Pertanika 8(2), 231 – 236 (1985)

Extraction of Microsomal Fraction from Midguts of Western Spruce Budworm *Choristoneura occidentalis* F., and Rat Liver, and their Ability to Expoxidize Aldrin

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Key words: Microsomal fractions; western spruce budworm; *Choristoneura occidentalis* F.; rat liver; epoxidation; aldrin.

ABSTRAK

Asingan mikrosoma telah disediakan dari bahagian usus tengah larva-larva western spruce budworm dan daripada hati tikus. Persediaan mikrosoma ini kemudiannya diasai untuk kandungan sitokrom P-450 dan aktiviti enzim epoxida aldrin. Asingan mikrosoma dari kedua-dua organisma ini memberi spektrum sitokrom P-450 yang jelas. Tetapi perbandingan relatif dengan kandungan protein menunjukkan bahawa kandungan sitokrom P-450 yang didapati dalam asingan mikrosoma dari usus tengah spruce budworm adalah 7 – 9 kali lebih rendah daripada kandungan dalam asingan yang didapati dari hati tikus. Kupayaan menukarkan aldrin kepada dieldrin menunjukkan terdapatnya aktiviti enzim epoxida pada kedua-dua asingan mikrosoma ini. Aktiviti kedua-dua asingan mikrosoma ini terencat dengan adanya piperonyl butoxide.

ABSTRACT

Microsomal fractions were isolated from midguts of western spruce budworm larvae and rat liver. The microsomal preparations were then assayed for cytochrome P-450 content and aldrin epoxidase activity. Both microsomal preparations showed distinct spectrum of cytochrome P-450. Relative to protein content, the cytochrome P-450 content of midguts of western spruce budworm was lower by 7 – 9 times compared to that of the rat liver. Epoxidase activity is present in the two microsomal preparations as indicated by their ability to convert aldrin to dieldrin. The activity of both microsomal preparations was inhibited by piperonyl butoxide.

INTRODUCTION

The oxidative enzymes known as microsomal oxidases play a central role in the metabolism of organic insecticides in animal body. The enzyme has an extremely broad spectrum of substrates and catalyze a wide variety of transformation, with cytochrome P-450 acting as the oxygen-activating enzyme component to carry out the reaction. Cytochrome P-450 and its implication as the terminal oxidase component has been discovered in mammalian and insect microsomes (Williamson and Schechter 1970).

Microsomal fractions of insect tissues and rat liver constitute a ready source of mixed-function oxidases (Wilkinson 1979; Wong and Terriere 1965). These microsomal oxidases of the microsomal fractions in several lepidopterous insects and in rat livers are known to parallel

that of epoxidase activity (Brattsten and Wilkinson 1973; Chan *et al.*, 1967; Wong and Terreire 1965). Therefore, aldrin, a model substrate to measure the total enzyme activity (by epoxidation) of mixed-function oxidases, is often used for comparison.

This paper reports on the isolation of microsomal fractions from midguts of western spruce budworm, an important lepidopterous insect pest, and testing of the activity of the enzyme that is involved in insecticide metabolism. The presence of the cytochrome P-450 spectrum and aldrin epoxidation were used to demonstrate the activity of the isolated enzyme fractions. The activity of the enzyme fractions from rat liver was also studied for comparison.

MATERIALS AND METHODS

Enzyme Preparation

The microsomal fraction was prepared from two sources; the midguts of last-instar larvae of western spruce budworm and the liver of six months-old male Wistar rats.

The larval midguts were isolated, sectioned longitudinally and rinsed in cold buffer solution (0.1 M phosphate, pH 7.6) to remove their contents, and then cleaned thoroughly in the fresh ice-cold buffer medium. The cleaned midguts (20 midguts/5 ml cold buffer) were homogenized for 15 min using a Potter-Elvehjem homogenizer fitted with a motor-driven teflon pestle. The homogenized samples were later combined for centrifugation at 0° to 4°C for 15 min at 10,000 *g*. The supernatant was filtered through glass wool and then recentrifuged at 0° to 4°C for 60 min at 105,000 *g* in a Sorval OTD 75B ultracentrifuge with a Ti60 fixed-angle rotor according to Yu and Terriere (1979). The supernatant was decanted and the microsomal pellets were resuspended in cold buffer solution, at the equivalent of 20 midguts/ml, by gentle homogenization.

The livers of rats, killed by decapitation, were perfused with the cold buffer solution by inserting a hypodermic needle into the portal vein. The perfused liver was then removed from the thoraxic cavity, washed thoroughly, and homogenized for 30 sec in cold buffer solution at 1 g/4 ml, using a VirTis blender. The homogenate was centrifuged for 30 min at 10,000 *g* and 0° and 4°C. Microsomes were prepared from the supernatant as described above, and finally buffer was added at the equivalent of 1 g wet liver weight/ml.

Protein concentration was estimated for each preparation by the technique of Lowry *et al.*, (1951). The microsomal suspension was either used immediately or stored frozen at −12°C, and used within a week without loss of the activity.

Cytochrome P-450 Determination

Cytochrome P-450 was measured as its carbon monoxide-binding complex according to Omura and Sato (1964). A 3-ml microsomal preparation was reduced with about 100 mg of sodium dithionite ($Na_2S_2O_4$). Carbon monoxide (CO) was then slowly bubbled through the reduced sample for about 1 min, and CO difference spectra were immediately determined with a Cary-14 recording spectrophotometer by scanning from 390 − 520 nm. The concentration was determined by the peak height of the spectrum between 450 and 490 nm (ΔOD450 − 490).

Epoxidation of Aldrin

The epoxidation of aldrin was done using a typical reaction mixture (Wong and Terriere 1965). Each incubation mixture contained 0.5 ml microsomal preparation, 1 ml of NADPH-generating solution (containing 2 μmoles of NADPH, 20 μmoles of glucose-6-phosphate dehydrogenase, and 25 μmoles of magnesium chloride, in phosphate buffer), and 5.5 ml of buffer. It was shaken in a Dubnoff metabolic shaker at 37°C for 10 min, before 10 μg of aldrin in 50 ml methyl cullusolve was added as substrade compound. The incubation times were 0, 15, 30 and 60 min. To one of the incubation media, 10^{-4}M piperonyl butoxide was added, and incubated for 60 min. The reaction was

TABLE 1
Protein and Cytochrome P-450 contents of microsomal preparations from midguts of spruce budworm larvae and rat livers used in aldrin epoxidation

Source of microsomal preparation	Preparation no.	Protein (mg ml^{-1})	Cytochrome P-450 (nmoles ml^{-1})	(nmoles ml^{-1} protein)
Midguts of spruce budworm larvae	1	2.13	0.27	0.127
	2	2.03	0.24	0.118
	3	2.05	0.27	0.132
	4	1.90	0.23	0.121
	5	1.95	0.23	0.118
	X ± S.D.	2.01 ± 0.09	0.25 ± 0.02	0.123 ± 0.006
Rat livers	1	10.00	9.56	0.956
	2	8.00	7.14	0.893
	3	12.50	9.78	0.782
	X ± S.D.	10.17 ± 2.25	8.83 ± 1.46	0.877 ± 0.088

stopped by shaking and extracting the incubation mixture with 20 ml of a 1 : 1 mixture of hexane and acetone. Two additional extractions followed, each with 20 ml of the same solvent mixture routed through the incubation flask. The combined hexane extracts were washed twice with 20% Na_2SO_4. The hexane phases were then stored over granular anhydrous Na_2SO_4 at $-20°C$ until analysed within a week.

The extracts were analysed using a Tracor MT220 gas chromatograph, equipped with ^{63}Ni EC detector. Injections were done into a U-shaped Pyrex glass column, 183 × 0.3 cm I.D., packed with 2% OV1 *plus* 6% OV210 on Chromosorb W"H.P.", 80 − 100 mesh. The carrier gas was nitrogen with a flow rate of 70 ml/min. Other operating parameters were: injector port, 150°C; column oven, 225°C; detector, 245°C.

Extraction efficiency was checked by adding 10 μg of aldrin and 20 μg of dieldrin to an insecticide-free incubation medium and extracted immediately. All extracts were suitably diluted with redistilled hexane before the injection. A standard solution containing aldrin and dieldrin in hexane was used to prepare a standard curve each time the extracts were analysed.

RESULTS AND DISCUSSION

The classical method for preparing microsomal fractions is to centrifuge the mitochondria-free supernatant at about 105,000 *g* for 1 hour. Using this method, the microsomes could be isolated from homogenates of midguts of western spruce budworm larvae and of rat livers to make up the microsomal preparations. The typical spectral difference curves of the cytochrome P-450-CO complex of the microsomes from the two species are shown in *Figures 1a* and *1b*. They clearly show that absorption maxima to be at 450 nm in both cases, thus indicating an almost complete absence of the degredation product cytochrome P-420, suggesting a fully active sample of the cytochrome. The protein and cytochrome P-450 contents were determined as index of "activity". Results are shown in Table 1. Since the main purpose was to test if the microsomal fractions activated insecticide *in vitro*, no attempt was therefore made to compare the specific contents of the protein and the cytochrome P-450 in the preparations from the two sources. It was enough to note the consistency of the preparations within each source, as each source of the preparations will represent a replicate in the epoxidation work done later in the study.

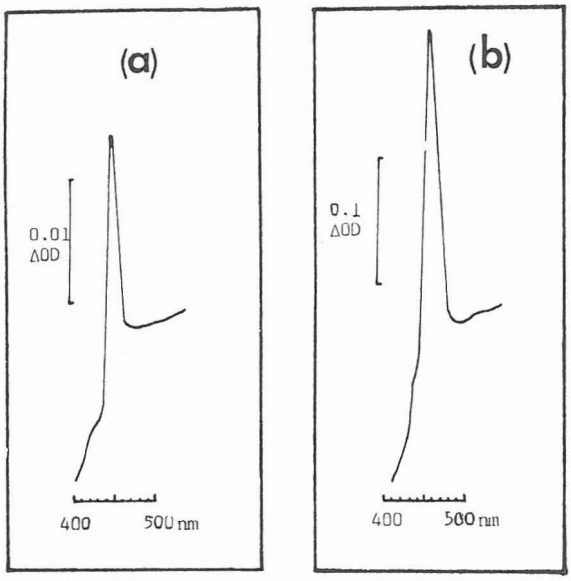

Fig. 1. Spectral difference curve of cytochrome P-450-CO complex of microsomes from (a) midguts of western spruce budworm larvae, and (b) rat liver.

Several workers have reported the isolation of microsomal fractions from the midguts of some other lepidopterous larvae. It was interesting to note that the content of cyrochrome P-450 based on μmoles/mg protein obtained from the western spruce budworm larvae in the present preparations was about the same as that obtained from the southern armyworm by Brattsten and Gunderson (1981), but was two or three times lower than those obtained from alfalfa looper and cabbage looper larvae, respectively (Farnsworth *et al.*, 1981), and about two times higher than that obtained from the variegated cutworm (Yu *et al.*, 1979).

Incubation of the microsomal preparations with the NADPH-generating system to test for expoxidation of aldrin *in vitro* gave significant results (Table 2). Incubation of up to 60 min did not result in reduced recovery. At least 88% of the added substrate compound was recovered, either epoxidized to dieldrin or as unchanged parent compound. The conversion occurred very rapidly: at least 71% of the total recovery after 15 min of incubation was dieldrin. At 15 min and 60 min after the incubation, the conversion levels were, 73.23% and 82.96% respectively of the total recoveries from rat liver MFO, and 71.14% and 79.36% for those incubated with MFO of midguts of spruce budworm larvae. There was no evidence that a product other than an epoxide (dieldrin) was produced. The chromatogram obtained only showed two distinct

TABLE 2
In-vitro aldrin epoxidation by microsomal preparations at 37°C

		Recovery (X)[1]					
		Midgut of western spruce budworm			Rat liver		
Incubation time (min)	Aldrin added (μg)	Aldrin (μg)	Dieldrin (μg)	%[2]	Aldrin (μg)	Dieldrin (μg)	%[2]
0	10	9.63	0	0	9.19	0	0
15	10	2.69	6.63	71.14	2.65	7.25	73.23
30	10	1.99	7.50	79.03	2.09	7.50	78.21
60	10	1.99	7.65	79.36	1.69	8.23	82.96
60[3]	10	8.80	0	0	8.50	1.99	18.97

[1]N = 2.

[2]Dieldrin, % of aldrin and dieldrin.

[3]Incubation with 1×10^{-4}M piperonyl butoxide.

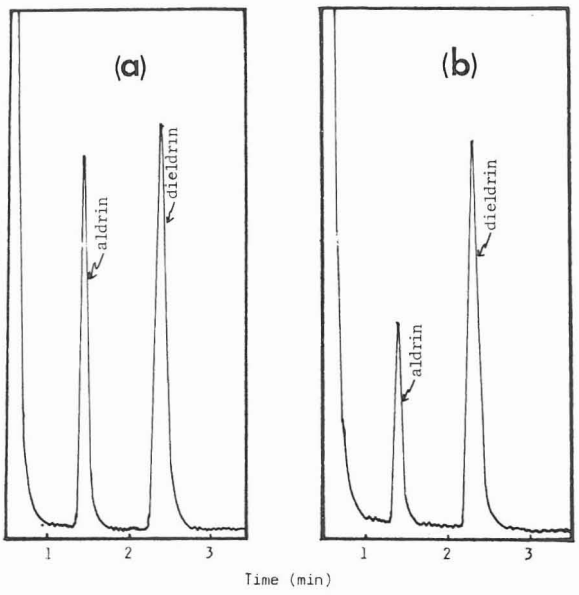

Fig. 2. Chromatograms of aldrin and dieldrin peaks obtained from (a) standard solution, and (b) in-vitro *aldrin epoxidation using NADPII-generating system.*

peaks of aldrin and dieldrin comparable to that of the standard solution *(Figure 2)*. It is also important to note that the extraction technique applied gave between 95% to 100% recovery for both chemicals. The data in Table 2 also indicate that the conversion of aldrin was inhibited when 1×10^{-4}M piperonyl butoxide was included in the incubation medium. Thus, it is evidence that epoxidase activity is present in the microsomal preparations.

Dahm *et al.*, (1962) stated that piperonyl butoxide is one of the most effective insecticide synergists, inhibiting *in vitro* microsomal activation of several OPs at 1×10^{-4}M. Nevertheless, Shishido *et al.*, (1972) found that is only depressed the metabolism of an OP (diazinon) by the microsomes-NADPH enzyme systems prepared from rat liver and American cockroach fat body. However, piperonly butoxide at 1×10^{-4}M was reported to inhibit activation of parathion by microsomes prepared from American cockroach fat body (Nakatsugawa and Dahm 1965). Thus the degree of inhibition of metabolism by piperonyl butoxide would depend on the insecticide exposed and the MFO source.

The results obtained in the present study were comparable with those reported by several other workers. Williamson and Schecter (1970) showed that the rate of aldrin epoxidation by MFO obtained from whole larvae of tobacco budworm was rapid for 20 min and reached a maximum after 40 min. Wong and Terriere (1965) obtained similar results with rat liver microsomes. Using diazinon as the substrate, Shishido *et al.*, (1972) found that the rate of the oxidative metabolism was higher with rat liver MFO than with that of American cockroach fat body.

CONCLUSION

Using the conventional method of preparation, microsomal fractions were able to be isolated from the midguts of western spruce budworm larvae. The microsomal preparations and those obtained from rat liver have in common in that they show the presence of cytochrome P-450 (with spectral difference peak at 450 nm), they epoxidize aldrin with the requirements of NADPH-generating system, and are inhibited by piperonyl butoxide. Since liver microsomes have been shown to perform a variety of biological oxidations, it seems likely that microsomal enzymes in the midguts of western spruce budworm larvae would also catalyze a wide variety of transformations in metabolism of insecticides.

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(Received 14 January, 1985)