

Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana*

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A toxic protein, bassiacridin, was purified from a strain of the entomopathogenic fungus *Beauveria bassiana* isolated from a locust, using chromatographic methods. The final toxic fraction contained between 0.1 and 0.3% of the proteins of the crude extract. Bassiacridin showed no affinity for ion exchangers, was characterised as a monomer with a mol. wt of 60 kDa and an isoelectric point of 9.5, and exhibited β -glucosidase, β -galactosidase and N-acetylglucosaminidase activities. Injection of fourth instar nymphs of *Locusta migratoria* with the pure protein at relatively low dosage (3.3 μ g toxin g body wt⁻¹) caused a rate of mortality near to 50%. The effects of the crude and pure fractions were characterized at tissular and cellular levels. The formation of melanised spots on tracheae and air sacs and of melanised nodules in contact with the fat body was observed in injected locusts. Alterations of the fine structure of epithelial cells of tracheae, air bags, and integument were also revealed. The insecticidal protein showed a specific activity against locusts. Bassiacridin is different from the other macromolecular toxins of entomopathogenic fungi already described. Microsequencing of peptides generated by trypsin digestion of bassiacridin confirmed that it is a novel molecule and showed that it exhibits a probably limited similarity with a chitin binding protein from yeast.

INTRODUCTION

The entomopathogenic fungus *Beauveria bassiana* has been used as microbial agent to control many important insect pests worldwide, and has provided the only practical microbial control of locusts and grasshoppers which have few known viral or bacterial diseases (Mason & Erlandson 1994, Lomer *et al.* 2001, Quesada-Moraga 2002). This fungus is a key regulatory factor in populations of the Moroccan locust *Dociostaurus maroccanus* in Spain, and field trials show promise for *B. bassiana* as a biocontrol agent of this important insect pest (Hernández-Crespo & Santiago-Álvarez 1997, Jiménez-Medina, Aldebis & Santiago-Álvarez 1998). Isolate EADm 90/2-Dm of *B. bassiana*, obtained from *D. maroccanus* in the breeding area of La Serena (Spain), is currently being developed as a microbial insecticide to control this pest (Santiago-Álvarez *et al.* 2002).

Entomopathogenic fungi secrete a wide range of toxic metabolites in artificial culture and, in a few cases, *in vivo* (Mazet, Hung & Boucias 1994, Vey 1998, Vey, Hoagland & Butt 2001). Most of the fungal toxins

described are low molecular weight (mol. wt) secondary metabolites, mainly cyclic peptides, showing antibiotic or insecticidal properties (Strasser, Vey & Butt 2000, Vey *et al.* 2001). In contrast, information concerning high mol. wt fungal toxins is scarce, and to our knowledge, the only macromolecular compound that has so far been purified to homogeneity is hirsutellin A, a molecule with ribonucleic activity produced *in vitro* by *Hirsutella thompsonii* (Mazet & Vey 1995, Liu *et al.* 1996).

A major hindrance to the development of entomopathogenic fungi as mycopesticides has been that 5–10 d are required after application to kill an insect pest. Thus, a key aim of most recent work has been to increase the speed of kill and thus improve commercial efficacy, mainly by optimising production, stability and application of the inoculum (St Leger & Screen 2001). But another promising approach is to add genes encoding for insecticidal molecules to the fungus to reduce lethal times, as already performed by St Leger *et al.* (1996) who constructed an improved myco-insecticide overproducing the cuticle-degrading Pr1 protease required for pathogenesis. This approach requires precise information on the mechanisms involved in pathogenesis and host death, which are key components amenable to improvement via biotechnology (Hegedus & Khachatourians 1995, St Leger

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& Screen 2001). With this aim, investigation of high molecular weight toxins is of great interest as they may play an important role in fungal pathogenesis. Moreover, since protein synthesis generally requires the expression of a single gene, high mol. wt fungal toxins may be simpler to produce by recombinant DNA techniques than the cyclic peptides, whose synthesis requires the activity of large multifunctional enzymes (Peeters, Zocher & Kleinhauf 1988).

Quesada & Vey (2003) recently showed that filtrates of isolate 90/2-Dm of *B. bassiana* and their dialysates were toxic upon injection to *Locusta migratoria* nymphs. The toxicity was also present when the molecules were precipitated by 90% saturation of ammonium sulphate and desalted by gel filtration on Sephadex G-25. It was therefore suggested that the biologically active compounds were proteinic, although they were not identified. As *B. bassiana* is known to produce low mol. wt compounds such as beauvericin and bassianolide (Vey *et al.* 2001), toxicity was expected in dialysates prepared from filtrates, yet the dialysates had no lethal effect on the insect host.

In agreement with these previous findings, this study focused on the purification and characterisation of a high molecular weight toxin secreted by *B. bassiana* isolate EABb 90/2-Dm. This toxin seems to be specifically active against locusts and its effects at tissular and cellular levels have been studied on locust tissues and on insect cells cultivated *in vitro*. This is the first report of the purification of a high mol. wt toxin from *B. bassiana*.

MATERIAL AND METHODS

Insects

Newly emerged fourth instar *Locusta migratoria* nymphs (mean weight 361 ± 31 mg) from our long established healthy colony were used as the insect host. New locusts from other laboratories were regularly introduced into this colony to maintain the vigour of the insects.

For specificity studies, the toxic fractions were also injected into the other locust species *Schistocerca gregaria* and *Dociostaurus maroccanus*, into larvae of the lepidopterans *Galleria mellonella* and *Spodoptera littoralis* and into the coleopteran *Tenebrio molitor*.

Newly emerged fourth instar *S. gregaria* nymphs were obtained from our long established healthy colony. Newly emerged fourth instar nymphs of *D. maroccanus* were obtained from the stock colony of Cándido Santiago Álvarez (University of Córdoba). The nymphs were maintained under controlled conditions 13:11 h L:D photoperiod, at 26 ± 4 °C and 40–60% rh. Wooden cages (50 × 50 × 50 cm) were used to maintain populations of nymphs, with a 60 W bulb inside supplying extra heat during the light period. The locusts were fed with dry wheat bran and wheat

(*Triticum* sp.) seedlings (Quesada-Moraga & Santiago-Alvarez 2000).

G. mellonella larvae were reared to sixth instar (L6) in a rearing room with ultraviolet sterilization and maintained at 25 °. Larvae were confined to glass jars and maintained on an artificial diet composed of 250 g mixed honey (Ickowicz, Bollène, France); 220 ml glycerol (Quarrechim, Béziers, France); 340 g wheat meal (local supplier); 100 g yeast powder (Fisher Scientific Labosi, Elancourt, France); 50 g pure bee wax (Ickowicz, Bollène, France) and 1.75 g nipagin (VWR International, Fontenay sous bois).

S. littoralis larvae from our laboratory colony were reared in plastic boxes divided into 3 × 2.5 × 2 cm individual compartments (Mino-Gaillard, France), at 25 °, 65% rh, and 16:8 h L:D photoperiod. They were maintained on an artificial diet composed of 170 ml distilled water; 28 g corn flour (INRA-UPAE, Jouy en Josas, France); 7.5 g brewer's yeast (Fisher Scientific, Labosi); 7 g wheat germ (local supplier); 5 g agar (CNI, Rouen); 1 g ascorbic acid (VWR International); 0.4 g nipagin (VWR International); 0.3 g benzoic acid (VWR International) and 0.28 g formaldehyde (VWR International).

Larvae of the mealworm beetle *T. molitor* were reared on wheatmeal at 25 °, with 65% rh in a dark room.

Fungal strain and cultivation

The *Beauveria bassiana* EABb 90/2-Dm isolate was obtained from Cándido Santiago-Alvarez (University of Córdoba, Spain). It was isolated from a nymph of the Moroccan locust, *Dociostaurus maroccanus* (*Orthoptera: Acrididae*), in the breeding area of 'la Serena' in Badajoz (Spain) in 1990 and characterised according to morphology of conidiophores and conidia. Recent molecular studies performed at the University of Córdoba based on the comparison of ITS 1 and 2 indicate that among the studied isolates, 90/2 Dm differs from other *B. bassiana* strains isolated from the same locust, related locust species, and coleopteran and lepidopteran insects (Maranhao 2003).

Strain EABb 90/2-Dm was deposited in the Spanish Collection of Culture Types (CECT; Edificio de Investigación, Campus de Burjasot, 46100 Burjasot, Valencia) with the accession no. CECT 20371. Slant multispore cultures of strain 90/2 were grown on malt agar (MA Biokar Diagnostic, Beauvais, France) at 25 ° in the dark and then stored at 4 °. Conidial suspensions were prepared by scraping conidia from well sporulated 21 d old slant cultures into sterile distilled water, estimating their concentrations using a haemocytometer. Viability of conidia was checked before preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all the experiments, germination rates were higher than 90%. To prepare a primary culture, 1 ml of a suspension of conidia (adjusted to 1×10^7 conidia ml⁻¹) was inoculated into 25 ml of Adamek's liquid medium: 40 g

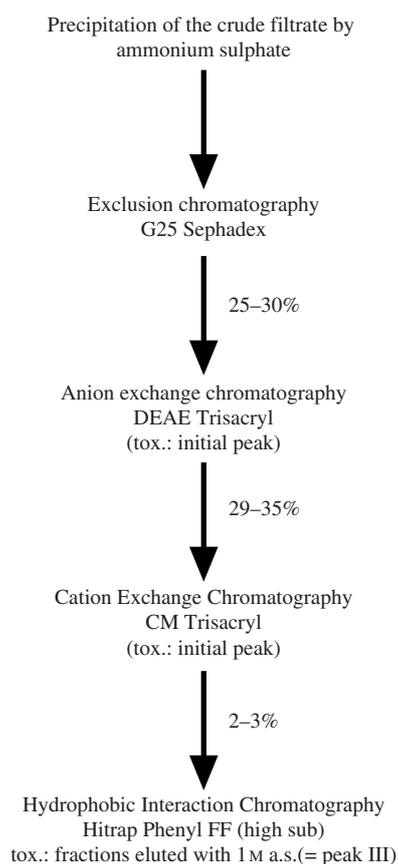


Fig. 1. Diagrammatic representation of the purification steps of bassiacridin. Numbers indicate protein yield of the toxic fraction (in %) for each step. Tox., fractions toxic for *L. migratoria*; a.s., ammonium sulphate.

glucose (Sigma-Aldrich, St Quentin Fallavier, France), 40 g yeast extract (Biokar Diagnostics), 30 g corn steep liquor (Sigma-Aldrich, St Quentin Fallavier, France), and 1000 ml distilled water. This was placed in a 100 ml Erlenmeyer flask and cultured at 25 ° on a rotatory shaker (CFL 1092) at 110 rpm for 4 d. To inoculate secondary cultures for large-scale growth of the fungus, 2 ml of the primary culture were transferred into 250 ml of the same medium in a 1 L Erlenmeyer flask, and cultured on a TR 125 INFORS shaker at 25 ° and 110 rpm for 7 d, before removing the mycelial material by filtration through Whatman N°. 3chr filter paper (VWR International).

Purification procedure

At each step of the purification (Fig. 1) the fractions obtained during the chromatographic runs were tested for their biological activity by injection into fourth instar *Locusta migratoria* nymphs.

Step 1. Precipitation of the crude filtrate by ammonium sulphate, and G25 Sephadex exclusion chromatography

Proteins from the filtrate of isolate EABb 90/2-Dm, precipitated with 90% of saturation of ammonium

sulphate, were collected by centrifugation at 10 000 g for 30 min. The precipitate was submitted to a gel filtration through a Sephadex G-25 (Pharmacia) column (2.5 × 30 cm) in 50 mM Tris/HCl buffer at pH 8.0. Seven ml fractions were collected, and those corresponding to the initial peak were pooled and concentrated before being injected.

Step 2. DEAE Trisacryl ion exchange chromatography

The desalted toxic fraction was applied to a DEAE Trisacryl (IBF) column (2.5 × 16 cm) pre-equilibrated with 50 mM Tris/HCl buffer at pH 8.0. The column was washed and bound material was then eluted stepwise with the above buffer containing 0.1, 0.2, 0.3, 0.5 and 1 M of NaCl at a constant flow rate of 1.0 ml min⁻¹. Four ml fractions were collected, and those from each peak were pooled and concentrated.

Step 3. CM Trisacryl ion exchange chromatography

The pooled toxic fractions from the DEAE column were dialysed against 50 mM sodium acetate buffer pH 5.0, and further resolved by CM Trisacryl (IBF) ion exchange chromatography (2.5 × 16 cm) column in 50 mM sodium acetate buffer, pH 5.0. The bound material was eluted stepwise with the same buffer containing 0.1, 0.3, 0.5 and 1 M of NaCl at a constant flow rate of 1.0 ml min⁻¹. Four ml fractions were collected, and those from each peak were pooled and concentrated.

Step 4. Hitrap phenyl FF (high sub) hydrophobic interaction chromatography

The pooled active CM Trisacryl fractions were dialysed against 50 mM Tris/HCl buffer at pH 8.0 with 2 M ammonium sulphate and further applied to a Hitrap Phenyl FF (high sub) hydrophobic interaction chromatography (0.7 × 2.5) column from Amersham Bioscience, Saclay, France. The column was washed with the same buffer at a flow rate of 0.5 ml min⁻¹ and the bound materials were eluted stepwise with the 50 mM Tris/HCl buffer at pH 8.0 containing 1.5, 1.0, 0.5 and 0 M of ammonium sulphate at the same constant flow rate. Fractions of one ml were collected, and those from each peak were pooled, concentrated and evaluated for insecticidal activity.

Gel electrophoresis and isoelectrofocusing

Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate (SDS-PAGE) according to Laemmli (1970) on a 12% acrylamide slab gel (stacking gel: 5%) with a Mighty Small II SE 250 apparatus (Hoeffer Scientific Instruments, San Francisco). Aliquots of 15 µl of solution from each peak were loaded into each well and gels were silver stained using the Bio-Rad Silver Stain Kit. The mol. wt

of the protein was determined by measuring its mobility in comparison to a series of molecular mass standards in the range of 14.4–97.4 kDa (Bio-Rad). The purity of Bassiacridin was determined by native PAGE on a 8–12% gradient gel in the presence of 1.5 M Tris-HCl buffer at pH 8.8 (Hames 1990). The protein was loaded in the gel and electrophoresed using tris-glycine buffer, pH 8.3. Gels were silver stained as above.

The determination of the isoelectric point of the pure insecticidal protein was performed with the same equipment, and with 1% ampholytes (Bio-Lyte 3/10 or 3/5; Bio-Rad) by procedures described in the manufacturer's handbook. Gels were loaded with Protein Test Mixture 9 (Serva) used as a standard and silver stained.

Determination of total protein concentration

Total protein concentration was determined with the Bio-Rad Protein Assay based on the method of Bradford (1976). Bovine serum albumin was used as the standard.

Determination of enzyme activity

The enzymatic activities of the pure toxin were examined with the API ZYM semi-quantitative micro method system according to the manufacturer's instructions (BioMeyrieux, Marcy l'Étoile, France). Each microcupule containing dehydrated chromogenic enzyme substrates was inoculated with 65 μ l of pure toxin at 0.1 g l⁻¹. The assayed enzymatic activities were: alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The reaction in each microcupule was scored from 0 to 5 depending of its intensity.

Toxicity of the sources of fungal molecules

To assess the toxicity of the different chromatographic peaks, their fractions were pooled and concentrated at 4 ° by introducing the sample into a molecular porous membrane (Spectra; VWR International) with a cut-off of 6–8 kDa for globular proteins, and by embedding the membrane in polyethylene glycol 20000 (Fluka; Sigma-Aldrich, St Quentin Fallavier, France). By using a Desaga microinjector, 8 μ l of each peak were injected through the intersegmental membrane between the 2nd and 3rd abdominal segments to newly emerged fourth instar of *Locusta migratoria*. Control nymphs were injected with the corresponding chromatographic peak of the Adamek's liquid medium.

Batches of 10 nymphs, maintained in plexiglas-framed cages (12 \times 12 \times 17 cm) with mosquito-mesh

sides and roof, were placed in incubators at 28 °, 60% rh under a 16:8 h L:D photoperiod. Each treatment was replicated three times with 10 nymphs in each replicate, and the whole experiment was repeated four times. All locusts were fed fresh wheat seedlings. Nymphal mortality was recorded every 24 h, for 10 d post-injection.

Dead insects were dissected under a binocular microscope to observe effects of the injected molecules on insect tissues. Observations for the presence of any chromatic changes or morphological alterations were made on tracheae, air sacs, digestive track, fat body, muscle, cuticle and genitalia. Samples of tissues showing effects were also examined with a Nikon Microphot-FXA optic microscope.

Nymphs of *Schistocerca gregaria* and larvae of *Galleria mellonella*, *Spodoptera littoralis* and *Tenebrio molitor* were injected as above. Nymphs of *S. gregaria* were maintained as those of *L. migratoria*. Larvae of *G. mellonella* and *T. molitor* were maintained at 25 ° in sterile 0.5 L glass jars and those of *S. littoralis* were reared in plastic boxes divided into 3 \times 2.5 \times 2 cm individual compartments (Mino-Gaillard, Izernore, France). Each treatment was replicated three times with 10 nymphs/replicate and the whole experiment was repeated four times.

Electron microscopy

Organs were cut into fragments of 2 mm and fixed overnight at 4 ° with 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. After three rinses with sodium cacodylate buffer, the fragments were post-fixed for 90 min at 4 ° in 0.2% osmium tetroxide and dehydrated in a series of acetone baths from 40 to 100%. Cells from single cell suspensions were collected by centrifugation at 3000 g for 15 min, and fixed following the same procedure. After infiltration overnight with an epon/acetone mixture (1 v/1 v) they were embedded in Epon 812. Ultrathin sections were prepared with uranyl acetate and lead citrate, and examined using a Zeiss EM 10 microscope at 75 Kv. Glutaraldehyde, sodium cacodylate, osmium tetroxide and Epon (Fluka) were purchased from Sigma-Aldrich.

Microsequencing: comparison with data from databases

The protein was characterised by microsequencing of internal peptides by Edman degradation: a Coomassie stained spot from a SDS-Page was digested by trypsin according to Rosenfeld *et al.* (1992). Resulting peptides were fractionated by reverse phase HPLC on RP8 and RP18 columns (2 \times 10 mm), eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid, monitoring the eluate by UV spectroscopy (220 nm). Peptides were sequenced on a Procise sequencer (Applied Biosystems, Foster City, CA) with the pulsed liquid program.

The results obtained were analysed with the Infobiogen Network Service (Villejuif, France) which is the central French server (www.infobiogen.fr). The sequences were compared to other proteic sequences described in different databases: Swissprot, a general protein bank; gp_pl:*. (genpep restricted to plants); Sptr_fun:*, a specific bank restricted to fungi. Comparisons were performed with the programs Fasta and Search. Besides the current methods of alignment of sequences, another method was applied to the data provided by the Genpep bank. The comparison was oriented towards the search for proteins exhibiting amino acid motives similar to relatively uncommon motives found in the *B. bassiana* toxic protein. A first selection was performed using the DDFLK sequence and then, among the proteins selected, a second step of comparison was carried out using the DYYY, or alternatively the GDTC motives.

Insect cell line

In vitro experiments were performed with Sf-9 cells derived from the original Sf-9 cell line obtained from *Spodoptera frugiperda*, currently cultivated in our laboratory. Sf-9 cells were grown in Wheaton spinners (i.e. cell fermentors) in E.X.T.R.A. SBR medium (Eurobio, France) without serum. In most of the experiments, the cells were harvested after 4 d.

Cell toxicity assay

The micro assays were performed in 96 well Falcon plates, with suspensions of cultivated cells adjusted to 5×10^6 cells ml^{-1} . 50 μl of cell suspension and 50 μl of the tested fraction, diluted with tissue culture medium to the indicated concentrations, were added to each well. Two controls were used: one containing 50 μl of cell suspension and 50 μl of culture medium, the other 50 μl of the cell suspension and 50 μl of sterile distilled water. Morphological evolution and cell proliferation were observed regularly with an inverted microscope. The detection and quantification of cytotoxic effects of different peaks were based on the Promega Cell Titer 96 kit. The MTT test, which provides a rapid and convenient method for determining the cell proliferation rate, is based on cellular enzymatic capacity to convert a tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), into the formazan product that is easily detected using a 96 well plate reader. Toxic macromolecules were tested at different final concentrations and the cells were incubated for 72 h with these compounds. Their capacity to convert MTT into formazan was tested following the kit instructions. After 4 h incubation the cells were harvested and kept at 4 ° overnight. Finally, the absorbance of each well was measured with a microtiter plate reader at 540 nm and a reference wavelength of 690 nm. The data concerning absorbance at 540 nm allowed comparison of the proliferation rate of cells submitted to the different treatments.

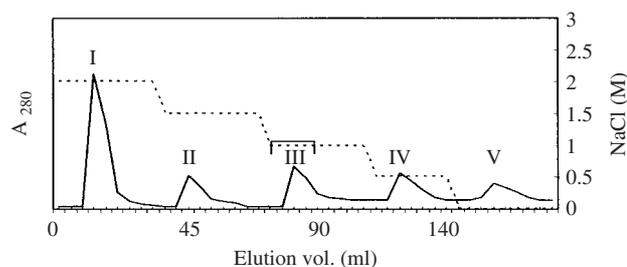


Fig. 2. Hydrophobic Interaction chromatography on Hitrap Phenyl FF (high sub) of the toxic fraction containing metabolites not bound to CM-Trisacryl column. After application of the sample, the column was washed with 50 mM Tris/HCl buffer at pH 8.0 with 2 M ammonium sulphate at a flow rate of 0.5 ml min^{-1} (peak I). The bound material was eluted stepwise with the 50 mM Tris/HCl buffer at pH 8.0, containing 1.5, 1.0, 0.5 and 0 M of ammonium sulphate. These steps of elution enabled collection of peaks II, III, IV and V. The fractions indicated with the bar were toxic to *Locusta migratoria* nymphs.

Statistical analysis

Mortality data were analysed with one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means in Statistics for Windows (Anon. 1996). Percentage mortality caused by the active CM peak I on different insect hosts has been corrected for mortality in the controls using Abbott's formula (Abbott 1925).

RESULTS

Purification of the toxic protein present in the culture filtrates

The toxic metabolites contained in the crude filtrate of *Beauveria bassiana* EABb 90/2-Dm were precipitated with ammonium sulphate and eluted from a Sephadex G-25 column (Fig. 1). The injection of $26 \mu\text{g g body wt}^{-1}$ of the Sephadex G-25 fraction caused a highly significant percentage mortality of 91.7 ($F_{1,22} = 2667$; $P < 0.001$). These results confirmed that the secreted toxins were high mol. wt compounds and consequently, their purification was pursued following the steps described in Material and Methods.

Three peaks were obtained after application of the desalted active fraction to a DEAE-Trisacryl column. These three peaks were injected individually to *Locusta migratoria* fourth instar nymphs at a dose of $26 \mu\text{g g body wt}^{-1}$ of total protein. The first peak eluted from the column with 50 mM Tris buffer caused significant 62.5% mortality ($F_{3,48} = 410.6$; $P < 0.001$).

This peak, containing material which exhibited no affinity for the anion exchanger, was further resolved by CM-Trisacryl. This separation resulted in three further new fractions. The first peak eluted with 50 mM sodium acetate buffer was significantly toxic, and caused a 51.6% mortality ($F_{3,48} = 547.1$; $P < 0.001$).

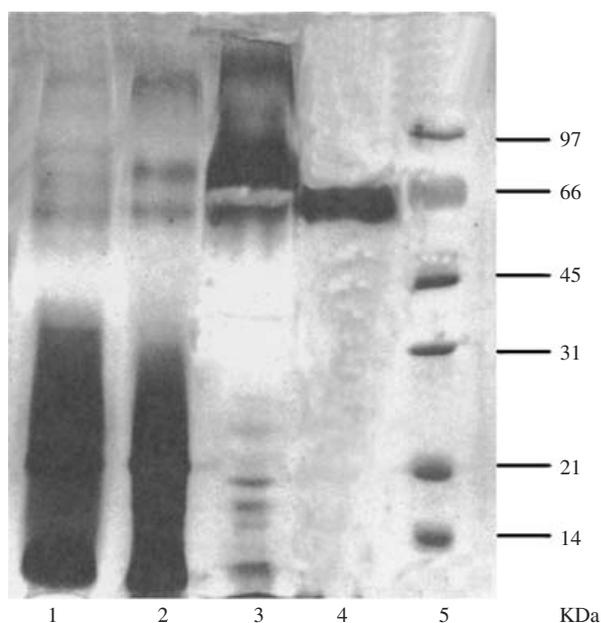


Fig. 3. Analysis of the purification steps of bassiacridin by SDS-PAGE (12% acrylamide) and silver staining. Lanes: 1, Sephadex G-25 pool; 2, DEAE trisacryl initial peak; 3, CM trisacryl initial peak; 4, Hitrap Phenyl FF (high sub) peak III (bassiacridin); 5, Molecular Weight markers, in kDa. The samples (15 μ l) applied to the gels contained between 1 and 10 μ g of protein.

when injected to *L. migratoria* nymphs at a dose of 26 μ g g body wt⁻¹.

Finally, the active CM fraction was applied to a Hitrap Phenyl FF column (Fig. 2). The insecticidal activity was significantly retained on peak III eluted with the 50 mM Tris/HCl buffer containing 1 M of ammonium sulphate ($F_{5,66} = 140.7$; $P < 0.001$). Injection of nymphs with peaks I, II, III, IV and V (Fig. 2) at a dose of 3.3 μ g g body wt⁻¹ gave mortality rates of 4.6, 5.0, 38.3, 6.2 and 5.8% respectively. Mortality in controls of the different bioassays ranged between 0.8 and 4.6%. The purified toxic fraction contained between 0.1 and 0.3% of the total protein of the crude extract.

Characterisation of the protein

Fig. 3 shows the results of SDS-PAGE controls performed at different purification steps of bassiacridin. The observation of the bands revealed on polyacrylamide gels under denaturing conditions suggested that the final product of the separation is a single polypeptide chain with mol. wt of 60 kDa. Furthermore, electrophoresis under non-denaturing conditions also revealed a single band, indicating that the native protein is composed of only one copy of the peptidic chain. Analysis of this protein by isoelectric focusing revealed a single protein band with the apparent isoelectric point of 9.5 (data not shown).

Six internal peptides of those obtained after digestion of the Coomassie stained spot from the SDS-Page by

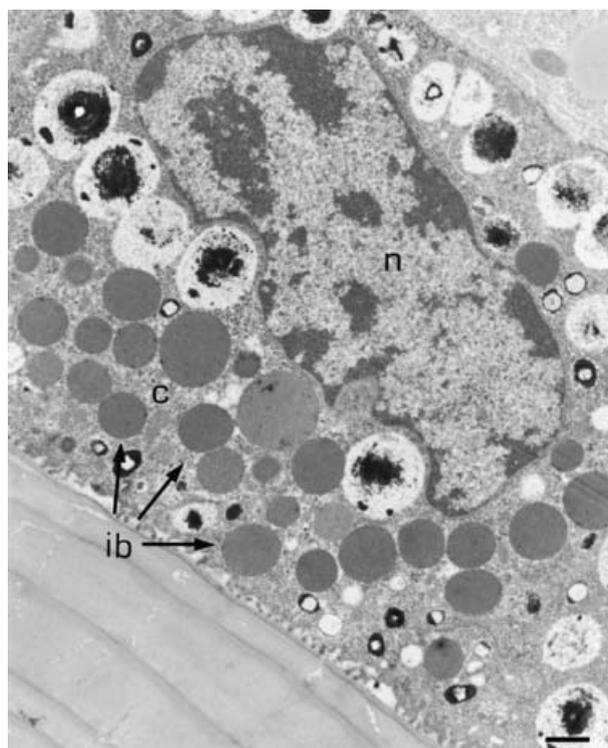


Fig. 4. TEM micrograph of the integument of fourth instar *Locusta migratoria* showing the effect of the Sephadex G-25 toxic fraction (26 μ g g body wt⁻¹) on hypodermic cells. Note the development of lytic inclusion bodies (ib). C, cytoplasm; n, nucleus. Bar = 1.0 μ m.

trypsin were micro sequenced. Their sequences were determined to be: (1) YGAFSTDDFLK; (2) TFTVS-EFTSWNPAVGK; (3) AFNLEQLVSWNPAIGK; (4) AHAGDTCDSK; (5) DXTGIWVDYXXIAPVGP-TP; X being a non-identified amino acid; and (6) NQFNFTSLDDFLK.

No significant homology was revealed when these sequences were compared by standard methods (*cfr* Material and Methods) with the amino acid sequence of other proteins described in different data bases. After application of another two step strategy to the general bank Genpep, based on the specific consideration of relatively unusual motives, a homology was revealed between bassiacridin and one molecule, a chitin binding protein of the yeast *Pichia etchellsii*. However, the degree of homology between the two proteins could not be determined. The motives DDDFLK, DYYY, and GDTC found in the peptides obtained by proteolysis of bassiacridin, and used for the comparison, were also present in the sequence of the yeast chitin binding protein at the level of the amino acids 288–292, 695–698, and 121–124 respectively.

Among the 19 enzyme activities detectable by the API ZYM system, we observed β -galactosidase, β -glucosidase, and N-acetylglucosaminidase with the purified protein. The results concerning these enzymes were considered as positive as the intensity of the reaction was noted as at least 3 (max. of 5). No other

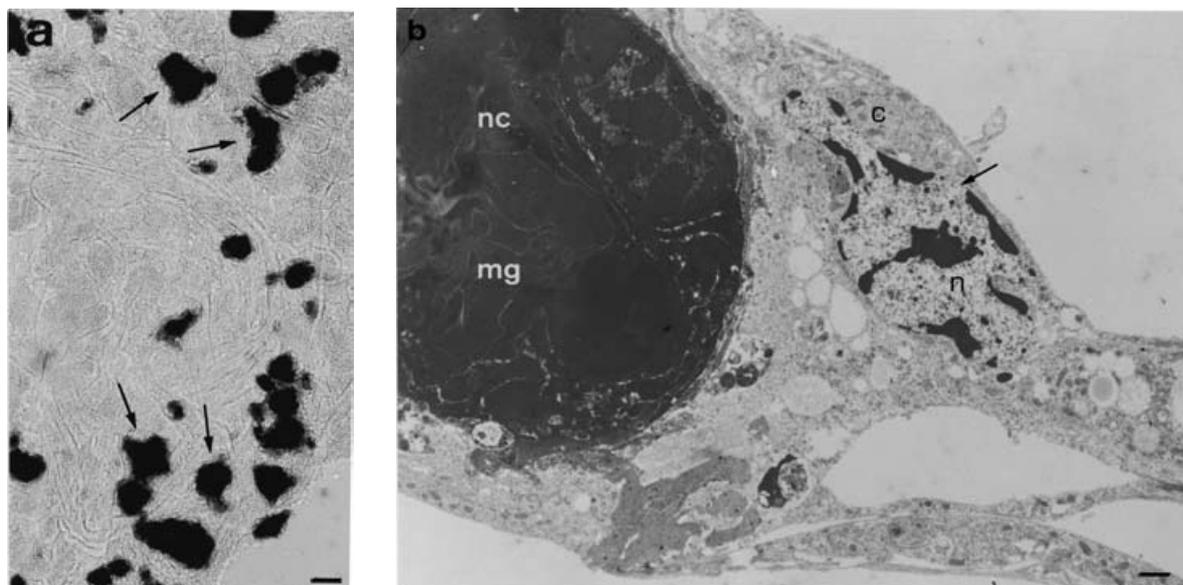


Fig. 5. Structure and ultrastructure of melanised nodules. (a) Nodules (arrows) in the fat body of fourth instar *Locusta migratoria* nymphs 96 h after injection of $26 \mu\text{g g body wt}^{-1}$ of the Sephadex G-25 toxic fraction. Bar = $20 \mu\text{m}$. Fresh mount of tissue in glycerin; observation with the light microscope. (b) Fine structure of a melanised nodule after injection of bassiacridin $3.3 \mu\text{g g body wt}^{-1}$. The nodule exhibits a central necrotic and melanotic granule (mg), and a few apparently healthy cells (\rightarrow) at its periphery. C, cytoplasm; nc, necrotic cell. Bar = $1 \mu\text{m}$.

enzymatic activity, including that of phosphatases, esterases, aminopeptidases and proteases (trypsin and chymotrypsin activities) was observed.

Characterisation of cytotoxic effects in insect tissues and cells

The fractions exhibiting an insecticidal activity also showed clear cytotoxic effects under our experimental conditions. Observation of tissues of cadavers under binocular and light microscopes revealed melanised dark spots on tracheae, air sacs, and melanised nodules in the fat body in contact with the cuticle. In ultra thin sections of the epithelial cells of the integument, dense autolytic cytoplasmic inclusion bodies appeared in great numbers and may reach a large size (Fig. 4). Observation of sections of nodules with light microscopy (Fig. 5a) revealed that these structures consisted of the accumulation of necrotic haemocytes, identified as granular haemocytes in the early steps of the toxic effect. This accumulation of necrotic granulocytes was surrounded by healthy cells forming a thin envelope (Fig. 5b). In the trachea and air sacs, a melanisation spread initially around the taenidium into the layers of cuticular material (Fig. 6a–c), and then, a melanisation and necrosis process extended progressively to the full tracheal epithelial cell (Fig. 6d). In some cases, the recruitment of other cells (probably haemocytes), which became necrotic, led to the formation of dense masses attached to the basal lamina.

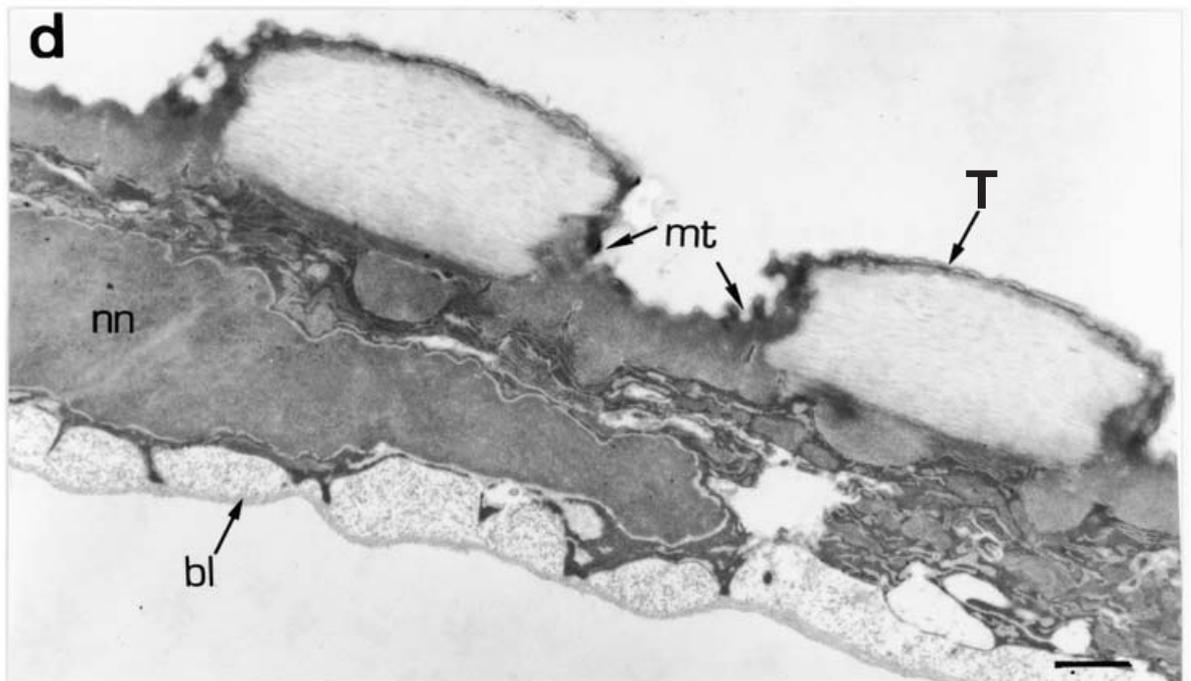
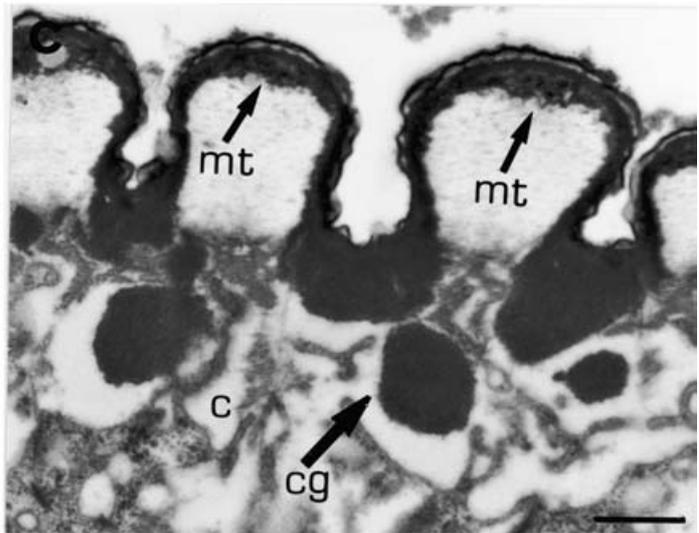
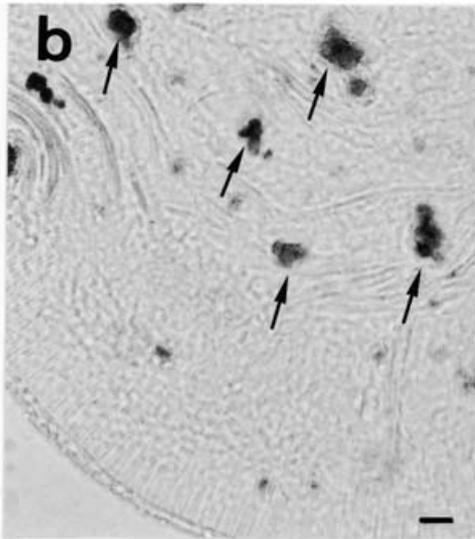
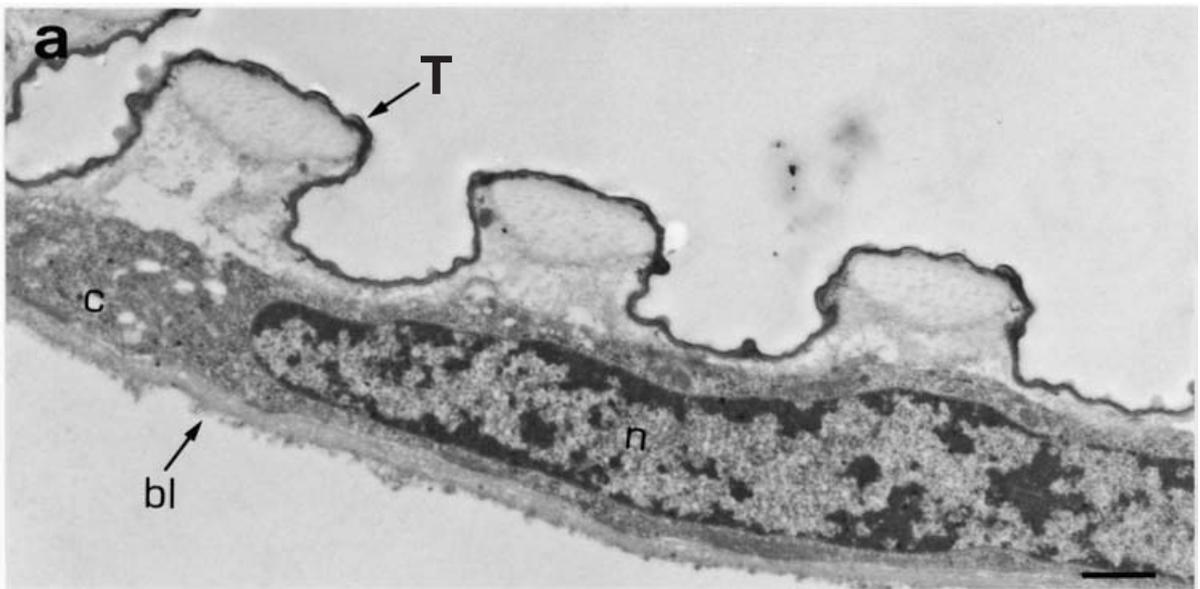
Cytotoxic effects on the Sf-9 cell line

In vitro experiments using the MTT cytotoxicity bioassay showed that the Sf-9 insect cells were sensitive

to the toxic protein. Concentrations of 80 and $60 \mu\text{g ml}^{-1}$ reduced significantly the metabolic activity of the Sf-9 cells within 72 h post-treatment ($F_{4,34} = 17.10$; $P < 0.001$) and ($F_{4,29} = 21.22$; $P < 0.001$), respectively. Morphological changes were also detected by observation with phase contrast within 36–48 h. In contrast, concentrations of 40 and $10 \mu\text{g ml}^{-1}$ did not impair the metabolic activity of cells compared with controls. Electron microscopy data showed that the Sf-9 cells treated with the purified toxin at 80 and $60 \mu\text{g ml}^{-1}$ became hypertrophied and internal organelles (particularly mitochondria) and cell membranes were disrupted. The cytoplasm of the cells was also submitted to an intense vacuolisation consisting of the accumulation of small rounded vacuoles (Fig. 7a–d).

Toxicity of bassiacridin to other insect hosts

Bassiacridin was injected at a dose of $2.8 \mu\text{g g}^{-1}$ to fourth instar nymphs of the locusts *Locusta migratoria*, *Schistocerca gregaria* and *Dociopterus maroccanus*, and to fifth instar larvae of the lepidopterans *Galleria mellonella* and *Spodoptera littoralis*, and of the coleopteran *Tenebrio molitor*. The host significantly impacted mortality ($F_{5,66} = 129.86$; $P < 0.001$). Bassiacridin was not toxic to *S. littoralis* and *T. molitor*, whereas it was slightly toxic to *G. mellonella* causing 16.6% corrected mortality. In contrast, it was equally toxic to the migratory locusts *L. migratoria* and to the desert locust *S. gregaria*, with mortality rates of 42.5 and 38.3% respectively, and slightly more toxic to the Moroccan locust *D. maroccanus*, causing 49.2% mortality.



DISCUSSION

As a result of the present study, a novel high-molecular-weight fungal toxin from culture filtrates of *Beauveria bassiana* strain EABb 90/2-Dm (CECT 20371) was purified and characterized. The yield of pure product, bassiacridin, corresponded to 0.1–0.3% of the total protein content of the crude extract, which is rather similar to that of 0.075% obtained for the glycoprotein of *Beauveria sulfurescens* (Mollier *et al.* 1994) and for hirsutellin A (0.035%) according to Mazet & Vey (1995).

As the dialysate obtained from active filtrate caused no significant mortality (Quesada-Moraga & Vey 2003), and as a unique toxic metabolite was isolated among macromolecules, it can be concluded that only one macromolecular compound plays a significant role in the toxicity of isolate *B. bassiana* 90/2-Dm. Small toxic molecules seem not to contribute to toxicity, perhaps because they are not produced in Adamek's medium. The data obtained may be also explained by the specific susceptibility of the orthopteran hosts, which are highly resistant to certain fungal cyclic peptides due to an active detoxification (Lange *et al.* 1991). In contrast, culture filtrates of *Hirsutella thompsonii* and *B. sulfurescens* contained several toxic molecules, the most active ones being hirsutellin A and a partially purified glycoprotein, respectively (Mollier *et al.* 1994, Mazet & Vey 1995). Hirsutellin A showed an affinity for cation exchangers, while bassiacridin had affinity neither for anion nor cation exchangers and seemed to be unglycosylated as it did not bind to the Con A Sepharose. The isoelectric point of bassiacridin, 9.5, is lower than that of the hirsutellin A, 10.5, although the two proteins are basic if compared with proteins produced by *Aspergillus species* (Sacco, Drickamer & Wool 1983, López-Ontín *et al.* 1984, Fernández Luna *et al.* 1985). Bassiacridin has a mol. wt (60 kDa) higher than those of the hirsutellin A (15 kDa) and of the *Aspergillus* toxins (Sacco *et al.* 1983, López-Ontín *et al.* 1984, Fernández-Luna *et al.* 1985, Mazet & Vey 1995), but lower than that of the *B. sulfurescens* glycoprotein (100–200 kDa) (Mollier *et al.* 1994). We had previously shown that the Sephadex G-25 desalted fraction of the crude filtrate of *B. bassiana* EABb 90/2-Dm was susceptible to heat treatment while Hirsutellin A was reported to be thermostable (Mazet & Vey 1995, Quesada-Moraga & Vey 2003).

The biochemical properties reported here demonstrate that bassiacridin is a novel molecule different from previously described toxic proteins of entomopathogenic fungi. Moreover, the microsequencing of bassiacridin and the comparison of the amino acid sequences by current methods has not revealed a homology with other molecules. These conclusions emphasize the interest in cloning and sequencing of the Bassiacridin gene as already performed with that of hirsutellin A (Boucias, Farmerie & Pendland 1998). For that, the peptide 5 (GIWVDYY), which includes some amino acids with a non degenerated or weakly degenerated code, provides a good tool to prepare homologous synthetic nucleotides which could be labelled and used as probes for the cloning of the gene.

Concentrations of 3.3 µg g body wt⁻¹ of bassiacridin caused 30–40% mortality levels in *L. migratoria* nymphs. This activity is slightly lower than that of hirsutellin A (LD₁₀₀ 1.0 µg g body wt⁻¹), and much lower than that of the glycoprotein of *B. sulfurescens* (LD₁₀₀ 0.01 µg g body wt⁻¹). Among the six assayed hosts, the major toxicity of bassiacridin observed was against locusts (*Orthoptera*; *Acrididae*), the protein being equally toxic to *L. migratoria* and *S. gregaria* and more toxic to the Moroccan locust, *D. maroccanus*, the original host of isolate 90/2-Dm. In contrast, injection assays demonstrated that it was toxic neither to the coleopteran *T. molitor* nor to the lepidopteran *S. littoralis*, whereas it was slightly toxic for *G. mellonella*, considered to be a very sensitive host (Zimmermann 1986). Interestingly, we had previously tested a series of *B. bassiana* strains isolated from locusts and from the soil for their production of compounds toxic for *L. migratoria* but only isolate 90/2-Dm yielded positive results (Quesada-Moraga & Vey 2003). This suggests that bassiacridin could be specifically secreted against acridids, although it is not produced by all strains of *B. bassiana* pathogenic to locusts. This specificity has motivated our proposal of the name bassiacridin for this compound.

The observation of locust tissues after injection of bassiacridin revealed moderate alterations of the fine structure of hypodermal cells. However, the most obvious effects occurred at the level of tracheae and air sacs, which exhibited melanised spots. Ultrastructural studies showed that if the cuticular material of these organs became pigmented, their epithelial cells were also altered and finally became necrotic. The action of

Fig. 6. Effect of toxin treatment on tracheae and air sacs. (a) Tracheal epithelial cell from a control nymph. Bar = 1 µm. (b) Melanised dark spots (arrows) on air sacs of fourth instar *L. migratoria* nymphs 96 h after injection of 26 µg g body wt⁻¹ of the Sephadex G-25 toxic fraction. Bar = 20 µm. (c) TEM micrograph showing the effect of bassiacridin 3.3 µg g body wt⁻¹ on the fine structure of the air sacs of fourth instar nymphs of *L. migratoria* 96 h post-injection. Bar = 1 µm. Bl, basal lamina; c, cytoplasm; cg, dense cytoplasmic granule; n, nucleus; mt, melanisation at the periphery of the taenidium; T, taenidium. (d) Section of the tracheal wall showing the extreme melanisation of its cuticle, and a complete necrosis of the hypodermic cells. Bar = 1 µm. Bl, basal lamina; c, cytoplasm; cg, dense cytoplasmic granule; n, nucleus; nn, necrotic nucleus; mt, melanisation at the periphery of the taenidium; T, taenidium.

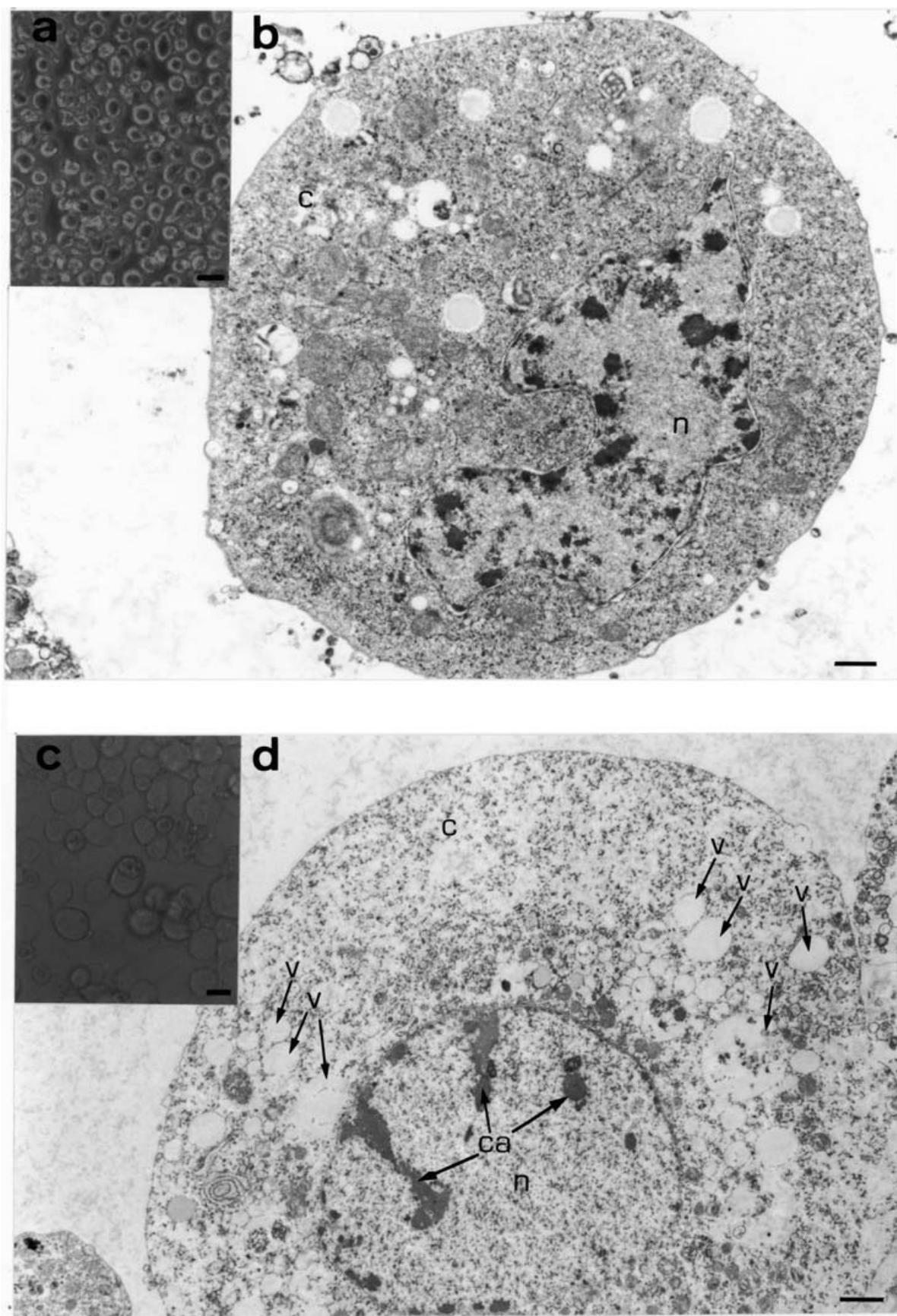


Fig. 7. *In vitro* cytotoxic effect of bassiacridin. Light (a) and TEM (b) micrographs of Sf-9 control cells. Light (c) and TEM (d) micrographs of Sf-9 cells incubated 72 h with $60 \mu\text{g ml}^{-1}$ of bassiacridin. The treatment with the proteic toxin induces a reduction in electron density of the cytoplasm (c) and the development of more vacuoles than in controls (v). The nuclei (n) of treated cells exhibited a clear nucleoplasm and only a small number of chromatin aggregates (ca). Bars = $1 \mu\text{m}$.

prophenoloxdase leading to the formation of melanins was observed only on the respiratory organs and in the small brown nodules formed in contact with the fat body. This is in agreement with literature reports indicating that phenoloxdases are present in the cuticle of the integument and tracheae of insects, and in the haemolymph as precursors (Ashida & Yamazaki 1990, Ashida & Brey 1995). There is no general melanisation of the haemolymph leading to the production of toxic quinones which could cause unspecific alterations of the different organs of the host. It has not yet been determined if bassiacridin can directly activate the prophenoloxdase in the cuticle of the tracheae and air sacs, or if the induction is indirect (activation by compounds released by intoxicated cells).

Cytotoxic effects of bassiacridin were also revealed *in vitro* by the inhibition of proliferation of insect cells of the Sf-9 cell line. This insect cell line is of mesodermic origin, being derived from the envelope of the ovaries. The threshold of activity on Sf-9 cells correspond to 40–50 µg ml⁻¹, around ten fold higher than that of destruxins A and D in *Bombyx mori* cells (Dumas *et al.* 1994). However, the cytopathic effect of bassiacridin occurred very quickly after the beginning of the treatment (36 h). It is now essential to know the mechanisms by which bassiacridin interacts with the host's tissues and cells, and to elucidate its role in the pathogenesis of infections caused by *B. bassiana*. The comparison of the amino acid sequences has revealed a tentative homology between bassiacridin and a yeast chitin-binding protein. One of the priorities would consequently be to determine whether bassiacridin has chitin-binding properties, and which mechanisms are involved in this activity.

The mechanisms involved in the effect on target cells such as epithelial cells (receptors, signalling pathway activated, aspect of the cellular metabolism impaired) have also to be identified. Preliminary assays indicate that haemolymph extracted from 90/2-Dm strain which infected *L. migratoria* nymphs is toxic to the same host (data not shown). This indicates that the insecticidal molecule seems to be produced *in vivo*, and also suggests its possible role in pathogenesis. Therefore, the orthopteran species could possess appropriate receptors/transport mechanism for this toxic protein, or could lack the detoxification mechanisms exhibited by non-orthopteran organisms.

By using a specific antiserum, it should be possible to confirm if bassiacridin is responsible for the toxicity of haemolymph, and, therefore, if it is produced *in vivo* and has a role in pathogenesis. Lethal times of the 90/2-Dm strain could be reduced from 6–7 d (Jiménez-Medina *et al.* 1998) to lower values by adding additional copies of the gene encoding for this toxic protein, and hopefully by overproducing its production. Furthermore, *per os* activity of this toxic protein has to be studied on a broad range of different hosts. The molecule should also pose only limited risks for the environment. Such investigations could enable

bassiacridin to become the active ingredient of a novel class of biopesticides.

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