In Situ Production of Hydrolytic Detoxifying Enzymes by Symbiotic Yeasts in the Cigarette Beetle (Coleoptera: Anobiidae)

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ABSTRACT The ability of symbiotic yeast found in larvae and adults of Lasioderma serricorne (F.) to detoxify xenobiotics hydrolytically in situ was investigated. The symbionts of larvae and adults hydrolyzed 1-naphthyl acetate, a reaction that was inhibited by DEF (S,S,S-tributyl phosphorotrithioate, Chem Service) and mercuric chloride. The yeasts also accumulated tannic acid, which was hydrolyzed to gallic acid. Thus, in addition to nutritional contributions that were previously demonstrated, these symbionts are capable of assisting the survival of the host by detoxifying xenobiotics.

KEY WORDS Insecta, Lasioderma serricorne (F.), symbiont, detoxification

Intracellular symbiotic microorganisms that are found in insects can produce amino acids, lipids, vitamins, or other nutrients for their hosts (Koch 1960). However, the contribution of these symbionts appears to be more than just nutritional. The enlarged size of symbionts in insecticide-resistant aphids has caused speculation that the symbionts contribute to the resistance through detoxification of the insecticide (Amiressami & Petzold 1976a,b; Amiressami 1980). The involvement of intracellular and extracellular symbionts of herbivorous insects in detoxification of plant allelochemicals may be widespread (Jones 1984). However, actual detoxification of any xenobiotics by intracellular symbionts has never been demonstrated.

The cigarette beetle, Lasioderma serricorne (F.), contains intracellular yeasts in specialized tissues (mycetomes) at the junction of the foregut and midgut (Jurzitza 1979), a fact that was discovered nearly 100 years ago for the closely related Stegobium panicum (F.) (Karawaiew 1899). These symbionts apparently contribute amino acids (Jurzitza 1969a,b), sterols (Pant & Kapoor 1963), and vitamins (Blewett & Fraenkel 1944, Pant & Fraenkel 1950) to their hosts. L. serricorne feeds on many dried plant products including tobacco, straw, seeds, and pepper (Metcalf et al. 1962). These plant substances contain a variety of toxins, which could be detoxified by oxidative, conjugating, or hydrolytic enzymes. For example, in addition to the toxin nicotine, tobacco contains a number of potentially toxic esters including scopalamine, polyphenolics, and flavonoid glycosides (Hegnauer 1973). The cigarette beetles must be able to detoxify these chemicals in order to survive. Several insect species can hydrolyze appropriately structured plant toxins as a means of detoxification (Dowd in press a). Thus, the ability of the yeasts of L. serricorne to hydrolyze model xenobiotics may determine their potential involvement in detoxification in their host.

As a result of research on the factors that affect enzyme activity in microorganisms apart from the native environment, a careful consideration of the methods to be used to demonstrate the detoxification of xenobiotics by insect symbionts is necessary. For example, Ishikawa (1984) demonstrated that the synthesis of proteins by the symbiont from the aphid Acyrthosiphum pisum (Harris) is much more limited in the host than when the symbiont is incubated apart from the host. Enzyme activity can be dramatically affected (either increased or decreased) when the microorganisms are cultured apart from the host (Cole et al. 1985). In the case of aphid symbionts, Amiresammi (1980) suggested that histochemical techniques were necessary to demonstrate actual involvement of these organisms in insecticide resistance. Histochemical techniques have been successfully used to demonstrate the presence and location of hydrolytic enzymes in tissues (e.g., Pearse 1968). Therefore, I used histochemical assays to detect the hydrolysis of aromatic esters in the mycetomes containing the symbiotic yeasts from L. serricorne.

Materials and Methods

Chemicals. All chemicals, including the histochemical 1-naphthyl acetate esterase kit, were obtained from Sigma Chemical Company, St. Louis, except for DEF (S,S,S-tributyl phosphorotrithioate), which was obtained from Chem Service, West Chester, Pa.
Insects. A culture of *L. serricorne* was obtained from the USDA-ARS Stored Product Insect Research and Development Lab, Savannah, Ga. Insects were reared on a white flour/white corn meal/brewer's yeast mix (10:10:1.5) at 27 ± 1°C, 60 ± 10% RH, and a photoperiod of 14:10 (L:D). Feeding last instars or 1-wk-old adults (sex not determined) were used for assays. Aposymbiotic insects were obtained by surface-sterilizing the eggs for 1 min in 70% ethanol.

Assays. The mycetomes and associated gut tissue were dissected from larvae and adults in distilled water, placed on cover slips, and allowed to air dry before to fixation. Because the stability of the enzymes was unknown and I sought to examine enzyme activity under as near as natural conditions as possible, intact tissues (rather than thin sections) were used. Tissues were gently fixed according to suggested procedures (Sigma Diagnostics 1985).

Hydrolysis of 1-naphthyl acetate was used as an indication of general esterase activity. The method followed the procedures outlined in Sigma Chemical publication 90 (Sigma Diagnostics 1985). Briefly, fixed tissues were incubated in Trizmal 0.02 M buffer (pH 7.6) containing 0.04% 1-naphthyl acetate and 0.02% Fast Blue RR Salt (the diazo coupling reagent). Tissues were incubated for 5 min at 35°C, rinsed with distilled water, and air dried before they were mounted in Permount (Fisher Scientific, Fair Lawn, N.J.). When inhibitors were used for esterase assays, the tissues were preincubated with 10⁻⁴ M concentrations of the inhibitors (either DEF or mercuric chloride) before addition of the substrate. The hydrolysis of 1-naphthyl acetate by mycetome and midgut homogenates was determined by the method of Dowd & Sparks (1984) except the concentration of tissues was 20 per ml of buffer. Each tissue type was assayed twice in triplicate.

Because the host plants of *L. serricorne* often contain high levels of phenolic esters, tannic acid (tannin) was used to assay for the hydrolysis of a naturally encountered substrate. Penetration of the tannin into the symbionts was detected by incubating the fixed tissues in 0.04% tannin for 30 min, followed by a 1 min incubation in 2% ferric chloride. Ferric chloride reacts with tannin or other phenolic compounds to form a deep purple precipitate (Jenzen 1962). Hydrolysis of tannin was detected by complexing the potential product, gallic acid, with the Fast Blue RR Salt. When incubated with this diazo reagent, tannic acid forms a pink color (peak absorbance at 465 nm, extinction coefficient about 55,000 cm²/mol), and gallic acid causes an orange color (peak absorbance at 520 nm, extinction coefficient approximately 10,000 cm²/mol). These peak absorbances were determined with a spectrophotometer (Lambda 4B, Perkin-Elmer, Oakbrook, Ill.). This color change also could be used as an effective spectrophotometric assay for tannin hydrolysis. For the histochemical assay, the tissues were incubated for 30 min with 0.04% tannic acid under the same conditions used for 1-naphthyl acetate assay. They then were rinsed with distilled water, moved to a solution of 0.02% Fast Blue RR for 2 min, and then treated in the same manner as for the 1-naphthyl acetate hydrolyase assay.

To be consistent with standard histochemical methods (Vacca 1985), I used controls that consisted of tissues incubated without the substrate (negative controls). Because I wanted to observe the symbionts in intact tissue as well as free of tissue, a dehydration step before mounting was not used. Instead, the tissues were directly mounted to allow for tissue fracture to release some of the imbedded symbionts. All assays were done at least five times.

**Results and Discussion**

The mycetome area of all tissues reacted strongly with 1-naphthyl acetate and tannin (e.g., Fig. 1). At 1,000× magnification, 1-naphthyl acetate metabolism was observed clearly in the mycetomes; activity was especially concentrated within and around the yeast compared with controls (Fig. 2A and B), as indicated by deposits of the diazo complex. This activity was inhibited by mercuric chloride and DEF (Fig. 2C and D, respectively), and was much lower in aposymbiotic larvae (Fig. 2F). All reactions were similar in adults and larvae. The 1-naphthyl acetate was hydrolyzed more rapidly by the mycetome homogenates compared with the rest of the midgut (15.5 ± 0.4 and 10.9 ± 0.2 nmol/min per tissue, mean ± SEM, respectively). The rate of hydrolysis of 1-naphthyl acetate by aposymbiotic mycetomes was reduced to 3.8 ± 0.1 nmol/min per tissue.

The presence of the precipitate suggested that the 1-naphthyl acetate was hydrolyzed by the symbionts in the tissues of *L. serricorne*. The effectiveness of DEF and mercuric chloride (inhibitors of hydrolytic enzymes) in inhibiting the reaction also indicated that hydrolytic enzymes are involved in the metabolism. The insect tissues containing the symbionts also appeared to hydrolyze 1-naphthyl acetate. However, very little metabolic activity occurs in the cytoplasm of these cells (Jurritz 1979). Many species of yeast secrete hydrolytic enzymes into their environment (Ahearn et al. 1968). The symbionts of *A. pisum* transfer proteins into the host cells (Ishikawa 1984). Thus, much of the hydrolytic activity of the surrounding tissues may also originate from the symbionts.

Tannic acid, its metabolites, or both were concentrated in the mycetome tissues after 30 min compared with controls, as indicated by complexing with ferric chloride (Fig. 3A and B, respectively). Most of the product appeared to consist of gallic acid, as indicated by the intense orange color of the tissues (Fig. 1B). Compared with controls, the color was localized in the yeast (Fig. 4A and B, respectively) and was not seen in aposymbiotic...
Fig. 1. Top. (A) Localization of 1-naphthyl acetate hydrolysis in the gut of adult *L. serricorne*. FG, foregut, MG, midgut, M, mycetome (10×). (B) Localization of tannin hydrolysis in the gut of larval *L. serricorne*. MG, midgut, M, mycetome (10×).

Fig. 2. Middle. Metabolism of 1-naphthyl acetate by *L. serricorne* larval symbionts. (A) Control. (B) Substrate alone. (C) Preincubated with DEF. (D) Preincubated with HgCl₂. (E) Aposymbiotic. Arrows indicate yeast (1,000×, phase contrast).

Fig. 3. Bottom left. Uptake of tannin by *L. serricorne* larval symbionts. (A) Control. (B) With tannin. Arrows indicate yeast (1,000×).

Fig. 4. Bottom right. Hydrolysis of tannin by *L. serricorne* larval symbionts. (A) Control. (B) With tannin. (C) Aposymbiotic. Arrows indicate yeast (1,000×, phase contrast).
insects (Fig. 4C). All responses were similar in adults and larvae.

The hydrolytic enzymes produced by the symbionts appear to have an adaptive role in hydrolyzing potentially toxic phenolic esters that the insect may consume. These could include the hydrolyzable tannins, as well as the alkaloid ester scopolamine and a variety of flavonoids complexed with sugars. The symbionts from *L. serricorne* are thought to be members of the Taphrinaceae (Jurzitza 1979), the free living forms of which are pathogens on woody plants and ferns (Mix 1936). These plants often contain high levels of tannins (Swain 1979). Thus, these yeasts may have been predisposed to hydrolyzing phenolic compounds before evolution of their symbiotic relationship with *L. serricorne*, a factor that would promote the symbiotic relationship.

The glucose liberated by the hydrolysis of tannic acid may be used for nutrition by the symbiont, the host, or both, because the glucose liberated by the hydrolysis of salicyl aldehydes can be used by various Chrysomelidae (Rowell-Rahier & Pasteels 1986). The phenolic portion of the molecule may also be used by the symbionts. Many species of fungi are able to hydrolyze tannins (Haslam 1966), and some can use tannic acid as a sole carbon source (Knudson 1913). Other fungi can use related phenolic compounds (Hashimoto 1973) including flavonoids (Westlake & Spencer 1966) as a sole carbon source. Cultures of the yeast from *L. serricorne* also can use flavonoids, tannic acid, and gallic acid as sole carbon sources (Dowd in press b). Nitrogen for amino acid synthesis in the cigarette beetle is apparently provided by uric acid obtained from the host (Jurzitza 1979).

Extracellular symbionts from insects also appear to be capable of detoxification. Cultures of extracellular microorganisms from bark beetles can convert terpenoids to pheromones or their precursors (Brand et al. 1975, 1976). Cultures of the extracellular microorganisms from the apple maggot, *Rhagoletis pomonella* (Walsh), now thought to be nonsymbionts (Rossiter et al. 1983), can metabolize a variety of insecticides (Boush & Matsumura 1967).

My study demonstrates that the intracellular yeasts of *L. serricorne* are actually capable of detoxifying chemicals as they naturally occur in the host. When the yeasts are absent, development in the presence of flavone and tannic acid is significantly retarded compared with controls (Dowd in press b).

Detoxifying activity of the symbiotic yeast from *L. serricorne* occurs in larvae and adults. Yeasts are transmitted to the surface of the eggs from tissues in the reproductive tract of the female (Jurzitza 1979), so they do not need to be present in the gut to ensure transmission. Because feeding by adults is very limited, the presence of symbiotic yeast in adults is difficult to justify on a nutritional basis (Milne 1963). However, because the insects do consume foodstuffs, they may encounter toxic compounds that must be detoxified. Activity of the symbionts in hydrolyzing 1-naphthyl acetate and tannin in the adult suggests one possible reason adults still contain symbionts in the gut region. Thus, this study demonstrated that the symbionts of *L. serricorne* produce enzymes that could hydrolytically detoxify allelochemicals that are encountered in the natural environment. This information suggests that they could assist the insect in surviving on toxic substrates, and as suggested by other studies, also could assist the insects in metabolizing other xenobiotics, such as mycotoxins or insecticides.

**Acknowledgment**

I thank W. R. Halliday, USDA–ARS Stored Product Research and Development Laboratory, Savannah, Ga., for providing the culture of *L. serricorne*.

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Received for publication 21 January 1988; accepted 21 October 1988.