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INSECTICIDE ACTIVITY OF SURFACTINS AND ITURINS FROM A BIOPESTICIDE *BACILLUS SUBTILIS* COHN (S499 STRAIN)

L.K. ASSIÉ[1], M. DELEU[2], L. ARNAUD[1], M. PAQUOT[2], P. THONART[3], Ch. GASPAR[1] & E. HAUBRUGE[1]

[1] Unité de Zoologie générale et appliquée
[2] Unité de Chimie Biologique et Industrielle
[3] Unité de Bio-industrie,
Faculté universitaire des Sciences agronomiques de Gembloux, B-5030 Gembloux, Belgium

ABSTRACT

Surfactin C_{14}, surfactin C_{15}, and iturin C_{15} are lipopeptides purified from *Bacillus subtilis* (S499 strain). They were incorporated to artificial diet of the fruit fly *Drosophila melanogaster* (Meigen) (Diptera, Drosophilidae) to assess their potential insecticide activity. Surfactins with long fatty acid chain (C_{14} and C_{15}) showed insecticide effect on the fruit fly, *D. melanogaster*. On the contrary, iturin was not toxic to fruit fly *D. melanogaster*. At 100ppm, surfactin C_{14} and C_{15} showed respectively 85.4 and 92.6 % adults mortality after one-day exposure. F1 progeny fly emergence inhibition by C_{14} and C_{15} were respectively 79.8 % and 91.3 %. To check whether the biocide activity of lipopeptides was due to their surface-active properties, detergent Triton X100, SDS, CTAB and Tween 80 were tested. No adult mortality was recorded with the detergents but Triton X100 and SDS showed F1 progeny emergence inhibition similar to that of surfactins. We showed that there was a dose-response activity with surfactin C_{15}.

Keywords: *Bacillus subtilis*, lipopeptides, surfactin, insecticide, toxicity, *Drosophila melanogaster*

INTRODUCTION

Surfactins and iturins are lipopeptides produced by *Bacillus subtilis* Cohn (Delcambe, 1965a; Delcambe, 1965b).
The general structure is a peptide ring of seven amino acids linked to a fatty acid chain. The length of the fatty acid chain can vary from C_{13} to C_{16} for surfactins and from C_{14} to C_{17} for iturins, giving different homologous compounds and isomers (n, iso, anteiso) for each lipopetide (Besson *et al.*, 1977; Peypoux *et al.*, 1973; Peypoux *et al.*, 1984) (Peypoux *et al.*, 1979; Peypoux *et al.*, 1991).
Surfactins have high surface and interface activity (Deleu *et al.*, 1999b; Deleu *et al.*, 1999c; Maget-Dana & Ptak, 1992a; Razafindralambo *et al.*, 1998) and develop different important biological properties including antiviral, antibacterial, and hemolytic activities (Bernheimer &Avigad, 1970; Vollenbroich *et al.*, 1997a; Vollenbroich *et al.*, 1997b).
The biological activity of surfactin directly relies on its interactions with biomembranes, as shown by several studies using different membranes models and analysis techniques (Deleu *et al.*, 2001; Deleu *et al.*, 1999a;

Gallet *et al.*, 1999; Grau *et al.*, 1999; Maget-Dana &Ptak, 1992b;1995; Sheppard *et al.*, 1991; Thimon *et al.*, 1993).
Iturins were tested with success in different veterinary applications (De Keyser *et al.*, 1960) for their antimycotic properties. More recent studies (Pusey *et al.*, 1988; Pusey &Wilson, 1984) showed that *Bacillus subtilis* could be a potential biocontrol agent of crop diseases as harmful fungi and bacteria. Gokte and Swarup (1988) also showed that cells and spores of *B. subtilis* were toxic to nematodes *Meloidogyne incognita, Heterodera cajani, Heterodera avenae* and *Anguina tritici*. Moreover, Lazare *et al.* (1996) reported the potential insecticide activity of spores and metabolites of *B. subtilis* on *Drosophila melanogaster* and their oviposition reduction ability on *Callosobruchus maculatus*.
In this work, we investigate the insecticide activities of iturins and surfactins using *D. melanogaster* as insect model. Their activity was compared to these of classical detergents in order to determine the relationship between the surface-active properties and the insecticide activity.

MATERIALS AND METHODS

Bacteria cells culture

Lipopeptides were produced from a culture medium of *Bacillus subtilis* as described by Jacques *et al.*, (1999). The cells were grown in 20 liters fermenter (Biolafitte, Poissy, France) at 30°C for 72 hours with the medium. The stirrer speed was 300 rpm and aeration rate was 0.3 vvm. The pH was maintained at 7.0 during culture.

Extraction of lipopeptides and purification

Surfactins with a β-hydroxy fatty acid chain of 14 or 15 carbons atoms (S_{C14} or S_{C15}) and iturin with a β-amino fatty acid chain of 15 carbons atoms (I_{C15}) were produced and purified as described previously (Razafindralambo *et al.*, 1993).
Primary structure and purity of the different lipopeptides were ascertained by analytical RP-HPLC (Chromspher 5 μm C18 column, 0.46 x 25 cm, Chrompack, Middelburg, The Netherlands), amino acid analysis, and electrospray mass spectrometry (Fininigan MAT 900 ST) measurements. Purity was superior to 95 %.

Insect culture

All insects were obtained from a laboratory strain (*meCS*) of *Drosophila melanogaster* originated from Canton (USA). The *meCS* strain had no prior exposure to insecticide and were cultured on standard corn meal medium

at 25±1°C; 60%±5 RH. At these conditions, the fly development time were scaled at 14±2 days.

Toxicity bioassays

Lipopetides were dissolute in bicarbonate buffer 0.1 M and incorporated to the fly artificial diet at different concentrations. A control was performed with bicarbonate buffer. Thirty unsexed fly (5-10 days old) were placed into each 50ml-jar containing 10 g of artificial diet mixed with purified lipopeptides and closed with wadding. The effect of lipopetides on adult mortality and F_1 progeny reduction were observed.
Effects of classical detergents Tween 80 (Sigma n° 9005-65-6), Triton X-100 (Sigma n° 92046-34-9), SDS (sodium dodecyl sulfate, Fluka n° 71725), and CTAB cationic (Sigma n° 57-09-0) were also tested to verify whether or not there was relationship between the insecticide activity of purified lipopetides and their surface-active properties. Detergents were incorporated to the fly diet at 100 ppm.
For all trials, assessments of adult mortality and F_1 emergence inhibition were performed as follow: After 24 hours, all flies were removed and the number of killed adults was recorded. From day 10 after adult removal, the infested diets were observed each 2 days intervals and the number of emerged adults were recorded.

RESULTS

The results of biocontrol activity of surfactins, iturins and the other commercial detergents are showed.

Toxicity bioassays

Surfactin toxicity to *D. melanogaster* at a concentration of 100 ppm showed high toxicity on adult flies (table 1). The surfactin C_{14} showed 85.4 % adults mortality whereas surfactin C_{15} showed 92.6 % adults mortality after 24 hours exposure but data were not significantly different ($p>0.05$, Tukey's pair means test). It appeared that surfactins inhibited significantly the F_1 progeny emergence (i.e. table 1). Tukey's pair means comparison showed a significant difference between controls and treated ($P<0.05$). On the other hand, there was significant biological activity difference between C_{14} and C_{15} ($P = 0.001$; $F= 25.51$).
Iturin C_{15} failed to show insecticide activity either on the adult or on the eggs and the larva of *D. melanogaster* (i.e. table 1)

Table 1: Toxicity of surfactins and iturins on *Drosophila melanogaster*. Both iturins and surfactins were tested at 100 ppm. SC_{14} and SC_{15}: surfactin with a fatty acid chain containing respectively 14 carbons and 15 carbons; IC_{15}: iturin with a fatty acid chain containing 15 carbons

Lipopeptides	% of adult mortality*	% emergence reduction*
SC_{14}	85.4 ± 9.2	79.5 ± 10.0
SC_{15}	92.6 ± 6.0	99.6 ± 0.2
IC_{15}	0	2.0 ± 4.8

* (mean ± SE)

The effect of surfactin C15 concentration were studied (Figure 1). At 10 ppm and 50 ppm, no adult mortality was recorded nevertheless. However, the inhibition of emergence of F_1 progeny was positively correlated with concentrations.

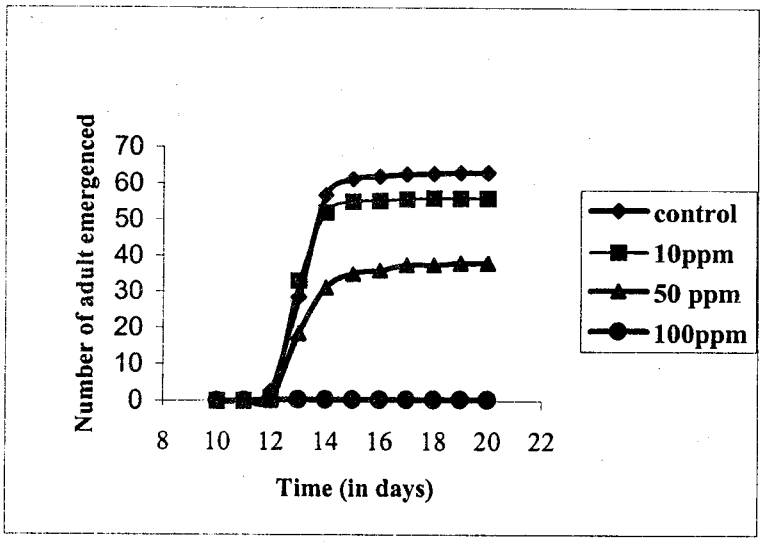

Figure 1: Doses-response effect of surfactin SC_{15} on F1 progeny emergence of *Drosophila melanogaster*

Table 2 shows toxicity results for four classical detergents. None of them was toxic to adults. However, the Triton X-100, SDS and TWEEN 80 showed high F_1 generation adult emergence inhibition (98.86±0.7 %, 71.4±9 % and 51.2±20 %). CTAB showed relatively low F_1 progeny emergence inhibition (8.3±26 %).

Table 2: Toxicity of several commercial detergents on *D. melanogaster* at 100 ppm.

Detergents	% of adult mortality	% of emergence inhibition*
Triton X 100 (non ionic)	0	98.9 ± 0.7
CTAB (cationic)	0	8.3 ± 26.0
SDS (anionic)	0	71.4 ± 9.0
Tween 80 (non ionic)	0	51.2 ± 20.0

* (mean ± SE)

DISCUSSION

The insecticide activity of two lipopeptides from *Bacillus subtilis*, surfactin and iturin, was evaluated.

According to our results, surfactins show a pronounced insecticide activity while iturin has no effect. A difference of behavior between the two molecules has already been demonstrated for other biological activities like hemolytic, antifungal and antibacterial activities (Maget-Dana *et al.*, 1992; Thimon *et al.*, 1992), and for several physico-chemical properties like surface activity (Razafindralambo *et al.*, 1997) and foaming properties (Razafindralambo *et al.*, 1998).

According to Hourdou and Besson (1994), these differences are mainly due to the difference in their primary structure. Indeed, although the two lipopeptides are cyclic heptapeptides linked to a fatty acid, three main differences exist in their structure: (i) the nature of the β function of the fatty acid, (ii) the absence of tyrosyl residue in surfactin, and (iii) the ionic nature of the peptide ring (surfactin is anionic while iturin is non ionic).

Two toxic effects of surfactin on *D. melanogaster* were found. First, an insecticide effect against adult *D. melanogaster* and, second, it was found to reduce the F_1 progeny emergence.

The toxicity of surfactins to adults of *D. melanogaster* certainly was caused by surfactin consumption by the insect but the target and the mode of action inside insect remained unknown and was not documented. As lipopeptides were incorporated to the diet, it insured topical contact also.

One of the mechanism suggested to be involved in the different biological activities of surfactin (cytolytic effect on yeast and fungi (Vater, 1986); antitumoral effect (Kameda *et al.*, 1974), and antiviral activity (Itokawa *et al.*, 1994) is its ability to disorganize the cell membrane physical properties and to cause metabolism disruption. Other metabolites from the genus *Bacillus*, especially δ-endotoxin, from *Bacillus thuringiensis*, has been shown to cause structure disorganization and atrophy of the midgut of insect leading to their death two to five days following the toxin consumption (Gill *et al.*, 1992; Huber *et al.*, 1981). Flies mortality caused by surfactins occurred earlier (24h) leading to two hypotheses. (i) Surfactins do not act the same manner like δ-endotoxin or their target is different. (ii) A similar mechanism could be involved in the insecticide activity of surfactin

towards the insects. If this assumption is consistent, the swiftness of toxicity observed with surfactins should certainly build on the chemical differences between lipopeptides and the δ-endotoxin (protein). The *Bt* toxin is a protoxin, which must be activated in the midgut of insect before binding to the target while surfactin of *B. subtilis* could be immediately active.

The activity of surfactin towards the F_1 progeny emergence suggests that surfactin acts on the ovaries or is toxic to eggs and/or to larva of insect. Surfactins could act by integrating the insect larva or eggs and by disturbing the membrane permeability as reported by Gokte and Swarup (1988) with the nematodes. This assumption was strengthened by the fact that eggs, larva and pupae were both in permanent contact with the surfactin treated media. The target of the surfactin is not easy to be clearly defined. While assessing the potential control of strains of *B. subtilis*, Lazare *et al.* (1996) had assumed that the target of *B. subtilis* and its metabolites could be either toxic to the larva or the eggs. In fact, it reduced the reproductive output but there were not consistent proof of real target.

The F_1 progeny emergence reduction caused by surfactins should not build on the F_0 population mortality. In fact, F_0 mortality occurred 24 hours after treatment; but we found that flies led eggs less than one hour after the contact with the diet (unpublished data). They were assumed to have laid eggs before their death. Moreover, the assumption was consistent by comparing surfactin activities to that of the detergents (i.e. Triton X 100, Tween 80, SDS). No F_0 mortality occurred with detergents but they induced the reduction of F_1 progeny.

By comparing the activity towards the F_1 progeny of the two tested surfactins (SC_{14} and SC_{15}), we can suppose that the fatty acid chain exerts a significant role. A higher number of carbon atoms in the fatty acid chain increases the activity of surfactin. The critical micellar concentration (CMC) of surfactin, which reflects its detergency property, is also affected by the carbon atom number of its fatty acid chain (Razafindralambo, 1996). A higher number of carbon atoms decreases CMC, or in other terms, increases the detergency ability of the surfactin. The correlation between the insecticide activity towards the F_1 progeny emergence and the detergency property can suggest that a detergent effect is at the origin of the mechanism. The same hypothesis has already been suggested for the mechanism of iturin on erythrocytes (Thimon *et al.*, 1992). The dependence of the insecticide activity towards the concentration is another argument for this hypothesis. A concentration of 100 ppm which is above the CMC (CMC of surfactin C15 is 19μM according to Razafindralambo (1996)) is more efficient.

However, the detergent effect is not the only mechanism involved in the insecticide activity of the surfactin towards the F_1 progeny emergence since no direct relation exist between the CMC of classical detergent ($CMC_{triton\ X\text{-}100}$ = 0.30mM; $CMC_{tween\ 80}$ = 0.012 mM, CMC_{CTAB} = 1.0 mM; CMC_{SDS} = 2.30mM according to Black (2002) and their effect on the reproductive output.

The interaction between the surfactant and the cellular membrane of the eggs or of the larva can also have an effect by perturbing the physical properties of the membrane. Consequently, the ionic nature and structure of the surfactant can also play an important role.
It emerged from this work that the structure of the lipopeptide has a crucial role in their insecticide activity. The fatty acid chain of the surfactin is partially involved in their mechanism of action. Further investigations are necessary to understand the exact mechanism of action of surfactin as insecticide and to locate the target in insect cells or organs.

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