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Coronatin-2 from the entomopathogenic fungus *Conidiobolus coronatus* kills *Galleria mellonella* larvae and incapacitates hemocytes

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Abstract

Coronatin-2, a 14.5 kDa protein, was isolated from culture filtrates of the entomopathogenic fungus *Conidiobolus coronatus* (Costantin) Batko (Entomophthoramycota: Entomophthorales). After LC–MS/MS (liquid chromatography tandem mass spectrometry) analysis of the tryptic peptide digest of coronatin-2 and a mass spectra database search no orthologs of this protein could be found in fungi. The highest homology was observed to the partial translation elongation factor 1a from *Sphaerosporium equinum* (protein sequence coverage, 21%), with only one peptide sequence, suggesting that coronatin-2 is a novel fungal protein that has not yet been described. In contrast to coronatin-1, an insecticidal 36 kDa protein, which shows both elastolytic and chitinolytic activity, coronatin-2 showed no enzymatic activity. Addition of coronatin-2 into cultures of hemocytes taken from larvae of *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), resulted in progressive disintegration of nets formed by granulocytes and plasmatocytes due to rapid degranulation of granulocytes, extensive vacuolization of plasmatocytes accompanied by cytoplasm expulsion, and cell disintegration. Spherulocytes remained intact, while oenocytes rapidly disintegrated. Coronatin-2 produced 80% mortality when injected into *G. mellonella* at 5 µg larva^{-1}. Further study is warranted to determine the relevance of the acute toxicity of coronatin-2 and its effects on hemocytes *in vitro* to virulence of *C. coronatus* against its hosts.

Keywords: insecticidal protein, mycotoxin, cytotoxicity, HPLC, LC–MS/MS

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Introduction

Entomopathogenic fungi are natural ecological regulators of insect populations. In recent years, crop protection has been trending toward integrated pest management using fungi as insecticides and more environmentally friendly alternatives to chemical pesticides (Purwar & Sachan, 2006; Srivastava *et al.*, 2011). Although several species of entomopathogenic fungi are employed as biological control agents of insect pests, mycopesticides are still niche products (Shah & Pell, 2003; Faria & Wraight, 2007; Glare *et al.*, 2012). The major limitations to the use of entomopathogenic fungi, i.e., their relatively low efficacy and the length of time it takes for the fungus to kill target insects, might be overcome by the construction of transgenic fungi which overexpress their own enzymes or produce insect host molecules for the augmentation of virulence (St. Leger *et al.*, 1996; Fan *et al.*, 2012). Insect-derived molecules which regulate critical physiological processes, for instance trypsin-modulating oostatic factor derived from *Aedes aegypti*, and the *Manduca sexta* diuretic hormone and *Solenopsis invicta* β-neuropeptide expressed in *Beauveria bassiana* (Balsamo) Vuillemin (Acomycota: Hypocreales) increase the virulence of the fungus toward insects (Keyhani, 2012).

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Alternatively, new toxic compounds might be isolated from the members of Entomophthorales that are known to be rapid killers of insects (Samson *et al.*, 1988).

The overall process of insect infection by all entomopathogenic fungi is a multi-step process. Fungi attack insect hosts via attachment to the exoskeleton, which represents the first and possibly the most important barrier to fungal infection, followed by the production of enzymes for the degradation and penetration of the insect cuticle (Gillespie *et al.*, 2000; Fang *et al.*, 2012; Ortiz-Urquiza & Keyhani, 2013). The combination of the enzymatic degradation of the cuticle and mechanical pressure of the growing hyphae, allows the cuticular barrier to rupture and reach the nutrient-rich contents of the insect hemocoel. During the colonization phase, the pathogen produces a range of secondary metabolites. Some of the metabolites might be toxic and/or suppress the host's immune system, resulting in mycosis and, ultimately, the death of the host (Clarkson & Charnley, 1996; Vilcinskas *et al.*, 1999).

The susceptibility or recalcitrance of various insect species to fungal invasion results from several factors, including differences in the structure and composition of the exoskeleton, the presence of antifungal compounds in the cuticle (fungistatic fatty acids, phenoloxidases and melanins), and the efficiency of the cellular and humoral defense reactions of the invaded insect (Vilcinskas & Götz, 1999; Boguś *et al.*, 2007, 2010; Gołębiowski *et al.*, 2011). The host defense system of insects relies on several innate cellular and humoral reactions that are securely interrelated. Upon the aggressor's invasion, the onset of enzymatic cascades leads to confined blood clotting and melanization, involving the production of cytotoxic molecules Hoffmann (1995). Intruders face a battery of cell defenses, including the phagocytosis of small pathogens and the formation of multicellular layers that encapsulate large intruders and limit damages by forming a physical barrier between the self and non-self (Sugumaran, 1998). Key humoral elements include highly fungitoxic phenoloxidase, reactive oxygen species and antimicrobial peptides (Hajek & St. Leger, 1994; Gillespie *et al.*, 2000; Ottaviani, 2005; Wojda *et al.*, 2009).

Several lines of evidence indicate that entomopathogenic fungi have evolved additional strategies to evade host immune defenses. They can release immunosuppressive extracellular proteases and secondary metabolites, such as beauverolides, cyclosporins, cytochalasins and destruxins (Vilcinskas & Götz, 1999; Fröbius *et al.*, 2000; Gillespie *et al.*, 2000; Castrillo *et al.*, 2005; Schrank & Vainstein, 2010). During the colonization of the hemocoel, hyphal bodies of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales), mask the cell wall by coating it with collagen-like material (Wang & St. Leger, 2006). However, the effects of fungal metabolites on hemocytes, immunocompetent insect blood cells, remain underexplored to date.

In light of the need for further research to identify and characterize new metabolic toxins from entomopathogenic fungi and their effects on the immune system of insects, we initiated an experiment on an isolate of *Conidiobolus coronatus* (Costantin) Batko (Entomophthoramycota: Entomophthorales), a cosmopolitan soil fungus that causes the rapid death of susceptible insects, presumably due to the secretion of toxic metabolites (Prasertphon & Tanada, 1969; Domsch *et al.*, 1980; Boguś & Scheller, 2002; Boguś *et al.*, 2007; Wieloch *et al.*, 2011). Of 43 insect species tested, 25 were infected by *C. coronatus* after exposure to sporulating colonies (Boguś *et al.*, 2012). Larvae of *Galleria mellonella* have been shown to be an excellent model organism for *in vitro* toxicology and pathogenicity testing (Harding *et al.*, 2013). This paper describes the successful isolation of coronatin-2, an insecticidal 14.5 kDa protein. Coronatin-2 kills *G. mellonella* hemocytes cultured *in vitro*, but its mode of action is unlike that displayed by coronatin-1, which was isolated earlier from the same material (Wieloch *et al.*, 2011).

Materials and methods

Fungus

Conidiobolus coronatus (Entomopthorales), isolate number 3491, originally isolated from *Dendrolaelaps* spp., was obtained from the collection of Professor Bałazy (Polish Academy of Sciences, Research Center for Agricultural and Forest Environment, Poznań). The isolate induced 100% mortality in *G. mellonella* larvae exposed to sporulating colonies (Boguś *et al.*, 2012).

The fungal cultures were routinely maintained in 90 mm Petri dishes at 20°C with cyclic changes of light (L:D 12:12), stimulating sporulation (Callaghan, 1969) on a Sabouraud agar medium with the addition of homogenized *G. mellonella* larvae to a final concentration of 10% wet weight. Sporulating colonies were briefly washed with sterile water in order to harvest the conidia. One hundred microliter portions of suspension each containing around 50 conidia, were used for inoculations. Cultivation was performed at 20°C in 500 ml Erlenmeyer flasks containing 250 ml of minimal medium (MM) as described by Bania *et al.* (2006) but without shaking. Four weeks after inoculation, the mycelia were removed by filtration through Whatman no. 1 filter paper and the cell-free filtrate served for coronatin-1 (Wieloch *et al.*, 2011) and coronatin-2 preparations.

Insects

Galleria mellonella was cultured and maintained as described previously (Wieloch *et al.*, 2011). Five-day-old last instar larvae with weights of 190–210 mg were used for the experiments. They were exposed to a fungal pathogen to check the pathogenicity potential of fungal colonies; they received injections of protein fractions and chlorfenvinphos, or were bled to obtain hemocytes.

The larvae were exposed for 18 h to fully grown 7-day-old sporulating *C. coronatus* colonies. The insects which were exposed for 18 h to sterile Sabouraud agar medium served as controls. The condition of the exposed and control larvae were monitored daily. The exposure of tested insects to a *C. coronatus* colony for 18 h was found to be the most efficient method resembling the natural infection process (Wieloch & Boguś, 2005).

The toxicity of protein fractions was tested by injecting 5 µl of tested sample through the insect proleg into the hemocoel of ten *G. mellonella* larvae. Before the injection, the larvae were water-anaesthetized through submersion in tap water for 5–10 min. This procedure has no effect on the survival of the larvae. Control larvae were injected with 5 µl distilled water or bovine serum albumin (BSA; Sigma) solutions. BSA concentrations in solutions injected into control larvae were the same as the concentrations of fungal proteins (fp) used in the tests. Commercial insecticide, chlorfenvinphos (Sigma), was used as a positive control. Insecticide dissolved in dimethyl sulfoxide (DMSO; Sigma) was injected into *G. mellonella* larvae at concentrations ranging from 0.0001 to 50 µg larva^{-1} in order

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to establish LD_{50}. Twelve groups of larvae, each consisting of ten larvae, were formed. Each tested larva obtained 5 µl of chlorfenvinphos solution, while ten control larvae obtained the same amount of DMSO. Three independent experiments were performed on 390 larvae in total. After injection, the insects were transferred to clean Petri dishes with food and kept in their growing conditions.

Primary hemocyte preparation

Before bleeding, larvae were washed with distilled water and then immersed briefly in 70% (v/v) ethanol to sterilize their surfaces. Larval hemolymph was collected from incisions of the proleg. Freely dripping hemolymph was collected into sterile polypropylene 1.5 ml centrifuge tubes preloaded with Grace insect medium (GIM, Sigma), pH 6.6, supplemented with 75 µg ml^{-1} gentamycin and 1 µg ml^{-1} phenylothiourea (PTU, Sigma) to prevent melanization. The hemolymph was gently mixed with GIM. The hemolymph suspensions were immediately transferred to sterile 48-well cell culture plates (Corning) and incubated at 30°C. Each well contained 10 µl of fresh hemolymph in 300 µl of supplemented GIM. During the first 8 h, the condition of the primary hemocyte cultures was monitored at 15 min intervals with the use of an Olympus inverted microscope; afterwards, inspections were performed daily. Coronatin-2 isolated from *C. coronatus* post-incubation filtrates, was added to the primary hemocyte cultures at a concentration of 117 µg ml^{-1}.

Coronatin-2 purification

The cell-free filtrate was dialyzed (membrane cut-off 12,000) against three changes of distilled water at 3°C for 48 h, lyophilized and suspended in a minimal volume of distilled water, and afterwards sterilized by filtration (Corning syringe filter 0.22 µm) and applied to a Shodex KW-803 column fitted to a Waters HPLC (high performance liquid chromatography) system. The column was equilibrated and eluted with a 50 mM KH_2PO_4 buffer supplemented with 0.1 M KCl, pH 6.5. Elution was carried out at a flow rate of 0.5 ml min^{-1}. Chromatographic peaks, detected at 280 nm, were individually collected, dialyzed against distilled water and lyophilized. The collected fractions were assayed for protein concentration, enzymatic activity and toxicity against *G. mellonella* larvae. Fraction No. 6 was applied on a Microcon Ultracel-30 Membrane (Millipore). Samples of 300 µl each were centrifuged for 15 min at 5000 *g* and 2°C according to the producer's manual. The purity of protein fractions was checked by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Determination of protein concentration

Protein concentration was determined with the Protein Assay (Bio-Rad) and directly at 280 nm according to Schleif & Wensink (1981). BSA was used as the standard.

SDS–PAGE and molecular mass determination

Gel electrophoresis was carried out by Laemmli's method (1970), using 15% separating gel and 9% stacking gel with 0.1% SDS (Sigma), pH 8.9. Silver staining according to Morrissey (1981) was used to visualize protein bands. Molecular weight standards (Fermentas), mixtures of native proteins ranging in size (14.4–116 kDa, 10–60 kDa and 14.4–66.2 kDa, respectively) served as reference proteins. The apparent M_r (relative molecular mass) value of purified protein was calculated from semi-log plots of standard protein M_r vs. migration distances.

LC–MS/MS analysis

Coronatin-2 was analyzed using the LC–MS/MS (liquid chromatography tandem mass spectrometry) technique with the Nano-Acquity LC system (Waters) and LTQ Orbitrap Velos mass spectrometer (Thermo Electron Corp.) in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Protein in the excised polyacrylamide band was reduced 30 min at 56°C with 100 mM ditiotreitol (DTT; Sigma), alkylated 45 min in a darkroom at room temperature with 0.5 M iodoacetamide (Sigma) and digested overnight with trypsin (10 ng ml^{-1}; Sigma) at 37°C according to Shevchenko *et al.* (2007). Peptide mixtures were applied to a RP-18 trapping column (nanoACQUITY Symmetry® C18-Waters) using water containing 0.1% fluoroacetic acid (FA; Sigma) as a mobile phase and then transferred to a nano-HPLC RP-18 column (nanoACQUITY BEH C18-Waters) using an acetonitrile gradient (0–30% AcN in 45 min) in the presence of 0.1% formic acid with a flow rate of 250 nl min^{-1}. A column outlet was directly coupled to the ion source of mass spectrometer operated in data-dependent mode such that up to ten most abundant precursors were subjected to collision induced dissociation (threshold = 1E6, isolation width = 3 Da, normalized collision energy = 40%, activation $q = 0.25$, activation time = 30 ms) and HCD (threshold = 1E5, isolation width = 3 Da, activation time = 10 ms, m/z 300–1800). Other mass spectrometer parameters were the following: an electrospray voltage of 2,0 kV, capillary temperature 230°C, resolution of 15,000 and 7500 was defined for parent and daughter ions, respectively. A blank run ensuring there was no cross-contamination from previous samples preceded each analysis.

Protein identification

The acquired LC–MS/MS raw data were processed using a Mascot Distiller (v. 2.5, Matrix Science) followed by a Mascot Search (Mascot Server v.2.4, Matrix Science) against the NCBInr database, restricted to fungus (6,373,884 protein sequences). The search parameters included the following: mass tolerance for precursor 30 ppm, product ions mass tolerance 0.1 Da, trypsin enzyme specificity (one missed cleavage site allowed); a fixed modification: carbamidomethylation (C); variable modifications oxidation (M) and Carboxyethyl (K). Peptides with a Mascot Score exceeding the threshold value calculated by the Mascot procedure were considered to be positively identified.

Enzymatic assays

The activities of elastase, chymotrypsin, N-acetyloglucosoaminidase (NAGase), chitinase and lipase were measured in the *C. coronatus* homogenized mycelium, post-incubation filtrate and a purified coronatin-2 fraction. The enzymatic activities were measured spectrophotometrically (BioMate spectrophotometer) and fluorimetrically (Hitachi F-4500 spectrofluorimeter) using suitable synthetic substrates (all from Sigma). A unit of enzymatic activity was defined as the amount of enzyme

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that caused an increase in absorbance (ΔA) or fluorescence (ΔF) by 0.1 min^{-1}. Elastolytic activity was measured using the chromogenic substrate N-Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide in a 100 mM Tris–HCl buffer, pH 8.5. The reactions were performed in polystyrene cuvettes containing the appropriate amount of proteinase, 0.25 mM substrate concentration and reaction buffer to a final volume of 1 ml. Progress curves at A_{410}, following the initiation of a reaction through the addition of substrate, were followed using the BioMate spectrophotometer. Chymotrypsin activity was measured using fluorogenic substrate Ala-Ala-Phe-7-amido-4-methylcoumarin in 10 mM Tris–HCl buffer, pH 7.5. The reactions were performed in quartz glass cuvettes containing the appropriate amount of enzyme preparation, 0.27 mM substrate concentration, and reaction buffer to a final volume of 1 ml. Fluorescence data were read at $Ex = 380$ and $Em = 460$ nm, respectively. Chitinase activity was measured using 0.013 mM concentration of fluorogenic substrate 4-Methylumbelliferyl β-D-N-N′-diacetylchitobioside in 10 mM Tris–HCl buffer, pH 7.5. Fluorescence was read at $Ex = 340$ and $Em = 450$ nm. NAGase activity was measured using 0.3 mM concentration of chromogenic substrate 4-nitrophenyl-N-acetyl-β-D-glucosoaminide in 10 mM Tris–HCl buffer, pH 7.5. Absorbance was read at 405 nm. Lipase activity was measured using 0.13 mM concentration of fluorogenic substrate 4-methylumbelliferyl oleate in 10 mM Tris–HCl, pH 7.5. Fluorescence was read at $Ex = 340$ and $Em = 450$ nm.

Statistics

The obtained results (insect mortality and enzyme activities measured in fungal fractions) were tested by the parametric *t*-test and analysis of variance (ANOVA) at significance levels of $P \leq 0.01$ and ≤ 0.05. Data were checked for normality prior to analyses. The STATISTICA software (StatSoft Polska) was used in all statistical analyses, including the evaluation of data normality, performing a nonlinear regression, and calculating LD_{50}.

Results

Pathogenicity of C. coronatus

Conidiobolus coronatus infection was manifested in *G. mellonella* larvae by insects' immobilization and the appearance of black spots on the cuticle at the termination of exposure to the fungal colony. A few hours later, spots spread and joined with neighboring spots. The cuticle of dying insects was completely black. All larvae exposed to *C. coronatus* ($n = 60$) were dead around 48 h after contact with the fungus.

Preparations for the coronatin-2 isolation

Conidia from a fungal colony were used to inoculate 5 liters of MM as described in the Materials and methods section. The post-incubation filtrate was dialyzed, lyophilized and dissolved in a small amount of sterile water. Consequently, a suspension was obtained containing 43.5 mg of fp released from the mycelium into the incubation medium. Injections of crude filtrate preparation into *G. mellonella* larvae at concentrations of 5 and 11.5 μg fp $larva^{-1}$ resulted in 90 and 100% mortality, respectively. In contrast, injections of mycelium homogenates at the protein concentrations of 5 and 12 μg fp $larva^{-1}$ resulted in 10 and 50% mortality, respectively. The mortality of controls ($n = 10$ for each tested concentration) that received BSA

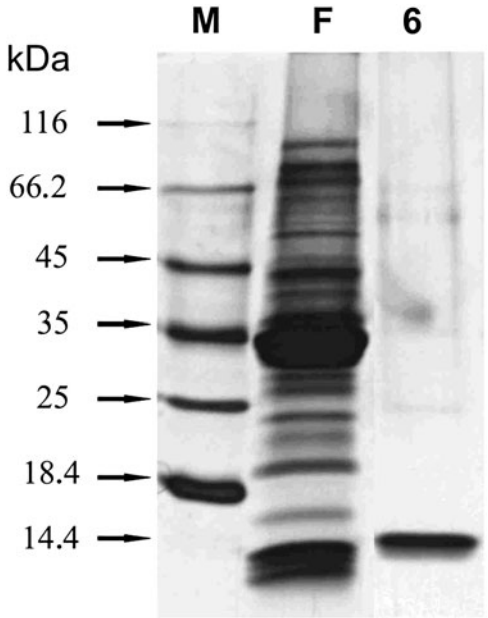

Fig. 1. SDS–PAGE analysis of proteins accumulated in the culture medium (MM) during *C. coronatus* cultivation. M, molecular weight markers; F, proteins released by the fungus into the incubation medium; 6, fraction No. 6 obtained after the HPLC purification.

solutions at the same concentrations as the fp (5 and 12 μg $larva^{-1}$) was 0 and 10%, respectively. In an additional negative control consisting of larvae injected with 5 μl of sterile water ($n = 10$), no mortality was noticed. A positive control consisted of larvae injected with chlorfenvinphos at concentrations ranging from 0.0001 to 50 μg $larva^{-1}$ ($n = 10$ for each concentration; all concentrations tested in three independent experiments). The toxicity of chlorfenvinphos to the test insects was dose dependent. Injection of 0.1 μg was sufficient to induce 90–100% mortality in all tested groups. Insecticidal activity of low chlorfenvinphos concentrations (0.0001, 0.0005, 0.001 and 0.005 μg $larva^{-1}$) differed significantly from the activity of high (1, 5, 10 and 50 μg $larva^{-1}$) and middle (0.05, 0.1 and 0.5 μg $larva^{-1}$) concentrations (*t*-value ranged from −2.91 to −6.6; *F*-value range: 0–21; df = 2,4; *P*-value range: 0.005–0.042). The LD_{50} with confidence interval was estimated to be 0.009 ± 0.003 μg $larva^{-1}$.

The same post-incubation filtrate was the source of coronatin-1, which was described earlier (Wieloch *et al.*, 2011), and coronatin-2. An SDS–PAGE analysis of fp accumulated in the culture medium revealed a predominance of proteins with molecular masses ranging from 30 to 35 kDa outnumbering both, low and high molecular weight proteins (fig. 1). The activities of all five tested enzymes (e.g., elastase, chymotrypsin, NAGase, chitinase and lipase) were present in the preparation obtained from the post-incubation filtrate, while no activity of chitinase and chymotrypsin was found in the mycelia homogenate (table 1). Elastolytic activity in the filtrate was 358 times higher than in the mycelium homogenate ($F = 559, 278.4$; df = 2,4; $P = 0.0002$), while the activity of NAGase was only 1.9 times higher in filtrates than in the mycelium ($F = 46.04$; df = 2,4; $P = 0.19$). The slightly higher lipase activity in the filtrate compared with the mycelium

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Table 1. Enzymatic activities present in mycelium homogenate, crude filtrate preparation and in coronatin-2 fraction, respectively.

	Mycelium (homogenate)	Post-incubation medium (filtrate)[1]	Coronatin-2
Elastase (ΔA/min/mg ± SD)	$8.6 \times 10^{-2} \pm 0.5 \times 10^{-2}$	30.8 ± 3.5	No activity
Chymotrypsin (ΔF/min/mg ± SD)	No activity	$15.5 \times 10^{-2} \pm 1.4 \times 10^{-2}$	No activity
NAGase (ΔA/min/mg ± SD)	$68.5 \times 10^{-2} \pm 7.9 \times 10^{-2}$	1.3 ± 0.5	No activity
Chitinase (ΔF/min/mg ± SD)	No activity	$17.6 \times 10^{-2} \pm 0.6 \times 10^{-2}$	No activity
Lipase (ΔF/min/mg ± SD)	$2.3 \times 10^{-2} \pm 0.9 \times 10^{-2}$	$5.0 \times 10^{-2} \pm 1.3 \times 10^{-2}$	No activity

[1]Data from Wieloch *et al.* (2011) (the same post-incubation filtrate was used to isolate coronatin-1 and coronatin-2).

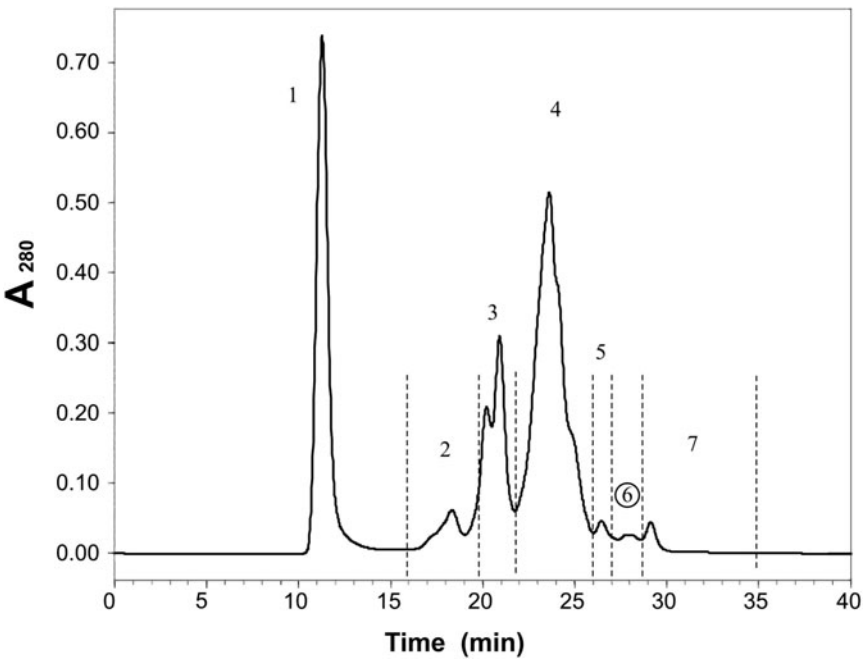

Fig. 2. The first step of the coronatin-2 purification. HPLC isolation: Shodex KW-803 column equilibrated and eluted with 50 mM KH2PO4 buffer supplemented with 0.1 M KCl, pH 6.5. Flow rate 0.5 ml min^{-1}. Peaks numbered 1–7 were collected and assayed for protein concentration, enzymatic activity and toxicity against *G. mellonella* larvae as described in the Materials and methods section.

homogenate was statistically insignificant ($F = 2.12$; df = 2,4; $P = 0.07$). In the filtrate, elastase activity was 23.7 times higher than the activity of NAGase ($F = 41.8$; df = 2,4; $P = 0.0002$), 199 times higher than the activity of chymotrypsin ($F = 64{,}049.6$; df = 2,4; $P = 0.0002$), 175 times higher than the activity of chitinase ($F = 310{,}000$; df = 2,4; $P = 0.0002$) and 616 times higher than the activity of lipase ($F = 74{,}417$; df = 2,4; $P = 0.0002$), respectively.

Purification of coronatin-2

The toxic metabolites present in the crude filtrate of *C. coronatus* retained by dialysis (molecular mass cut-off of 12 kDa) were excluded from a Shodex KW-803 column in seven peaks (fig. 2). The obtained fractions were dialyzed and lyophilized as described in the materials and methods section. After being dissolved in 100 µl sterile water, the protein concentration in each sample was determined, while the homogeneity of fractions was checked using SDS–PAGE. The insecticidal potentials of collected fractions were tested using ten *G. mellonella* larvae for each examined fraction.

When injected into *G. mellonella* larvae (fp 6.3 µg), fraction No. 6 induced 20% mortality. An SDS–PAGE analysis of fraction No. 6 showed a dominating polypeptide band about 14.4 kDa accompanied by few impurities (fig. 1). In the second step of purification, fraction No. 6 was applied on a Microcon Ultracel-30 Membrane and centrifuged. The sample obtained was analyzed using SDS–PAGE. The arrow in fig. 3 presents pure coronatin-2 as judged by SDS–PAGE. The behavior of coronatin-2 on polyacrylamide gels under denaturing conditions suggested a single polypeptide chain with a molecular mass of about 14.5 kDa. Coronatin-2 injected into *G. mellonella* larvae at concentrations of 1 and 5 µg $larva^{-1}$ ($n = 10$ for each concentration) resulted in 20 and 80% mortality, respectively. Mortality in the BSA controls which received the BSA solution at the same concentrations as coronatin-2 ($n = 10$ for each concentration) was not noticeable (0%).

The processing of LC–MS/MS data using the Mascot Distiller followed by a Mascot Search against the NCBInr database showed no conclusive protein identification. After LC–MS/MS analysis of the tryptic peptides derived from coronatin-2, the MS/MS spectra could not be matched with

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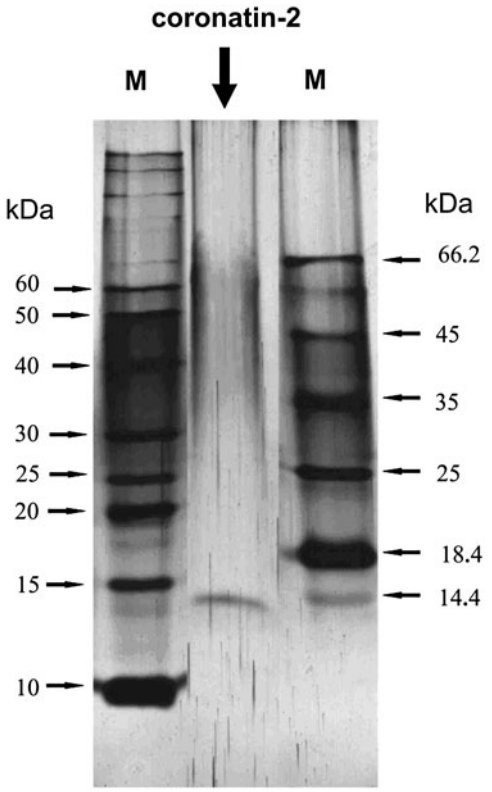

Fig. 3. SDS–PAGE analysis of the purified coronatin-2. M, molecular weight markers. Vertical arrow indicates coronatin-2.

any predicted proteins from the species *C. coronatus* (23 protein sequences in the database), genus *Conidiobolus* (57 protein sequences), order *Entomophthorales* (215 protein sequences) or even kingdom Fungi (6, 329, 343 protein sequences). The highest homology was observed to the partial translation elongation factor 1a, *Sphaerosporium equinum* (protein sequence coverage, 21%), with only one peptide sequence.

Cytotoxic activity of coronatin-2

A microscopic inspection of *G. mellonella* hemolymph, both freshly collected from insects as well as suspended in GIM, revealed the presence of all five morphologically and behaviorally distinct hemocyte types: prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids. Shortly after beginning the primary *in vitro* cultures of *G. mellonella* hemocytes, plasmatocytes forming numerous pseudopodia adhered to the bottom of the culture well. Thirty minutes later, the prohemocytes disappeared and, spherulocytes were freely floating in the culture medium while plasmatocytes and granulocytes formed clusters (fig. 4).

The addition of coronatin-2 into the primary *in vitro* cultures of *G. mellonella* hemocytes resulted in the rapid disappearance of granulocytes, the fracturing of oenocytes, and a massive expulsion of cytoplasm by plasmatocytes, accompanied by their vacuolization. Coronatin-2 stimulated pseudopodia atrophy and, consequently, the disintegration of clusters formed by cultured hemocytes. Phase contrast micrographs of hemocytes cultured in the presence of coronatin-2 show the extensive vacuolization of plasmatocytes followed by the disintegration of these cells, resulting in an openwork-like pattern observed 50 h after supplementing the culture medium with the fp. No hemocytes were observed exhibiting morphological symptoms of apoptosis (blebs on cell membrane), which is in contrast to the effects of coronatin-1. Spherulocytes in cultures with the addition of coronatin-2 remained unhurt.

Discussion

Entomopathogenic fungi that infect insects have received considerable attention from scientists for their potential in the biological control of pests. Insect pathogenic fungi have evolved to counter their host's defenses using a combination of enzymes to penetrate the cuticle and access the nutrient-rich contents of the hemocoel and a wide range of secondary metabolites that may suppress the host's immune system (Dubovskiy *et al.*, 2013). Research on toxic metabolites of entomopathogenic fungi is motivated by the importance of the analysis of their role in mechanisms of pathogenesis. Earlier investigations have revealed that the insecticidal activity of *C. coronatus* (the relatively little-investigated cosmopolitan soil fungus) may be due to the toxic metabolites (Prasertphon & Tanada, 1969; Boguś & Scheller, 2002). The insecticidal potential of coronatin-2 is significantly lower than that of commercial insecticide chlorfenvinphos, which acts on the nervous system of many organisms as an inhibitor of acetylcholinesterase and was used here as a positive control. Chlorfenvinphos injected into *G. mellonella* larvae efficiently induced the insects' death. LD_{50} was estimated as 0.009 ± 0.003 μg larva^{-1}, i.e., 0.045 ± 0.015 μg g^{-1} of the insect body, which is in accordance with the data presented in the report of the Advisory Committee on Pesticides (ACP, 1994). Due to the limited amount of pure coronatin-2 available, the estimation of LD_{50} was not possible. Coronatin-2 injected into *G. mellonella* larvae at concentrations of 1 and 5 μg larva^{-1} (i.e., 5 and 25 μg g^{-1} of insect body) resulted in 20 and 80% mortality, respectively. The mechanism of the action of chlorfenvinphos on target and non-target organisms is well known (ACP, 1994), while the contribution of coronatin-2 to the death of insects infected by *C. coronatus* needs to be examined.

The virulence of fungal entomopathogens involves several steps: conidial adhesion to the cuticle, germination and cuticle penetration, dissemination within the host, avoidance of host immune responses, and transmission from the host. Each step is influenced by a range of integrated intrinsic and external factors, which ultimately determine the pathogenicity (Clarkson & Charnley, 1996; Boguś *et al.*, 2007; Gołębiowski *et al.*, 2011). The forces responsible for the interaction between *C. coronatus* conidia and the cuticle of the insect are not known. In contrast to the *M. anisopliae*, *C. coronatus* does not produce an appressorium or any other specialized structure that firmly attach the fungus to the host epicuticle (Professor Andrzej Batko, personal communication).

The contribution of fungal enzymes in mycosis is well documented. The ability of parasitic fungi to invade susceptible insect species directly through the cuticle is mediated by their capacity to release digestive enzymes, which assist the penetration process (for review see Clarkson & Charnley, 1996; Khachatourians, 1996; St Leger & Bidochka, 1996; Vilcinskas & Götz, 1999). High activities of elastase, chymotrypsin, NAGase, chitinase and lipase, which we measured in the post-incubation medium, demonstrate the accumulation of cuticle-degrading enzymes released by growing hyphae of

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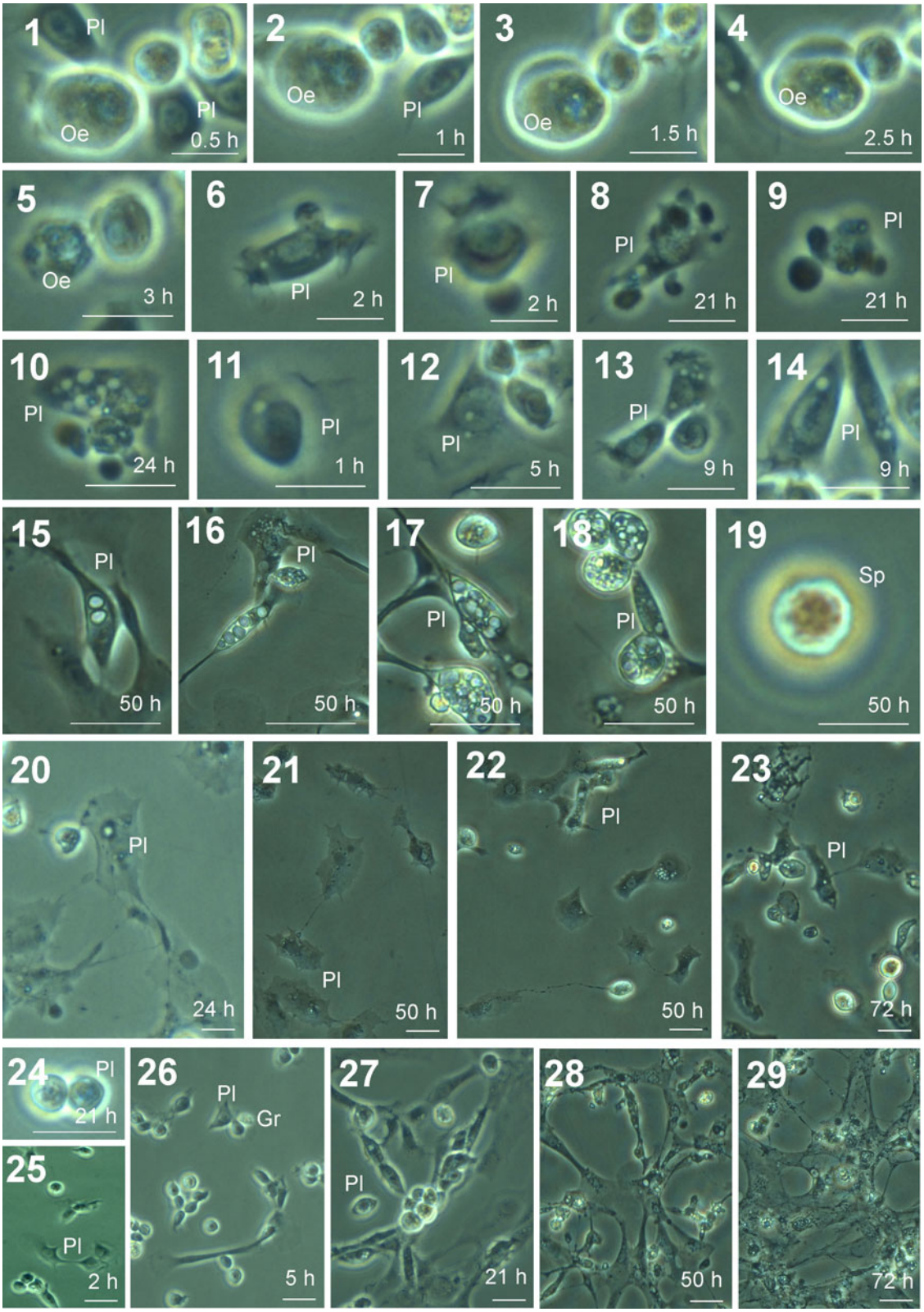

Fig. 4. Effects of addition of coronatin-2 (177 µg ml^{-1}) into primary *in vitro* cultures of *G. mellonella* hemocytes. Disintegrating oenocyte (1–5), plasmatocytes expelling cytoplasm (6–10), vacuolized plasmatocytes (11–18), unaffected spherulocyte (19), disintegrated hemocyte clusters (20–23), dividing control plasmatocytes (24), clustering control hemocytes (25–29). Oe, oenocyte; Pl, plasmatocyte; Sp, spherulocyte; Gr, granulocyte. Scale bars: 20 µm.

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C. coronatus. Proteo-, chitino- and lipolytic activities in the *C. coronatus* culture filtrates, as well as in the mycelial and spore homogenates, are dependent on growing conditions, being highest in liquid MM (Czygier *et al*., 2000; Wieloch & Boguś, 2007; Włóka & Boguś, 2010). Fatty acids present on insect epicuticles strongly affect the growth of *C. coronatus* and the ability of enzymes produced by this fungus to hydrolyze cuticle components (Gołębiowski *et al*., 2008, 2011; Boguś *et al*., 2010; Włóka *et al*., 2013, 2014). The extracellular presence of all of the enzymes that we tested reflects adaptation for the effective digestion of insect cuticle. It remains unclear why the lipase of *C. coronatus* grown in rich Luria Broth medium remains in an intracellular compartment, while in MM it is released into the culture medium. The behavior of the other examined enzymes is similar in both culture media (Wieloch & Boguś, 2007).

The involvement of the *C. coronatus* extracellular proteases in the invasion of the insect host was confirmed by several reports. *C. coronatus* secretes a range of proteinases: 6.8-kDa alkaline serine proteinase, 23-kDa proteinase I, 19-kDa proteinase II (probably resulting from the autoproteolysis of proteinase I), 27–28.5 kDa subtilisin-like proteinase, and a 30–32 kDa subtilisin-like serine protease (Sutar *et al*., 1991; Phadatare *et al*., 1992; Tanksale *et al*., 2000; Bania *et al*., 2006). The EST library of *C. coronatus* revealed three subtilisins (one highly similar to the aqualysin 1 precursor from *Thermus aquaticus*), a trypsin similar to Alp1 from *Cochliobolus carbonum*, a metalloprotease similar to MepB from *Aspergillus fumigatus* and an aspartyl protease similar to pepsinogen from *A. niger* (Freimoser *et al*., 2003). The insecticidal properties of fungal proteinases were described (Kucera & Samsinakova, 1968; Kucera, 1981). The participation of extracellular fungal proteases in the suppression of the insect immune system is well documented. Proteases from *B. bassiana* and *M. anisopliae* inhibit the phagocytic activity of plasmatocytes isolated from *G. mellonella*, and impair the attachment, spreading and formation of filopodia and cytoskeleton structures (Vilcinskas & Götz, 1999). The multiplicity of proteinases produced in cuticle-containing media suggests that *C. coronatus* has a plasticity in exploiting a broad range of proteins of various origins (Freimoser *et al*., 2003; Bania *et al*., 2006). Fungi belonging to the genus *Conidiobolus* are considered to be phylogenetically basal members of the Entomophthorales, with limited host ranges (Evans, 1989). *Conidiobolus coronatus* is, however, described as an opportunistic pathogen with a relatively wide host range (Dromph *et al*., 2001; Freimoser *et al*., 2003). *Conidiobolus coronatus* causes the rapid death of susceptible insects, most likely due to the secretion of toxic metabolites (Prasertphon & Tanada, 1969; Boguś & Scheller, 2002; Boguś *et al*., 2007; Wieloch *et al*., 2011). The factors determining the host specificity and virulence of *C. coronatus* remain poorly understood and, therefore, complementary data are required.

Surveys carried out in our laboratory have revealed that *C. coronatus* releases several insecticidal proteins into the culture medium; however, it is challenging to isolate and characterize them due to their very low level of accumulation (Boguś & Scheller, 2002; Wieloch, 2006). A 30-kDa insecticidal protein produced by *C. coronatus* was the first toxic compound isolated from any entomophthorealan fungus (Boguś & Scheller, 2002). Recently, we presented a successful isolation of coronatin-1, an insecticidal 36 kDa protein that displays both elastolytic and chitinolytic activities (Wieloch *et al*., 2011). In the present work, we describe the isolation of another insecticidal protein produced by *C. coronatus*. Coronatin-2, a 14.5 kDa protein judged on the basis of SDS–PAGE, seems to be a novel protein. Coronatin-2, in contrast to coronatin-1, presented no enzyme activity in our tests. LC–MS/MS analysis showed no homology of coronatin-2 with any known protein. Sequenced genome of the *C. coronatus* NRRL 28638 isolate (clinical source) encodes 10,572 proteins. How many proteins encodes genome of the isolate number 3491 (mite source) used in our studies, remains obscure. From 18 protein sequences (isolate NRRL 28638) with predicted molecular mass of 14.4–14.5 kDa, 13 sequences encode hypothetical proteins with no attributed function, while five sequences encode biogenesis of lysozyme-related organelles complex-1 subunit 1, cytochrome b5, ribosomal protein L18, DUF 1764-containing protein and a component of cytochrome P450, respectively (http://genome.jgi.doe.gov/Conco1). It seems improbable that any of these five proteins can possess insecticidal properties. Moreover, one cannot exclude that coronatin-2 might be initially synthesized as a high molecular weight precursor, which is subsequently cleaved and processed to give rise to the mature cytotoxic molecule. Only 23 proteins from *C. coronatus* were analyzed by MS, and no homology was found between their resulting mass spectra and that of coronatin-2. While peptide sequencing of coronatin-2 would be possible using the LC–MS/MS technique, this approach requires a relatively high amount of pure protein (1–10 μg), which was unavailable in the present study. The isolation of portions of coronatin-2 would be required before sequencing the protein and identifying the encoding nucleotide sequence.

We have provided evidence that coronatin-1 kills *G. mellonella* hemocytes cultured *in vitro*. Moreover, coronatin-1 incorporates into artificial planar lipid bilayers, forming potassium channels in a similar manner as ampullosporin A and alamethicin (Wieloch *et al*., 2011). However, coronatin-1's mechanism of action on insect hemocytes still remains obscure. Coronatin-2 also kills *in vitro* hemocytes from *G. mellonella*, but does not incorporate into artificial planar lipid bilayers (unpublished data). When added into *in vitro* culture of *G. mellonella* hemocytes, coronatin-2 induces the rapid disappearance of granulocytes, the cracking of oenocytes, and the pronounced expulsion of cytoplasm by plasmatocytes, accompanied by their vacuolization and disintegration. The expulsion of cytoplasm has been described in vertebrate oligodendrocytes, the myelin-producing cells of the central nervous system. The final stages of myelination involve the expulsion of cytoplasm to form compact myelin (O'Meara *et al*., 2011). The discarding of cytoplasm also occurs during differentiations of *Drosophila melanogaster* spermatids. Cystic bulges and waste bags, which contain cytoplasm expelled during spermatid individualization, display many features of apoptic corpses (Arama *et al*., 2003). More experiments are needed to establish whether coronatin-2 also induces cytoplasm expulsion by *G. mellonella* plasmatocytes in the apoptosis-like mechanisms, including the activation of caspases.

Like coronatin-1, coronatin-2 stimulates pseudopodia atrophy and, consequently, the disintegration of nets formed by cultured hemocytes, suggesting deep changes in their cytoskeletons. As reviewed by Vilcinskas & Götz (1999), healthy plasmatocytes from *G. mellonella* display *in vitro* filopodia formation accompanied by the formation of cytoskeleton structures such as bundled actin filaments, so-called stress fibers and a network of microtubules. In contrast, plasmatocytes treated with destruxins, cyclic peptide mycotoxins, remained round in shape and demonstrated a reduced capacity to adhere and spread on glass surfaces, which is a characteristic

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reaction of healthy cells. The incubation of isolated plasmatocytes with destruxins disturbs the intracellular distribution of actin and tubulin. Similar pathogenesis in the distribution of both filaments has been documented in plasmatocytes from *G. mellonella* larvae infected with *M. anisopliae* (destruxins are the most abundant secondary metabolites of this pathogenic fungus). The intervention of destruxins with subcellular structures of plasmatocytes impairs the cellular immune response of the insect, which is manifested by the reduction of phagocytic activity. Plasmatocytes isolated from *G. mellonella* larvae infected with *M. anisopliae* or *in vitro* treated with destruxins, exhibited characteristic symptoms of apoptosis: apoptic blebs on the cell membrane, swollen nuclei and clumped chromatin (Vilcinskas & Götz, 1999). *In vitro* assays with beauverolide L and cyclosporin A indicated no adverse effects on the attachment or spreading of isolated *G. mellonella* plasmatocytes. These two mycotoxins inhibit in dose-dependent manner phagocytic activity and induce cytoskeleton alterations that differed, however, from those observed in plasmatocytes from infected *G. mellonella* larvae or reported from other fungal secondary metabolites (Vilcinskas *et al*., 1997, 1999). FITC-phalloidin staining of hemocytes obtained from *C. coronatus*-infected *G. mellonella* larvae revealed that actin fibers in the cytoskeleton are unorganized (Ligęza-Żuber, unpublished). In order to determine the intracellular distribution of actin and tubulin in *G. mellonella* hemocytes treated with coronatin-2, it is necessary to isolate portions of this compound. In contrast to coronatin-1, the supplementation of the culture of *G. mellonella* hemocytes with coronatin-2 leaves spherulocytes unharmed and does not induce any morphological symptoms of apoptosis. It seems probable that *C. coronatus* is armed with numerous secondary metabolites, both proteins and low-molecular weight compounds, which selectively act on insect immunocompetent cells.

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