0014-4754/93/111024-03$1.50 + 0.20

A simple method for in vivo testing of glandular enzymatic activity on potential precursors of larval defensive compounds in *Phratora* species (Coleoptera: Chrysomelinae)

P. Soetens, J. M. Pasteels and D. Daloze[a]

Laboratoire de Biologie Animale et Cellulaire, CP 160/12 and [a]Laboratoire de Chimie Bio-organique, CP 160/07, Université Libre de Bruxelles, Avenue Franklin D. Roosevelt 50, B-1050 Bruxelles (Belgium)
Received 21 May 1993; accepted 17 August 1993

Abstract. The presence of glandular β-glucosidase and oxidase-specific activities, and the possible role of phenolglucosides and related compounds as precursors for larval defensive compounds, were directly demonstrated by introducing salicin, saligenin and helicin into the defensive glands of *Phratora* species. *Ph. tibialis* and *Ph. laticollis* that normally secrete endogenously-produced iridoid monoterpenes and do not use phenolglucosides from their food sources as precursors, were able to produce salicylaldehyde from helicin when the latter was introduced into the defensive glands.
Key words. Defensive secretion; β-glucosidase; oxidase; salicin; *Phratora*; Chrysomelinae.

Many phytophagous insects like Chrysomelidae, or leaf beetles, occur in large aggregations on their plant food source, which makes them particularly vulnerable to natural enemies. In the Chrysomelini, a tribe of the subfamily Chrysomelinae, defensive glandular secretions of both larvae and adults are particularly prominent[1]. The wide distribution of iridoid monoterpenes in larval secretions of many species of the subtribes Chysomelina and Phyllodectina, which feed on a great diversity of plants, suggests that these compounds represent the primitive form of the defensive secretions[2].

Some species (such as *Phratora vitellinae* and *Chrysomela* spp.), however, are specialized foliar feeders on Salicaceae (*Salix* and *Populus*) and produce salicylaldehyde. Pasteels et al.[3] demonstrated that salicin, a phenolglucoside widely present in the bark and leaves of Salicaceae, was used as substrate for the production of salicylaldehyde by larvae of *Phratora vitellinae* and *Chrysomela tremulae*. To derive salicylaldehyde from salicin, only two enzymes are needed: a β-glucosidase to hydrolyse salicin into saligenin and glucose, and an oxidase to transform saligenin into salicylaldehyde. The glucose produced is recovered by the larvae. Another species of Chrysomelina, *Gastrolina depressa*, secretes juglone which is probably derived from walnut, its food plant[4]. In this case, the same two classes of enzyme are needed. Pasteels et al.[5], postulated that these two enzymes are also involved in the biosynthesis of the primitive autogenous iridoid monoterpenes, and thus were already present in the ancestors of the species that now use plant compounds as precursors.

Nonspecific β-glucosidase activity was found in the defensive secretions of larvae using autogenous compounds or plant derived precursors for their defense. In contrast, oxidase activity is far more specific, and seems to be the determining factor in the nature of the defensive secretion[6]. A single change in oxidase specificity could allow host plant glucosides to be used as precursors[5,7].

The primary purpose of this work is to provide a method of assaying enzyme activity in larval defensive glands and to test potential precursors of defensive secretions. The comparison between different species of the genus *Phratora* which feed on Salicaceae, and which use phenolglucosides from plant origin for defense (*Ph. vitellinae*) or not (*Ph. tibialis*, *Ph. laticollis*), is useful for understanding mechanisms involved in the switch from de novo biosynthesis of chemical defenses to sequestration of plant-derived defense compounds.

Materials and methods

The study was carried out on third instar larvae of three Salicaceae specialist species of the genus *Phratora: Phratora vitellinae*, a salicylaldehyde producer, and *Ph. tibialis* and *Ph. laticollis*, both iridoid monoterpene producers. All species were reared on leaves of *Salix caprea*, which do not contain phenolglucosides, to eliminate host-plant influences on larval defense. We tested three commercially available compounds: the phenolglucoside salicin (from SIGMA®, 99.4% purity), which is known to be the main precursor of salicylaldehyde, saligenin (salicin aglycone, from SIGMA®, 99.0% purity) and helicin (salicylaldehyde β-D-glucoside, from ALDRICH®, 99.0% purity). All these compounds were dissolved in de-ionized water emulsified with TWEEN 60 (polyoxyethylene sorbitol mono-stearate, 0.11% volume) to reach concentrations equal to, or higher than, solubility (S) in pure water (from ref. 8): salicin = 50.00 μg/ μl (S = 43.48 μg/ μl), saligenin = 66.67 μg/ μl (S = 66.67 μg/ μl), helicin = 33.33 μg/ μl (S = 18.18 μg/ μl).

Table 1. Glandular secretions of the larval *Phratora* feeding on their normal host plant (from[2])

	Chemical nature of the defensive secretion	Host plants
Phratora vitellinae	S	*Salix, Populus*
Phratora laticollis	IM	*Populus*
Phratora tibialis	IM	*Salix*

IM, iridoid monoterpenes (major compound: plagiodial); S, salicylaldehyde.

The larvae of *Phratora* as well as all Chrysomelina larvae ooze secretions from 9 pairs of dorsal exsertile glands when they are disturbed. During discharge, the glandular reservoir is partly everted and covered by a drop of secretion. The latter was removed from larvae of each species using small pieces of filter paper. Then a small drop (approximately equal to the volume of the original drop: circa 15 nl) of the solution being tested was introduced onto the surface of the everted gland, using a thin, handmade glass capillary tube (tip internal diameter $\approx$40 µm). When the larvae inverted the glands, the introduced test solution was drawn into the glandular reservoir. Salicin was tested only on the salicylaldehyde producer, *Ph. vitellinae*, to verify the efficiency of the method. The emulsified water solution was used as control. Each test solution was given to larvae (see table 2 for details) of each species. After 24 h, glandular eversion was stimulated again, the secretions were removed using pieces of filter paper as before, and stored in 0.5 ml methanol at −17 °C. Secretions were analyzed by gas chromatography. 200 µl of the solution in methanol was concentrated by gentle evaporation under a nitrogen flow until 2 or 3 µl remained. 1 µl of this solution was injected directly in the chromatograph (column: PEG-HMW from Rescom™, 25 m, program temperature: 80 to 180 °C, 10 °C/min; detector: 220 °C; injector: 120 °C; total running time: 30 min). Salicylaldehyde was identified by its retention time and co-injection with an authentic sample. We checked that no peak of salicylaldehyde could be detected when either salicin or helicin was injected into the gas chromatograph under the same conditions. Iridoid monoterpenes of *Ph. tibialis* and *Ph. laticollis* have been identified by Pasteels et al.[2] and were not reinvestigated in this work.

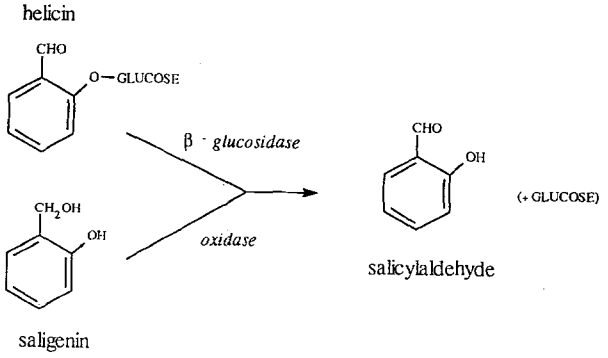

Figure. Enzymatic reactions for salicylaldehyde production from the two precursors helicin and saligenin.

Results and discussion

Compounds found in the larval secretions of the species studied, when feeding on their normal host-plant, are listed in table 1. Under these conditions, salicylaldehyde was only found in the defensive secretion of *Ph. vitellinae*. This species did not produce salicylaldehyde when fed on *S. caprea*, as already reported[9], or when the emulsified water solution alone was introduced into the gland. On the other hand, it was able to use all three precursors: salicin, saligenin and helicin to produce salicylaldehyde (cf. table 2). These results demonstrate the effectiveness of our method, and confirm the presence of both β-glucosidase and oxidase activity in the glandular reservoir in which the derivation of salicylaldehyde from host-plant salicin occurs. For both iridoid monoterpene producers, *Ph. tibialis* and *Ph. laticollis*, GC analysis revealed (cf. table 2) that they are able to use helicin to produce salicylaldehyde in addition to their normal iridoid monoterpene production. In contrast, these larvae could not use saligenin in this way. It has been postulated[5] that the β-glucosidase has a low substrate-specificity, and that the major biochemical factor determining the use of plant precursors, and therefore the chemical nature of the defensive secretion, is the oxidase specificity. Our results show the existence of a β-glucosidase activity (see table 2 and fig.) in the glandular reservoir, but no oxidase activity on saligenin could be detected.

Most species belonging to the genus *Phratora* are Salicaceae specialists, but the ability to use host-plant precursors has only previously been observed in *Ph. vitellinae*.

Table 2. Compounds detected in the secretions of *Phratora* larvae[a] 24 h after introduction of the test solutions into the glands

	Compounds introduced in the glands as water emulsified solution				
	control 1[b]	control 2[c]	helicin	saligenin	salicin
Phratora vitellinae	–	–	S	S	S
Phratora laticollis	IM	IM	S + IM	IM	not tested
Phratora tibialis	IM	IM	S + IM	IM	not tested

IM, iridoid monoterpenes; S, salicylaldehyde; - no IM or S detected.
[a]The larvae were fed upon *S. caprea*. 6–25 secretions were pooled for analysis. [b]No solution introduced into the glands. [c]Emulsified water alone.

However, the use of salicin as a precursor of their defensive secretions may confer two advantages on larvae. First, it provides them with a metabolically less expensive defense than the autogenously synthesized iridoid monoterpenes secreted by the other species. Second, it enables them to mobilize the glucose released through the hydrolysis of salicin[3]. If the specificity of the oxidase is one prerequisite for the use of plant phenolglucoside precursors by the larvae, it is not the only one. Obviously, these precursors must also reach the gland reservoir. They clearly do not in the monoterpene producer, *Ph. tibialis*. Indeed, when the larvae of this species were fed upon *S. caprea* to which helicin was added, salicylaldehyde could not be detected in its glandular secretions, whereas high quantities of salicylaldehyde but no helicin were present in the faeces. This result demonstrates that ingested helicin is not transported into the glands of *Ph. tibialis*, but hydrolyzed (probably in the gut) and excreted. In contrast, when *Ph. vitellinae* was fed with helicin, large amounts of salicylaldehyde were found in its defensive secretion, but neither salicylaldehyde nor helicin could be detected in the faeces, indicating that all the ingested helicin was transported into the glandular reservoir and hydrolyzed.

The technique described herein allows an easy in vivo testing of glandular enzymatic activity towards natural or synthetic potential precursors of defensive compounds in chrysomeline larvae which possess exsertile defensive glands.

Acknowledgements. We are grateful to the Belgian Fund for Joint Basic Research (grant # 2.451390) and to the Communauté française de Belgique (Arc 93–3318) for financial support. Soetens is indebted to the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture for the award of a fellowship. We thank A. Foggo, M. Rowell-Rahier and S. Duffey for critical reading of the manuscript.

1 Price, P. W., Insect Ecology, Wiley, New York, 1984.
2 Pasteels, J. M., Rowell-Rahier, M., Braekman, J.-C., and Daloze, D., Biochem. Syst. Ecol. *12* (1984) 395.
3 Pasteels, J. M., Rowell-Rahier, M., Braekman, J.-C., and Dupont, A., Physiol. Ent. *8* (1983) 307.
4 Matsuda, K., and Sugawara, F., Appl. Ent. Zool. *15* (1980) 316.
5 Pasteels, J. M., Duffey, S., and Rowell-Rahier, M., J. chem. Ecol. *16* (1990) 211.
6 Duffey, S., and Pasteels, J. M., unpubl. results.
7 Pasteels, J. M., and Rowell-Rahier, M., Ent. Gener. *15* (1991) 227.
8 Windholz, M. (Ed.), The Merck Index, Merck & Co. Inc. 1976.
9 Rowell-Rahier, M., and Pasteels, J. M., in: Proc. 5th int. Symp. Insect-Plant Relationship, p. 73. Eds Visser, J. H. and Minks, A. K., Centre for Agricultural Publishing and Documentation, Wageningen 1982.