Insect Immunity

ISOLATION FROM THE LEPIDOPTERAN HELIOTHIS VIRESCENTS OF A NOVEL INSECT DEFENSIN WITH POTENT ANTIFungal ACTIVITY*

(Received for publication, November 2, 1998, and in revised form, December 22, 1998)

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Lepidoptera have been reported to produce several antibacterial peptides in response to septic injury. However, in marked contrast to other insect groups, no inducible antifungal molecules had been described so far in this insect order. Surprisingly, also cysteine-rich antimicrobial peptides, which predominate in the antimicrobial defense of other insects, had not been discovered in Lepidoptera. Here we report the isolation from the hemolymph of immune induced larvae of the lepidopteran Heliothis virescens of a cysteine-rich molecule with exclusive antifungal activity. We have fully characterized this antifungal molecule, which has significant homology with the insect defensins, a large family of antibacterial peptides directed against Gram-positive bacteria. Their presence has been reported from many insect orders and also from scorpions and molluscs (8, 9). Hitherto, they had not been isolated from Lepidoptera and Diptera. They are devoid of cysteine residues and consist of two α-helices linked by a short hinge (for a review, see Ref. 6). Ceporins are frequently C-terminally amidated. They kill Gram-positive and Gram-negative bacteria. The group of cysteine-rich peptides contains members with 1–4 disulfide bridges with molecular masses ranging from 2 to 6 kDa. The most widespread of insect antimicrobial compounds, the insect defensins, belong to this group. They contain six cysteine residues involved in three disulfide bridges (7). Defensins are active against Gram-positive bacteria. Their presence has been reported from many insect orders and also from scorpions and molluscs (8, 9). Hitherto, they had not been isolated from Lepidoptera. In addition to these antibacterial cysteine-rich peptides, two cysteine-containing peptides with antifungal activity have been characterized so far from insects: drosomycin from the dipteran Drosophila melanogaster (10) and thanatin from the hemipteran Podisus maculiventris (11). In response to septic injury, larvae and adults of Drosophila produce considerable amounts of drosomycin, a 5-kDa peptide with eight cysteine residues that are engaged in the formation of four intramolecular disulfide bridges. Drosomycin is inactive against bacteria. It shows significant homology with plant defensins, e.g. ReAFP2 from Raphanus sativus (10). Thanatin is a 2-kDa peptide exhibiting both fungicidal and bactericidal activities. The third group of antimicrobial peptides from insects, the proline-rich molecules, have been isolated from Hymenoptera, Diptera, and Rhoˆne-Poulenc Agro. The peptide was patented April 15, 1998 under the number FR9804933.

* This work was supported by CNRS, the University Louis Pasteur of Strasbourg, and Rhône-Poulenc Agro. The peptide was patented April 15, 1998 under the number FR9804933.

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‡ Cotton is grown in more than 60 countries and plays a major role in the economy of these countries. The demand for cotton has not fallen despite the rapid growth in synthetic fiber production since 1960. The major biotic yield constraints are animal pests and weeds, although in all growing regions there are also diseases causing losses of cotton yields. Cotton plants appear to have little inherent defense against attack by insect pests. In fact, many insect species have been reported on cotton, and some of these species are regarded as major pests that can destroy the plants in a few days. In most growing regions, the principal pests are larvae of lepidopteran species that attack the roots, leaves, flowers, and bolls. This is in particular the case for the following species: Pectinophora gossypiella (pink bollworm), Heliothis armigera (American bollworm), H. virescens (tobacco budworm), and Earias spp. (spiny bollworm). Each year, these species cost the cotton industry millions of dollars in yield loss and control measures (1). In this context, the knowledge of the immune response mechanisms of the lepidopteran larvae is of great interest. Indeed, these mechanisms can represent a potential target for controlling these pests. In the present study, we have chosen H. virescens as a biological model. The host defense of insects relies on cellular and humoral mechanisms. The cellular response consists of phagocytosis and encapsulation of invading microorganisms by blood cells (for a review, see Ref. 2). The humoral facet of the defense reactions of insects has been extensively studied, and it is now clear that it is essentially based on the synthesis of a battery of cationic antibacterial and antifungal peptides/polypeptides exhibiting broad activity spectra. These antimicrobial molecules are produced in the fat body (a functional equivalent of the mammalian liver) and released into the hemolymph (blood). Today, more than 150 antimicrobial peptides have been characterized from various insect species. Although they exhibit great structural diversities, certain common structural patterns are apparent, and the peptides are often grouped into four families: (i) cecropins, (ii) cysteine-rich peptides, (iii) proline-rich peptides, and (iv) glycine-rich peptides/polypeptides (for reviews, see Refs. 2–4). Cecropins, which were the first inducible antibacterial peptides to be characterized (5), are linear 4-kDa cationic peptides isolated from Lepidoptera and Diptera. They are devoid of cysteine residues and consist of two α-helices linked by a short hinge (for a review, see Ref. 6). Cercocrin are frequently C-terminally amidated. They kill Gram-positive and Gram-negative bacteria. The group of cysteine-rich peptides contains members with 1–4 disulfide bridges with molecular masses ranging from 2 to 6 kDa. The most widespread of insect antimicrobial compounds, the insect defensins, belong to this group. They contain six cysteine residues involved in three disulfide bridges (7). Defensins are active against Gram-positive bacteria. Their presence has been reported from many insect orders and also from scorpions and molluscs (8, 9). Hitherto, they had not been isolated from Lepidoptera. In addition to these antibacterial cysteine-rich peptides, two cysteine-containing peptides with antifungal activity have been characterized so far from insects: drosomycin from the dipteran Drosophila melanogaster (10) and thanatin from the hemipteran Podisus maculiventris (11). In response to septic injury, larvae and adults of Drosophila produce considerable amounts of drosomycin, a 5-kDa peptide with eight cysteine residues that are engaged in the formation of four intramolecular disulfide bridges. Drosomycin is inactive against bacteria. It shows significant homology with plant defensins, e.g. ReAFP2 from Raphanus sativus (10). Thanatin is a 2-kDa peptide exhibiting both fungicidal and bactericidal activities. The third group of antimicrobial peptides from insects, the proline-rich molecules, have been isolated from Hymenoptera, Diptera,

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Lepidoptera, and Hemiptera (for a review, see Ref. 3). They are active against Gram-negative bacteria, and for some members of this group activity against Gram-positive bacteria and fungi was also reported. Some of the proline-rich peptides are O-glycosylated (e.g., drosocin (12)). Finally, the glycine-rich peptides/polypeptides, which are mostly large (8–24 kDa), are active against Gram-negative cells. They include attacins, characterized in Lepidoptera and Diptera (13); sarcotoxins II (14); and diptericins from Diptera (15, 16).

As regards the order of Lepidoptera, to which belongs *H. virescens*, a number of inducible antibacterial peptides had already been isolated when we engaged in the present study. These include, in addition to the cecropins and attacins mentioned above, gloverin (17), moricin (18), and lebocins (19). Gloverin is a 14-kDa inducible cationic antibacterial polypeptide isolated from pupae of the giant silk moth *Hyalophora cecropia*. It contains a large number of glycine residues but is devoid of cysteine residues. Moricin is a 4-kDa antibacterial peptide isolated from *Bombbyx mori*. Neither moricin nor gloverin range into any of the four groups of antimicrobial peptides/polypeptides defined above. Finally, lebocins that were isolated from hemolymph of immunized *B. mori* are structurally related to proline-rich peptides. Interestingly, the unique threonine residue in each lebocin is O-glycosylated.

From *H. virescens*, our experimental model, the presence of cecropin-like and attacin-like molecules has been described, but no amino acid sequences have been published (20, 21).

Intriguingly, no cysteine-rich peptides have been reported so far from Lepidoptera, and no antifungal activities have been recorded from this order.

We present here the isolation of a novel inducible antifungal peptide, the structure of which shares similarities with insect defensins, with the *Drosophila* antifungal peptide drosomycin, and with antifungal plant defensins.

**MATERIALS AND METHODS**

**Insect Immunization and Hemolymph Collection**

Fifth instar larvae of the lepidopteran *H. virescens* were individually pricked with a 30-gauge needle dipped into a combined bacterial pellet obtained after centrifugation of 37 °C overnight cultures of *Micrococcus luteus* and *Escherichia coli* 1106. After 24 h, the insects were chilled on a bed of ice, and several drops (30 µl/larva) of hemolymph were recovered by sectioning an abdominal appendix and gently squeezing the abdomen. The hemolymph was pooled in ice-cold polypropylene tubes in order to remove the organic solvent and trifluoroacetic acid equilibrated with acidified water (0.05% trifluoroacetic acid). Elutions containing aprotinin as a protease inhibitor (20 μg/ml) were subsequently reconstituted with MilliQ water.

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**Purification Procedure**

Two ml of cell-free hemolymph, from untreated or bacteria-challenged larvae, were acidified to pH 3 with 0.1% (v/v) trifluoroacetic acid. The acidic extraction was performed for 30 min under gentle shaking in an ice-cold water bath. After centrifugation (10,000 × g for 30 min at 4 °C), the supernatant was loaded onto Sep-Pak C18 cartridges (Waters) equilibrated with acidified water (0.05% trifluoroacetic acid). Elutions were performed with 10, 40, and 100% acetonitrile in acidified water. All fractions were first concentrated in a vacuum centrifuge (Speed-Vac; Savant) in order to remove the organic solvent and trifluoroacetic acid and subsequently reconstituted with MilliQ water.

**First Step of Purification**

The two 40% Sep-Pak fractions from immunized and control insects were purified in parallel by reversed-phase HPLC1 on a semipreparative Aquapore RP-300 C8 column (250 × 7.0 mm, Brownlee) equili-

brated with 2% acetonitrile in acidified water. Elution was performed with a linear gradient of 2% to 60% acetonitrile in acidified water over 120 min at a flow rate of 1.5 ml/min.

**Second Step of Purification**

The fraction with antifungal activity was further applied on an analytical Aquapore OD-300 C18 column (220 × 4.6 mm, Brownlee) developed with a linear biphasic gradient of acetonitrile in acidified water from 2 to 22% over 10 min and from 22 to 32% over 50 min at a flow rate of 0.8 ml/min.

**Final Step of Purification**

The fraction that contained the antifungal activity was further applied on a narrow bore reversed-phase column (Delta Pak HPIC, 2 × 150 mm, Waters) equilibrated with 2% acetonitrile in acidified water and developed with a linear biphasic gradient of acetonitrile in acidified water from 2 to 24% over 10 min and from 24 to 44% over 100 min at a flow rate of 0.25 ml/min and at a temperature of 30 °C.

The first and second steps of HPLC purification were performed with a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector. The last step of purification was carried out at a temperature of 30 °C using an all PEEK Waters HPLC system (Waters model 626 pump) attached to a tunable absorbance detector (Waters model 486) and equipped with an oven. During the purification procedure, the internal diameter of the column was chosen according to the amount of peptide to be purified, while the modification of the gradient was necessary to increase peptide separation.

In all HPLC purification steps, the column effluent was monitored by absorbance at 225 nm to have the most appropriate signal/noise ratio. Fractions were hand-collected in order to have one individual peak per fraction. The presence of antimicrobial activity was detected using liquid growth inhibition assays under the conditions described below.

**Bioassays**

**Bacterial Strains**—The following are the bacterial strains used with their source (gifts from colleagues): *M. luteus* ATCC 270 from the Pasteur Institut Collection (Paris) and *E. coli* SBS 636 and *E. coli* 1106 from P. L. Boquet (Center d'Etudes Nucléaires, Saclay).

**Fungi and Yeast Strains**—The filamentous fungi and the yeast strains used in this study were generous gifts from different colleagues: *Aspergillus fumigatus* (H. Koenig, Laboratoire de Mycologie, Faculté de Médecine, Strasbourg, France), *Pseudomonas aeruginosa* (MIC 180420), *Fusarium culmorum* (MUCL 989), *Nectria haematococca* (Collection Van Etten 160-2-2), *Neurospora crassa* (CBS 327-54), *Trichoderma viride* (MUCL 19724) (Université Catholique de Leuven, Belgique), *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans* (H. Koenig, Laboratoire de Mycologie, Faculté de Médecine, Strasbourg, France), and *Szczecinomyces cerevisiae* (Société Transgène, Strasbourg, France).

**Antibacterial Assay**

During the various steps of purification, antibacterial activity was monitored by a liquid growth inhibition assay on the Gram-positive strain *M. luteus* and on the Gram-negative strain *E. coli* 363. Ninety μl of a suspension of a midlogarithmic phase culture of bacteria at a starting A600 of 0.001 in Poor Broth nutrient medium (1% Bacto-Tryptone and 0.5% (w/v) NaCl, pH 7.5) were added to 10 μl of fraction to analyze. Microbial growth was assessed by an increase in A600 after a 24-h incubation at 30 °C using a microtiter plate reader.

**Antifungal Assays and Determination of the Minimal Inhibitory Concentration**

Fungal spores (final concentration 104 spores/ml) were suspended in ½ Potato Dextrose Broth (Difco), and the yeast strains were suspended at a starting A600 = 0.001 in the yeast complete medium YPG (1% yeast extract, 1% peptone, 2% glucose). The medium was supplemented with tetracyclin (10 mg/ml) and cefotaxim (100 μg/ml), dispensed by aliquots of 80 μl into wells of a microplate containing 20 μl of either water or the fraction to be analyzed. Growth of fungi and yeast was evaluated after 24 h at 30 °C by optical microscopy and after 48 h by measuring the culture absorbance at 595 nm using a microplate reader.

In the conditions where the antifungal assay was performed in the presence of salt, the ½ Potato Dextrose Broth medium was prepared in trix-assisted laser/desorption time of flight mass spectrometry; MES, 4-morpholinoethanesulfonic acid.

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1 The abbreviations used are: HPLC, high pressure liquid chromatography; MIC, minimal inhibitory concentration; MALDI-TOF-MS, matrix-assisted laser/desorption time of flight mass spectrometry; MES, 4-morpholinoethanesulfonic acid.
phosphate-buffered saline, 137 mM NaCl.

The procedure used for the determination of the minimal inhibitory concentration (MIC) was identical to that for the antifungal assay. The MIC values are expressed as an interval (a–b), where a is the highest peptide concentration tested at which fungi or yeast are still growing and b is the lowest concentration that causes 100% growth inhibition.

Fungalic Assay

Spores of N. crassa were cultured in the presence of 0.04–20 μm peptide. After 48 h, the % Potato Dextrose Broth medium containing the peptide was removed and replaced by fresh medium. Two days later, the cultures were examined microscopically and spectrometrically.

Structural Characterization

Capillary Zone Electrophoresis

Peptide purity was ascertained by capillary zone electrophoresis. Analysis was performed on a model 270-HT capillary electrophoresis system (PE Applied Biosystems). Two nl of the sample were injected using vacuum to a 50 μm × 72-cm fused silica capillary and run in 20 mm citrate buffer at pH 2.5 under 20 kV from anode to cathode at 30 °C for 20 min. Migration was monitored at 200 nm.

Microsequence Analysis

Automated Edman degradation of the native and pyridylethylated peptides and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequencer (PE Applied Biosystems, model 473A).

Reduction and S-Pyridylethylation

One nmol of purified peptide was submitted to reduction and S-pyridylethylation. The procedure used was reported previously (22).

Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed on a Bruker BIFLEX™ (Bremen, Germany) mass spectrometer operating in a positive linear mode as described previously (23).

Enzymatic Digestions

Endoproteinase Lys-C Treatment

The pyridylethylated peptide (200 pmol) was treated with endoproteinase Lys-C (Achromobacter protease I, Takara, Otsu, Japan). Digestion was carried out at 37 °C for 6 h in 50 μl of 10 mM Tris-HCl (pH 9) in the presence of 0.01% Tween 20 at a peptide/enzyme ratio of 1:40 (w/w). The reaction was stopped by acidification with 1% trifluoroacetic acid, and the peptide fragments were separated on a narrow bore reversed-phase column (Delta Pak HPIC18, 2 × 150 mm, Waters). Elution was performed with a linear gradient of acetonitrile in acidified water (0.05% trifluoroacetic acid) from 2 to 60% over 80 min at 37 °C at a flow rate of 0.2 ml/min. The fragments were analyzed by MALDI-TOF-MS, and the enzymatically derived fragment corresponding to the C terminus of the protein was sequenced by Edman degradation.

Determination of the Cysteine Arrangement by Thermolysin Digestion

Eight μg of the native peptide were treated with thermolysin at a peptide/enzyme ratio of 1.2 (w/w) for 1 h in 0.1 mM MES buffer at pH 7.4 at 37 °C in presence of 2 mM CaCl₂. The digestion was stopped by adding 50 μl of 70% formic acid. The peptide mixture resulting from the enzymatic digestion was subjected to the same reversed-phase HPLC column as above. Elution was performed with a linear gradient of acetonitrile in acidified water from 2 to 50% over 100 min after an isotonic period at 2% acetonitrile during 10 min. The peptides generated by protease treatment were characterized by MALDI-TOF-MS.

Production of Recombinant Peptide

Strains and Media

The E. coli strain DH5α was used in the cloning procedures, and the S. cerevisiae strain TGY 48–1 was transformed with the expression plasmid pSEA2, and transformants were selected by growth at 29 °C on YNBG medium supplemented with casamino acids lacking uracil. For large scale peptide preparation, the culture medium was inoculated at a dilution of 1:50 with a 48-h preculture of the selected transformant, and an additional 48-h growth was performed at 29 °C. Yeast were harvested by centrifugation at 4000 rpm for 30 min at 4 °C. The supernatant was carefully decanted, and the pellet was discarded. The recombinant peptide was secreted from the cells. The supernatant was subjected to solid-phase extraction on an open column filled with C₁₈ reversed phase (preparative C₁₈, 125 Å, Waters; 48 g of phase/4 liters of supernatant), solvated with methanol, and further equilibrated with acidified water. Elution was performed with 40% acetonitrile in acidified water and lyophilized under vacuum, reconstituted in MilliQ water and then applied on a reversed-phase column (Aqua pre preparative C₁₈, 250 × 10 mm, Brownlee) equilibrated with 2% acetonitrile in acidified water. Elution was performed with a linear biphase gradient of acetonitrile in acidified water from 2 to 20% over 10 min and from 20 to 40% over 30 min at a flow rate of 3 ml/min. The fraction containing the peptide was lyophilized and kept as dry powder.

RESULTS

Isolation of the Antibacterial and Antifungal Peptides from Bacteria-challenged Insects—One hundred fifth instar larvae of H. virescens were challenged by injection of bacteria, and their hemolymph (2 ml) was collected after 24 h. In parallel, 2 ml of hemolymph from unchallenged larvae were treated under the same conditions. The hemocytes were removed by centrifugation, and the antimicrobial peptides were extracted from the cell-free hemolymph in acidic condition during 30 min in an ice-cold water bath under gentle shaking. The acidic extracts were then submitted to prepurification by solid phase extraction onto Sep-Pak C₁₈ cartridges. Elution was performed with 10, 40, and 100% acetonitrile in acidified water. After concentration, the various fractions obtained from the acidic extracts were tested for their antimicrobial activity by liquid growth inhibition assays against M. luteus, E. coli, and N. crassa. Only the 40% Sep-Pak fractions were found to contain antimicrobial activity and were further applied on an Aqaura RP-300 C₁₈ column. Elution was performed with a linear gradient of acetonitrile, and aliquots of the eluted frac-
tions were tested for their antimicrobial activity by liquid growth inhibition assays against the three test organisms. Several fractions from immune larvae were found to contain strong antifungal and/or antibacterial activity (Fig. 1); in contrast, weak activities were detected in the control experiment (data not shown). Here we have focused on the purification of the compound present in fraction A eluted at 28% acetonitrile in acidified water, which exhibited the strongest antifungal activity. The active molecule was further purified by a two-step purification procedure (see “Materials and Methods”). Pure antifungal compound was obtained, as judged by capillary zone electrophoresis (data not shown). MALDI-TOF-MS gave a single molecular mass at 4784.8 Da, confirming the purity of the antifungal molecule (data not shown).

Characterization of the Isolated Antifungal Peptide—One nmol of purified antifungal peptide was subjected to reduction and S-pyridylethylation and subsequently to MALDI-TOF-MS measurement. The obtained molecular mass at 5422.3 Da, in excess of 637.5 Da to the mass measured on the native molecule, corresponds to six pyridylethylated groups, suggesting the presence of six cysteine residues. One hundred pmol of the S-pyridylethylated peptide were then submitted to Edman degradation, and a partial 41-residue NH2-terminal amino acid sequence was obtained (Fig. 2). The mass calculated for this N-terminal sequence is lower by 333.4 Da than the measured molecular mass of the native peptide at 5422.3 Da. This clearly establishes that the sequence obtained by Edman degradation is only a partial sequence. To obtain the full sequence, we used endoproteinase Lys-C to cleave the pyridylethylated peptide after the Lys residues at positions 2, 23, and 28 under the conditions described under “Materials and Methods.” The digest was analyzed by reversed-phase HPLC (data not shown), and the peptide fragments were measured for their respective molecular masses by MALDI-TOF-MS. Five major peaks (2661.7, 2001.2, 2779.3, 2535.7, and 5422.5 in MH+) were obtained. The peak at 5422.5 MH+ (mass/charge) corresponds to the native molecule, while the peptide fragments yielding the molecular masses at 2778.3 and 2534.7 Da correspond to the predicted fragments from the N-terminal sequence. Only the fragments at 2661.7 and 2001.2 MH+ did not fit with any possible cleavage product, suggesting that both include the C-terminal end. The mass difference of 659.5 Da between these two fragments can be explained by the presence of the additional sequence Arg-Arg-Gly-Tyr-Lys (residues 24–28) in the N-terminal part of the 2661.7 MH+ fragment obtained by Edman degradation of the native peptide. To obtain the C-terminal sequence information, the shortest fragment was subjected to Edman degradation and the following sequence was obtained: Gly-Gly-His-Cys*-Gly-Ser-Phe-Ala-Asn-Val-Asn-Cys*-Trp-Cys*-Glu-Thr, where Cys* represents a pyridylethylated cysteine residue. The calculated mass of the sequence is in perfect agreement with the molecular mass of 2000.2 Da measured by MALDI-TOF-MS, establishing that there is no C-terminal amidation. The overlap observed with the N-terminal sequence confirmed the identity of this fragment as the C-terminal part of this 44-amino acid antifungal peptide. The full sequence of the molecule is shown in Fig. 2. The comparison between the calculated mass after Edman degradation (4791.3 Da) and the measured mass by MALDI-TOF-MS (4784.8 Da) indicates that the six cysteine residues are engaged in the formation of three intramolecular disulfide bonds. The data bank analysis (BLAST program in the data base Swiss-Prot; Ref. 26) showed that the peptide has significant sequence similarities with the Drosophila antifungal peptide drosomycin and insect defensins. Therefore, we assumed that the cysteine arrangement is identical to that of insect defensins (Cys1–Cys4, Cys2–Cys5, and Cys3–Cys6) (27), which is also conserved in the core of drosomycin (Cys2–Cys5, Cys3–Cys6, and Cys4–Cys7) (25). The disulfide array of the peptide was determined by thermolysis digestion, separation of the peptide fragments by HPLC, and measurement of their respective molecular mass by MALDI-TOF-MS. The major peak (peak 6 in Fig. 3) had a molecular mass of 4784.4 Da, corresponding to the native molecule. Four minor peaks (peaks 1–4 in Fig. 3) corresponding to the digest products yielded single molecular masses at 1184.2, 2192.9, 2355.7, and 2337.7 Da. Peak 1 yielded a molecular mass at 1184.2 Da, which is in agreement with the peptidic fragment Ile*-Cys7 linked to the fragment Tyr*-Ser34 through an intramolecular disulfide bridge between Cys7 and Cys32 (see inset in Fig. 3). The mass of peak 4 can be explained by admitting the connection of the fragment Tyr14–Gly26 to the fragment Val38–Thr44. This indicates that the disulfide bonds are Cys18–Cys30 and Cys22–Cys42. The mass difference between peaks 2 and 3 of 162.8 Da...
corresponds to a Tyr residue, and the masses obtained by MALDI-TOF-MS are in perfect agreement with the digestion products proposed in Fig. 3. Taken together, the data are compatible with the following disulfide array: Cys(1)–Cys(4), Cys(2)–Cys(5), and Cys(3)–Cys(6), which is identical to that determined for insect defensins and to the three internal disulfide bridges of drosomycin.

**Recombinant Synthesis of the H. virescens Antifungal Peptide and Analysis of Its Activity Spectrum**

In order to produce sufficient amounts of the peptide for detailed studies on its activity spectrum, we expressed a synthetic cDNA in the yeast *S. cerevisiae*. The synthetic gene was constructed by annealing and ligating six individual oligonucleotides to form a double-stranded oligonucleotide cassette. This cassette is preceded by the last five amino acids of the yeast mating factor MF₁ proregion to generate a HindIII site necessary for an in-frame fusion with the presequence, responsible for secretion of the peptide by the yeast strain. The MF₁ promoter directs the expression of the gene, and Kex2 endopeptidase cleaves the fusion protein after the dipeptide site Lys-Arg at the C terminus of the MF₁.

With this system, the average recovery of pure peptide was 2.5 mg/1 liter of culture medium. MALDI-TOF-MS showed that the recombinant peptide exhibits the same molecular mass as the native molecule, and capillary zone electrophoresis analysis confirmed its purity. The antifungal activity of recombinant and native peptide was compared by measuring their MIC on *N. crassa* and found to be identical for both molecules. The purified recombinant peptide was tested on a variety of bacterial and fungal strains. No antibacterial activity against 15 Gram-positive and 10 Gram-negative bacterial strains was detected even at concentrations of up to 50 μM. However, in marked contrast, the *H. virescens* antifungal peptide showed a strong antifungal activity against six fungal strains tested (Table I). In liquid growth inhibition assays, the pure recombinant peptide had marked activity against *N. crassa* (MIC = 0.1–0.2 μM), *F. culmorum* (MIC = 0.2–0.4 μM), and *N. haematococca* (MIC = 0.4–0.8 μM). The peptide was also found to be active against *F. oxysporum* (MIC = 1.5–3 μM), *T. viride* (MIC = 1.5–3 μM), and *A. fumigatus* (MIC = 6–12 μM). Further studies on its activity spectrum, we expressed a synthetic cDNA in the yeast *S. cerevisiae*. The synthetic gene was constructed by annealing and ligating six individual oligonucleotides to form a double-stranded oligonucleotide cassette. This cassette is preceded by the last five amino acids of the yeast mating factor MF₁ proregion to generate a HindIII site necessary for an in-frame fusion with the presequence, responsible for secretion of the peptide by the yeast strain. The MF₁ promoter directs the expression of the gene, and Kex2 endopeptidase cleaves the fusion protein after the dipeptide site Lys-Arg at the C terminus of the MF₁. With this system, the average recovery of pure peptide was 2.5 mg/1 liter of culture medium. MALDI-TOF-MS showed that the recombinant peptide exhibits the same molecular mass as the native molecule, and capillary zone electrophoresis analysis confirmed its purity. The antifungal activity of recombinant and native peptide was compared by measuring their MIC on *N. crassa* and found to be identical for both molecules. The purified recombinant peptide was tested on a variety of bacterial and fungal strains. No antibacterial activity against 15 Gram-positive and 10 Gram-negative bacterial strains was detected even at concentrations of up to 50 μM. However, in marked contrast, the *H. virescens* antifungal peptide showed a strong antifungal activity against six fungal strains tested (Table I). In liquid growth inhibition assays, the pure recombinant peptide had marked activity against *N. crassa* (MIC = 0.1–0.2 μM), *F. culmorum* (MIC = 0.2–0.4 μM), and *N. haematococca* (MIC = 0.4–0.8 μM). The peptide was also found to be active against *F. oxysporum* (MIC = 1.5–3 μM), *T. viride* (MIC = 1.5–3 μM), and *A. fumigatus* (MIC = 6–12 μM). Further
thermore, it showed a strong activity against two of the four yeast strains tested: *C. albicans* and *C. neoformans* at a MIC of 2.5–5 μM. The peptide was inactive against *C. glabrata*, up to 50 μM, and against *S. cerevisiae*, even at a concentration as high as 100 μM. The antifungal activity was tested in a similar assay on *N. crassa* at physiologic ionic strength (137 mM NaCl), and the MIC recorded for the peptide was identical to that of the classical tests, suggesting that the activity of the peptide is not affected by salts. In contrast, an activity reduction (4-fold) was observed for drosomycin in the presence of salts (data not shown). Finally, spores of the fungus *N. crassa* were incubated in the presence of various concentrations of the peptide (0.04–20 μM). After 48 h, the medium containing the peptide was replaced by fresh medium, and 2 days later no growth recovery had occurred when the peptide concentration during the initial incubation was 0.15 μM or higher. This result demonstrates that the peptide is fungicidal at this concentration. We propose the name of heliomicin for this novel antifungal peptide from *H. virescens*.

**DISCUSSION**

The data presented in this report establish that the larval hemolymph of a lepidopteran species, *H. virescens*, contains a strongly inducible cysteine-rich antifungal peptide. The molecule was purified to homogeneity and fully characterized at the level of its amino acid sequence by a combination of reversed-phase chromatography, capillary zone electrophoresis, Edman degradation, protease digestion, and MALDI-TOF-MS. The peptide, which was named heliomicin, consists of 44 residues and contains six cysteine engaged in three intramolecular disulfide bridges. Cysteine-containing antimicrobial peptides had so far not been reported from lepidopteran insects, although this order served for the first successful identification of antimicrobial peptides and namely the discovery of cercopins and attacins by Boman and associates (5, 13). Similarly, inducible antifungal activities had not yet been recorded in Lepidoptera.

Sequence comparison with the group of insect defensins shows that the *Heliophilus* antifungal molecule presents the same cysteine arrangement (Cys(1)–Cys(4), Cys(2)–Cys(5), and Cys(3)–Cys(6)) as that present in these antibacterial peptides (22, 28) (Fig. 4). In addition to the six cysteines, the residues strictly conserved between heliomicin and insect defensins are three glycines at equivalent positions (numbered 26, 29, and 30 in heliomicin). The sequence of heliomicin is also evocative of that of antifungal peptides recently reported from *Drosophila* (drosomycin; Ref. 25) and plants (e.g. *Rs*-AFP1 from *Raphanus sativus*; Ref. 29) (Fig. 4). In particular, heliomicin shares the cysteine array with these molecules. Note that drosomycin and *Rs*-AFP1 have in addition two external cysteines absent from defensins. Drosomycin and heliomicin also present a conserved cluster of four amino acids (Cys-Trp-Cys-Glu) located on the third β-strand at the C-terminal part of drosomycin (30). They also have in common the hydrophobic dipeptide Ala-Val at positions 11 and 12; three glycine residues at positions 5, 26, and 30; and a lysine at position 23 (numbering relative to heliomicin). As regards plant defensins, the residues conserved between heliomicin and *Rs*-AFP1 are restricted to the six cysteine residues and to two glycines at positions 5 and 30 (relative to heliomicin, as shown in Fig. 4).

Heliomicin presents the structural motif Cysβ. In this scaffold, an invariant Cys-(Xaa)₂-Cys sequence of a β-helix is linked to an Cys-Xaa-Cys sequence of a β-sheet. A similar motif named CSH (for cysteine-stabilized helix) was initially described on the basis of NMR studies by Tamaoki et al. in endothelin (31). This motif appears now to be commonly shared by a variety of antibiotics and toxins like short- and long-chain scorpion toxins (32), insect defensins (first report in *Phormia* defensin; Ref. 33), the *Drosophila* drosomycin (30), plant defensins (e.g. *Rs*-AFP1; Ref. 34), and γ-thionins (35). The three-dimensional structure has been established by NMR for some of these defense molecules, and we can anticipate that the lepidopteran peptide will adopt a similar conformation.

In order to obtain sufficient amounts of heliomicin for three-dimensional structure determination by two-dimensional NMR and for detailed studies on its activity spectrum, we have successfully expressed a synthetic gene in the eukaryote *S. cerevisiae*. The purified recombinant molecule exhibits the same characteristics as the native molecule and was used to establish the activity spectrum of the peptide. Heliomicin was found to be active against various fungal and yeast strains, but it had no activity against either Gram-positive or Gram-negative bacterial strains even at concentration as high as 50 μM, which corresponds roughly to 10 times the estimated concentration of heliomicin in immune challenged *H. virescens* larvae (Table I). We noted that heliomicin is active against most of the filamentous fungi tested at concentrations significantly lower than those of the *Drosophila* antifungal peptide drosomycin. Furthermore, the latter exhibits no antifungal activity against the yeast strains *C. albicans* and *C. neoformans*. Note that *Phormia* defensin exhibits also some activity against several fungi, but at concentrations ≥4 times higher than heliomicin. At physiological ionic strength, heliomicin exhibits the same activity as in the classical tests against the filamentous fungus *N. crassa*. This contrasts with the reduction of antimicrobial activity observed in the presence of salts for other antimicrobial peptides such as drosomycin, human β-defensins 1 and 2 (36), and lebocins (37). This indicates that the antifungal activity of heliomicin is not based on charge differences but may involve other mechanisms such as interactions with a receptor in the target organism.

In conclusion, septic injury in *H. virescens* results in the production of an antifungal peptide, heliomicin. Sequence comparison indicates that heliomicin is related to insect defensins and may represent the insect defensin prototype of Lepidoptera. However, the antifungal activity observed for the *Heliophilus* peptide prompts us to classify heliomicin in the group of antifungal peptides such as plant defensins and drosomycin.

**Acknowledgment**—We are grateful to Martine Schneider for capillary electrophoresis analysis.

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