so the reagents must be prepared as unbuffered or very weakly buffered solutions. To achieve a constant starting pH for the reaction, all solutions (pectin, indicator dye, water) were adjusted to pH 7.50 with concentrated (2 N) NaOH just before each trial was started. This adjustment must be made whenever the assay is run or calibrated.

A 0.5% (w/v) solution of citrus pectin (Sigma Chemical Co., St. Louis, MO) was prepared in distilled water by heating the mixture to 40 °C while continuously stirring. This solution could be used for 1 month when stored at 4 °C. A 0.01% (w/v) solution of bromothymol blue was prepared in 0.003 M, pH 7.5, potassium phosphate buffer.

Solutions of commercial pectin methyl esterase from orange peel (Sigma) were prepared in distilled water that had been previously adjusted to pH 7.5 with concentrated NaOH. This preparation of PME contained 50% protein and 50% salt as $(NH_4)_2SO_4$ and NaCl. The presence of these salts did not affect the activity of the enzyme, as was shown by comparing the activity of the enzyme before and after dialysis against distilled water.

The reaction was monitored at 620 nm in a Cary 14 recording spectrophotometer. The temperature was maintained at 25.0 °C with a circulating water bath. In a cuvette 2.00 mL of pectin was mixed with 0.15 mL of bromothymol blue and 0.83 mL of water, and the initial absorbance at 620 nm (A_{620}) of the mixture was determined vs. water. The initial A_{620}, approximately 0.28, remained constant until the enzyme was added, indicating that nonenzymatic hydrolysis was not occurring. The reaction was started by adding 20 μL of enzyme solution containing 1–20 μg of commercial PME, and the rate of decrease in A_{620} (ΔA_{620}/min) was recorded. The initial rate of the reaction was linear for about 1 min after the enzyme was added (Figure 1). For determination of kinetic constants, the volume of pectin was varied and water was added to maintain a total reaction volume of 3.00 mL.

The spectrophotometric assay was calibrated with a strong acid, HCl, and two weak acids, acetic acid and galacturonic acid (Sigma). In a cuvette 2.00 mL of pectin was mixed with 0.15 mL of bromothymol blue, between 0.10 and 0.85 mL of acid, and water to bring the final volume to 3.00 mL. The difference between the A_{620} of this mixture and the A_{620} of a mixture without acid was recorded as ΔA_{620}.

Other PME Assays. The *p*-nitrophenyl acetate assay for esterases (Huggins and Lapides, 1947) was used to determine the activity of commercial PME as a function of pH between pH 5.8 and 7.8. Substrate solutions containing 0.5 mM *p*-nitrophenyl acetate were prepared in 0.1 M phosphate buffers at each of the pH values to be tested. In each trial 100 μL of water containing 50 μg of commercial PME was incubated with 2.00 mL of substrate at the desired pH for 20 min at 30 °C. Following this incubation 7.00 mL of 0.1 M pH 7.0 phosphate buffer was added, bringing the reaction mixture to pH 7.0 ± 0.1. The amount of *p*-nitrophenol produced in the reaction was determined by measuring the absorbance at 400 nm vs. water. The amount of *p*-nitrophenol produced by nonenzymatic hydrolysis was determined by running a control reaction without enzyme at each pH.

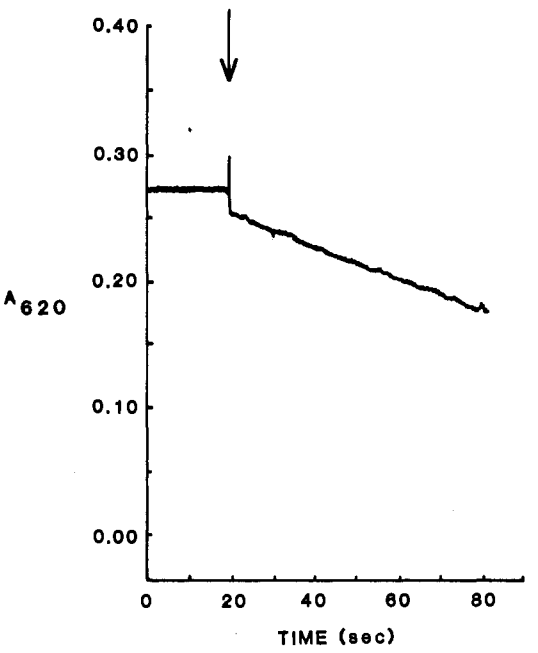

Figure 1. Absorbance change during the enzyme assay. Pectin (2 mL) was mixed with 0.15 mL of bromothymol blue and 0.83 mL of water, and the initial A_{620} was recorded. At the time indicated by the arrow, the reaction was started by the addition of 5 μg of PME (20 μL).

A titration assay for PME activity (Kertesz, 1937; Lee and Macmillan, 1968) was used to determine the effects of bromothymol blue on enzyme activity. The solutions of pectin, bromothymol blue, and enzyme were prepared as described above for the spectrophotometric assay. These solutions and water were adjusted to pH 7.50 just before each assay. For each trial 10.0 mL of pectin, 4.15 mL of water, and 0.75 mL of bromothymol blue were mixed together and placed on a pH meter equipped with a combination electrode (Model 91-15, Orion Research Inc., Cambridge, MA). The reaction was started by adding 100 μL of enzyme solution containing 50 μg of commerical PME. The reaction mixture was titrated with standarized NaOH to pH 7.50 at 1-min intervals for the first 6 min of the reaction. The rate of acid production was calculated from the rate of base consumption.

PME in Plant Extracts. Green apples, red grapes, tomatoes, bean sprouts, oranges, and cucumbers were obtained at a local supermarket. The apples, oranges, and cucumbers were peeled, and sections of the peeled tissue were homogenized in cold (4 °C) 8.8% (w/v) NaCl (Lee and Macmillan, 1968) on a Tissumizer homogenizer (Tekmar Co., Cincinnati, OH). The grapes, tomatoes, and bean sprouts were homogenized without peeling. The homogenates were centrifuged at 20000*g* for 10 min. The supernatants were collected, adjusted to pH 7.5 with NaOH, and assayed for PME activity by using the new spectrophotometric assay. Between 30 and 100 μL of extract was employed in the activity assay.

RESULTS

We have developed a sensitive assay for plant PME in which the acid produced (reaction 1) reacts with a pH indicator dye, causing a color change that is spectrophotometrically monitored. In order to select an appropriate indicator dye, the range of pH values to be measured had

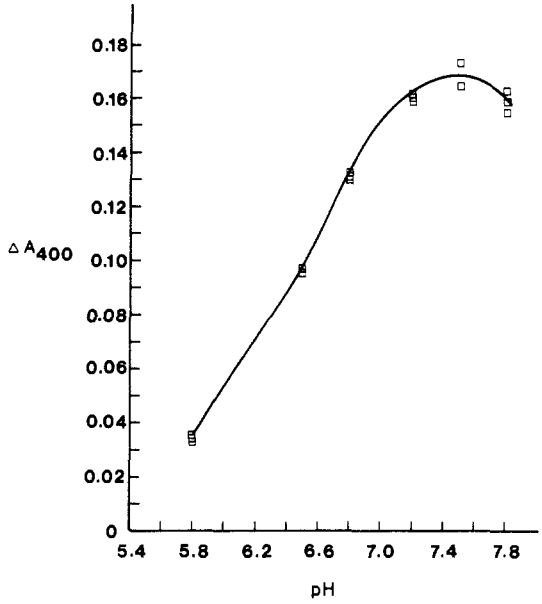

Figure 2. Hydrolysis of *p*-nitrophenyl acetate by PME as a function of pH. The activity of 50 μg of commercial PME at various pH values was determined by using the *p*-nitrophenyl acetate assay for esterases (Huggins and Lapides, 1947). The amount of *p*-nitrophenol produced was determined at 400 nm.

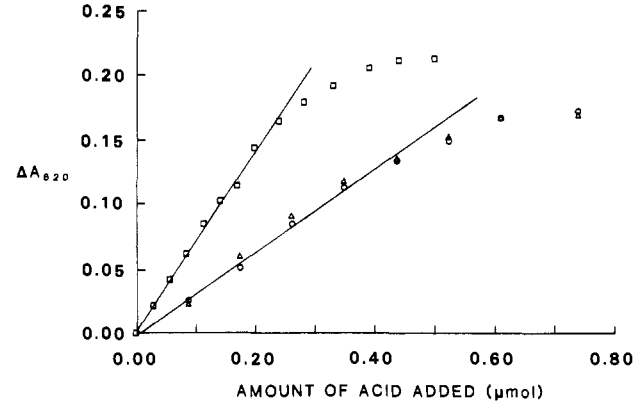

Figure 3. Calibration curve for spectrophotometric assay. Various amounts of 0.5 mM HCl (□), 0.87 mM acetic acid (△), or 0.87 mM galacturonic acid (monohydrate) (○) were added to assay mixtures containing pectin and bromothymol blue. The difference between the A_{620} of the mixture containing acid and the A_{620} of a similar mixture without acid was recorded as ΔA_{620}. The values shown are the means of three trials. The lines were drawn by the method of least squares. The linear portion of the curve for galacturonic acid is described by the equation ΔA_{620} = 0.328 (μmol of acid) − 0.003.

to be established. The *p*-nitrophenyl acetate assay for esterases (Huggins and Lapides, 1947) was used to determine the pH profile of commercial plant PME (Figure 2). The enzyme had maximum activity between pH 7.2 and 7.8; a similar optimum pH for plant PME has previously been established by using a titration method (Rexova-Benkova and Markovic, 1976). Those results suggested that the starting pH for the assay should be between pH 7.2 and 7.8; a starting pH of 7.50 was routinely used. A pH meter was used to determine the magnitude of the pH change during enzymatic hydrolysis of pectin. An unbuffered solution of pectin was adjusted to pH 7.5, and PME was then added. During the first several minutes of the enzyme-catalyzed reaction, the pH dropped to a value of pH 7.2. Therefore, bromothymol blue, which changes color from blue at pH 7.6 to yellow at pH 6.2 (Waser, 1966), was selected as the appropriate indicator dye for following the reaction.

Bromothymol blue does not affect the activity of PME. A titration assay of PME (Kertesz, 1937; Lee and Macmillan, 1968) was performed in both the presence and absence of bromothymol blue. The activity of 50 μg of commerical PME was 2.82 ± 0.38 μmol of acid produced/min in the presence of bromothymol blue and 2.84 ± 0.42 μmol of acid produced/min in the absence of bromothymol blue.

To maximize the sensitivity of the assay, the reaction should be monitored at the wavelength that gives the largest change in absorbance with change in pH. In order to choose the proper wavelength for the new assay the absorbance spectra of the completely protonated (pH <6.2) and completely deprotonated (pH >7.6) forms of bromothymol blue were compared. The deprotonated form of bromothymol blue (λ_{max} 620 nm) has a larger extinction coefficient than the protonated form of the dye (λ_{max} 430 nm). The largest peak in the difference spectrum was at 620 nm, and the enzyme activity was therefore monitored at 620 nm.

The bromothymol blue assay system was characterized with both strong and weak acids (Figure 3). Addition of a strong acid such as HCl caused a larger color change than the addition of an equal number of moles of a weak acid such as galacturonic acid. The strong acid is completely ionized, and each mole of acid produces 1 mol of hydrogen ion to react with the bromothymol blue; but, the weak acids are only partially ionized and thus produce fewer moles of hydrogen ion per mole of acid. Weak acids with similar pK_a values such as acetic acid and galacturonic acid produce the same color change on a molar basis (Figure 3).

Since galacturonic acid is the specific weak acid produced by PME, the spectrophotometric assay was calibrated by titration with galacturonic acid. The ΔA_{620} was linearly related to the amount of galacturonic acid added until a ΔA_{620} of approximately 0.12 was reached (Figure 3). The linear portion of the calibration curve was used to convert the ΔA_{620}/min observed for a given enzyme sample to micromoles of acid produced/minute during the enzymatic reaction. The buffering capacities of the pectin solutions differed slightly from each other and also changed as the solutions aged, so the assay was standardized daily.

The spectrophotometric assay, like the previously described titration assays (Kertesz, 1937; Lee and Macmillan, 1968), was performed in an unbuffered solution. Buffers minimize pH changes and therefore decrease the sensitivity of assays that monitor pH change. However, in the absence of buffer, the acid produced during the enzymatic reaction might cause changes in the enzyme activity. We tested the pH sensitivity of commerical plant PME using the *p*-nitrophenyl acetate assay for esterases (Huggins and Lapides, 1947). As seen in Figure 2, plant PME activity is essentially constant over the change in pH that occurs during the spectrophotometric assay (pH 7.5–7.2). Therefore, even in the absence of buffer, there is no significant change in enzyme activity due to acid production during the spectrophotometric assay.

The characteristics of commerical plant PME determined with the spectrophotometric assay were similar to those previously determined for plant PME using other assays. The specific activity of the commerical enzyme was 56.4 ± 7.6 μmol of acid produced/min per mg as measured with the titration assay and 59.0 ± 7.0 μmol of acid produced/min per mg as measured with the spectrophotometric assay. The activity measured with the spectrophotometric assay was linearly related to the amount of enzyme present until the activity exceeded 0.8 μmol of acid

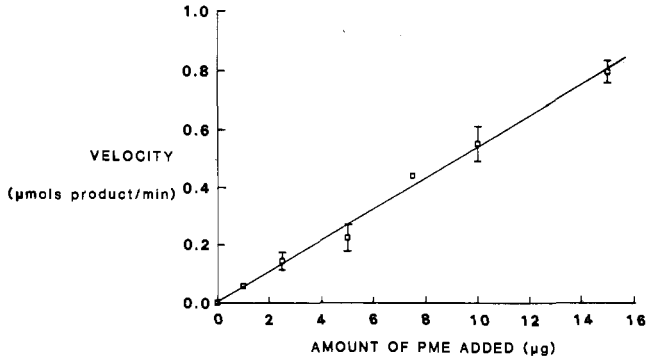

Figure 4. PME activity as a function of enzyme concentration. The activity of commercial PME was determined by using the spectrophotometric assay as described in the text. The values shown are the means and standard deviations of three trials, and the line through the values was drawn by the method of least squares. The average relative standard deviation over the range of activities shown was ±12%.

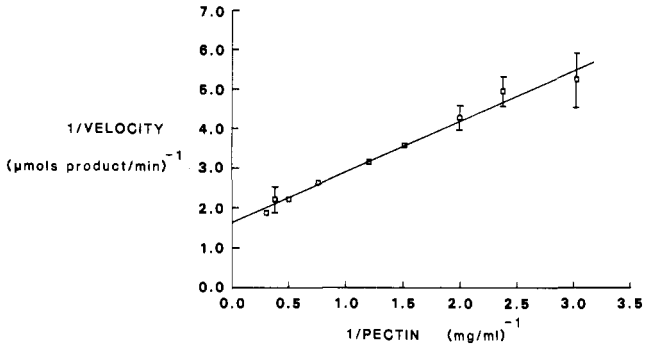

Figure 5. PME activity as a function of substrate concentration. The activity of 10 μg of commercial PME was determined with different substrate concentrations by using the spectrophotometric assay as described in the text. The values shown in the double-reciprocal plot are the means and standard deviations of three trials. The line was drawn by the method of least squares.

produced/min (Figure 4). For larger amounts of enzyme, the reaction occurred so quickly that the initial rate could not be accurately determined from the data obtained. The enzyme obeyed saturation kinetics, and a linear Lineweaver–Burk plot was obtained (Figure 5). From the plot, a K_m = 0.78 ± 0.03 mg/mL of pectin and a V_{max} = 0.61 ± 0.02 μmol of acid produced/min were determined for commerical PME. This K_m value agrees well with previously reported values, which range from 0.4 to 2.4 mg/mL of pectin (Rexova-Benkova and Markovic, 1976).

The new assay was also used to determine PME in crude extracts of several plant tissues (Table I). No change in the A_{620} was observed when the extracts were assayed in the absence of pectin, indicating that the activity measured in the crude extracts was not due to the presence of other esterases and their substrates in the extracts. Commercial pectin lyase (Sigma) did not interfere with the measurement of PME, so none of the activity in the crude extracts could be attributed to pectin lyase. Since less than 100-μL aliquots of the crude extracts were required for measurement of the PME activity, even the colored tomato and mung bean extracts could be assayed without interference from plant pigments.

DISCUSSION

The advantages of the spectrophotometric assay over previously described assays for PME are illustrated by comparing the convenience, specificity, and sensitivity of the methods. The spectrophotometric assay is a simple method, requiring smaller sample volumes and shorter

Table I. PME Activity in Extracts of Plant Tissues[a]

plant tissue	protein,[b] mg/mL	activity,[c] μmol of acid produced/min per mL
green apple	0.170	0.86 ± .03
red grapes	0.150	0.25 ± 0.3
tomato	0.305	6.32 ± .58
bean sprouts	0.205	1.98 ± .15
orange	0.145	3.80 ± .45
cucumber	0.085	1.48 ± .02

[a] The plant tissue (4.5 g) was homogenized in 15 mL of cold 8.8% (w/v) NaCl. The homogenate was centrifuged, and the supernatant was adjusted to pH 7.5 with NaOH. [b] Determined by a dye-binding method standardized with bovine serum albumin (Bradford, 1976). [c] Assayed with the spectrophotometric method as described in the text.

reaction times than the popular titration method (Kertesz, 1937; Lee and Macmillan, 1968) for determining PME. Only 2–3 min/trial and 2 mL of substrate/trial are required with the spectrophotometric assay, while 6–8 min/trial and 10 mL of substrate/trial are required with the titration assay. The spectrophotometric assay is much more convenient than the colorimetric method for methanol determination (Wood and Siddiqui, 1971), which requires sequential addition of several reagents during the 1.5-h sample preparation. Although sample preparation for determination of methanol chromatographically is simple (McFeeters and Armstrong, 1984), the instrumentation required makes the assay less convenient than the spectrophotometric assay. The *p*-nitrophenyl acetate assay (Huggins and Lapides, 1947) is a straightforward, simple assay, but it is a nonspecific assay that detects a variety of esterases. All of the other PME assays, including the spectrophotometric assay, utilize pectin as the substrate and are therefore specific for pectin-hydrolyzing esterases.

The new assay provides a sensitive, continuous method for determining PME. It can be used to detect activities as low as 0.020 μmol of acid produced/min. Methods for methanol determination (Wood and Siddiqui, 1971; McFeeters and Armstrong, 1984) are 10-fold more sensitive than the spectrophotometric method, and despite their inconvenience they might be better assays for samples containing very small amounts of enzyme. However, both methods for methanol determination are single-point assays, while the spectrophotometric assay is a continuous assay. The kinetic parameters of an enzyme-catalyzed reaction can be determined more accurately from continuous-assay data than from single-point data (Allison and Purich, 1983), so the spectrophotometric method should be employed for kinetic characterization of purified PME. The titration assay (Kertesz, 1937; Lee and Macmillan, 1968) is a continuous assay but is 10-fold less sensitive than the spectrophotometric assay. The *p*-nitrophenyl acetate assay, a single-point assay, is much less sensitive than any of the other assays, with a detection limit 100-fold higher than the spectrophotometric method.

The convenience, specificity, and sensitivity of the spectrophotometric assay make it a more generally useful method than the existing assays for plant PME. In addition, the lack of interferences from other esterases or from colored components makes the assay ideal for determining PME in crude plant extracts. This assay should be applied to a variety of studies of the role of PME in plant physiology and food science (Hobson et al., 1984; McLellan et al., 1985; Opermann et al., 1973).

The spectrometric assay not only will be valuable for determining plant PME but could also be modified for assaying a variety of other esterases, including fungal PME. The fungal PME generally have lower pH optima than the

plant enzyme (Rexova-Benkova and Markovic, 1976). The spectrophotometric assay would thus be set up at lower pH and an indicator dye with the appropriate pK_a selected. For example, those forms of PME with optimal activity at pH 5 could be conveniently assayed with bromocresol green as the indicator dye (Rexova-Benkova and Markovic, 1976; Waser, 1966). The potential adaptibility of the spectrophotometric assay should make it useful for characterizing the fungal pectin degrading enzymes important in plant disease (Cooper, 1983).

Registry No. PME, 9025-98-3; pectin, 9000-69-5.

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Influence of Extracts from Soybean (*Glycine max* (L) Merr.) Leaves on Hydrolytic and Glutathione *S*-Transferase Activity in the Soybean Looper (*Pseudoplusia includens* (Walker))

Patrick F. Dowd, Randy L. Rose, C. Michael Smith, and Thomas C. Sparks*

Methanol extracts of a resistant (PI227687) and a susceptible (Davis) soybean variety were incorporated into artificial diets that were fed to larvae of the soybean looper *Pseudoplusia includens* (Walker). Homogenates of insects fed on the diet containing the PI227687 extract generally had significantly lower rates of hydrolysis of *cis*-permethrin and fenvalerate, and significantly higher rates of conjugation of 1-chloro-2,4-dinitrobenzene relative to those insects fed on diets containing the Davis extract. Minor differences were noted for *trans*-permethrin and α-naphthyl acetate hydrolysis for insects fed on the two extract diet types. The rate of hydrolysis of acephate to methamidophos changed slightly and varied according to the leaves from which the extracts were made. Incorporation of coumestrol, a flavonoid occurring in the PI227687 extract, into the diet also resulted in lower rates of hydrolysis of *cis*-permethrin, fenvalerate, and acephate that were similar to those found for insects fed on diets containing the PI227687 extract.

INTRODUCTION

Host plant resistance and insecticide treatment are frequently combined as a strategy for insect control in integrated pest management programs (Adkisson and Dyck, 1980). However, the type of host plant fed upon by insects prior to treatment can affect insecticide toxicity (Maxwell, 1972). One possible explanation for this occurrence involves the modification of detoxification enzyme activity by plant allelochemicals. Plants have been shown to contain compounds that can either induce (Brattsten et al., 1977; Berry et al., 1980; El-Sebae et al., 1981; Farnsworth et al., 1981; Moldenke et al., 1983; Yu, 1982, 1983; Yu et al., 1979) or depress (Dowd et al., 1983a; Yu (1983) enzyme systems that may be involved in insecticide metabolism. Results of both laboratory (Dowd et al., 1983a) and field (Kea et al., 1978) studies have suggested that larvae of the soybean looper *Pseudoplusia includens* (Walker) fed on resistant soybean varieities may have altered levels of enzymes involved in insecticide metabolism.

While the effects of plant toxins on the induction or repression of microsomal mixed-function oxidases have received a great deal of attention, studies on the action of these plant toxins on hydrolytic and conjugative systems have been relatively limited (for review, see Dowd et al., 1983b). Hydrolytic and conjugative enzymes play a variety of roles in the metabolism of many insecticides, including pyrethroids and organophosphates (Dauterman, 1976; Yang, 1976). Therefore, we examined how the metabolism of several insecticides, and related substrates, was influenced by feeding larvae of *P. includens* on extracts of resistant and susceptible soybean varieties.

PROCEDURE

Chemicals. Radiolabeled *cis*- and *trans*-permethrin and 3-phenoxybenzyl alcohol (^{14}C label on the methylene carbon of the 3-phenoxybenzyl alcohol, sp act. 57 mCi/mmol) were a gift from FMC. Radiolabeled acephate (^{14}C label on the *S*-methyl group, sp act. 4.77 mCi/mmol) was

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