Determination of the disulfide array of the first inducible antifungal peptide from insects: drosomycin from *Drosophila melanogaster*

Lydia Michaut[a], Pascale Fehlbaum[a], Marc Moniatte[b], Alain Van Dorsselaer[b], Jean-Marc Reichhart[a], Philippe Bulet[a,*]

[a]*Institut de Biologie Moléculaire et Cellulaire, UPR 9022 Centre National de la Recherche Scientifique, Réponse Immunitaire et Développement chez les Insectes, 15 rue René Descartes, 67084 Strasbourg Cedex, France*
[b]*Laboratoire de Spectrométrie de masse Bio-Organique, Université Louis Pasteur, URA CNRS 31, Chimie Organique des Substances Naturelles, 1 rue Blaise Pascal, 67008 Strasbourg Cedex, France*

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Abstract Drosomycin is a 44-residue antifungal peptide with four intramolecular disulfide bridges which have been isolated from immune-challenged *Drosophila*. To produce adequate amounts of this peptide for 3D-structure analysis, studies on the mode of action and activity spectrum, we expressed a synthetic cDNA in *Saccharomyces cerevisiae*. For this purpose, we used the mating factor α gene and concomitantly overexpressed the *KEX2* gene to increase the yield of fully processed drosomycin. Using a combination of Edman degradation and mass spectrometry, we show that drosomycin shares the same array of intramolecular disulfide bridges than plant defensins, in addition to their sequence similarities.

Key words: Antifungal peptide; Yeast expression; Mass spectrometry; Disulfide array

1. Introduction

Insects are particularly resistant to microbial infections. Their host defense involves the rapid and transient synthesis of small-sized cationic peptides with antibacterial and/or antifungal properties. The peptides are produced in the fat body and secreted into the hemolymph, where they oppose the proliferation of microorganisms (reviewed in [1]). Drosomycin, a 44-residue cysteine-rich peptide with potent antifungal activity, was isolated from immune-challenged adults of *Drosophila* [2]. Interestingly, this peptide shares sequence similarities with plant defensins, a group of antifungal peptides which participate in the host defense of plants [3]. As plant defensins, drosomycin has eight cysteines engaged in four intramolecular disulfide bridges. It is surmised that the presence of these four bridges in the 44-residue peptide confers a highly compact structure, accounting for the remarkable resistance of drosomycin to protease and heat treatments.

Drosophila is a small insect, which hampers the isolation of acceptable amounts of native drosomycin for 3D-structure determination by NMR and detailed studies on its mode of action and activity spectrum. To overcome this problem, and in view of the interest in drosomycin in both basic and applied research, we have expressed under appropriate conditions its cDNA in *Saccharomyces cerevisiae*. The recombinant peptide was purified to homogeneity and found to have the same chromatographic behaviour and biological activity as the native molecule. We have established the disulfide bridges in parallel on recombinant and native drosomycin using enzymatic methods, automated microsequencing and mass spectrometry with a total of only 1 μg (200 pmol) per peptide. These results emphasize that the combination of enzymatic cleavage followed by matrix-assisted laser desorption mass spectrometry measurement and Edman degradation provide a rapid and sensitive method for determination of disulfide bridges on minute amounts of peptides, as already described [4,5]. The position of the disulfide bridges was found to be identical to that reported for plant defensins, reinforcing the idea that these molecules could be homologous. We have also extended, with recombinant drosomycin, our previous studies on the activity spectrum of this antifungal peptide.

2. Materials and methods

2.1. Strains and media

The *S. cerevisiae* strain TGY 48-1 used for drosomycin expression was described in [6]. Yeast complete medium was YPG (yeast extract 1%, peptone 1%, glucose 2%). Yeast cells were transformed using the lithium acetate method [7] and transformants were selected on YNBG medium (yeast nitrogen base 0.67%, glucose 1%) supplemented with 0.5% casamino acids, lacking uracil. The *Escherichia coli* media and cloning procedures were from standard methods [8].

2.2. Plasmids

The expression block contains the MFα1 promoter and preprosequence with a silent mutation in 3′ encoding a *Hin*dIII site to allow an in-frame fusion of the coding sequences. It was subcloned from pTG1895 [6] into an M13 phage, yielding M13JM132, from which it can be mobilized as an *Sph*I-*Bam*HI fragment. The yeast/*E. coli* shuttle vector pTG3828 has been described [9]. A 7 kb genomic fragment containing the KEX2 gene was cloned by complementation in *S. cerevisiae* and a *Bam*HI site was created by site-directed mutagenesis 220 bp downstream of the ORF encoding the endoprotease (Achstetter, T. and Ngyen, M., unpublished). Finally, the 3475 bp *Xho*I-*Bam*HI fragment containing the full-length *KEX2* gene was blunt-end ligated into the unique *Eco*RI site of pTG3828 to obtain pTG4812 in which transcription of *KEX2* is counterclockwise.

2.3. Synthetic gene construction and expression cassette assembly

A synthetic gene encoding the 44-residue drosomycin, N-terminally flanked by the last five amino acids of the MFα1 proregion, was synthesized by elongation of the two annealed oligonucleotides DRO-1 (5′GCGCGCAAGCTTGGACAAGAGGACTGCCTGTCCGGAAGATACAAGGGTCCCTGTGCCTGGACAACGAGACCTGTCGTCGTGTGTGCAAGGAGGAGGGA3′) and DRO-2 (5′GCAGCACACACGTTCCTCCTCCCTGCGAGGTCACCGGTGACGTCGGGGTCAGACTTCACGACCACGCTTCCTACGATTCCTAGGCGCGCG3′) with the large (Klenow) fragment of DNA polymerase I. The resulting 169 bp fragment (Fig. 1A) was digested by *Hin*dIII and *Bam*HI, ligated into M13JM132, yielding M13JM575, and sequenced using a T7 sequencing kit (Pharmacia). The expression cassette (Fig. 1B) was cloned as a 1.4 kb *Sph*I-*Bam*HI fragment in the

*Corresponding author. Fax: (33) 88 60 69 22.
E-mail: bulet@astorg.u-strasbg.fr

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yeast/*E. coli* shuttle vectors pTG3828 or pTG4812, yielding the two expression plasmids pJM576 and pJM600, respectively.

2.4. Transformant product analysis

Transformed cells were grown overnight in 10 ml of selective medium at 30°C. After centrifugation (12000×*g*, 30 min, 4°C), the supernatant was acidified to pH 5.2 with acetic acid and subjected to solid-phase extraction by reversed-phase chromatography on a Sep-Pak C_{18} cartridge (Waters) equilibrated with acidified water (TFA 0.05%). Elution was performed stepwise with solutions of 10% and 30% acetonitrile in acidified water. The 30% fraction was concentrated under vacuum (Savant) diluted in MilliQ water (Millipore) and applied onto an Aquapore OD300 C_{18} column (220×4.6 mm, Brownlee) equilibrated with acidified water. Elution was performed with a linear gradient of 7–57% acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min.

2.5. Large-scale purification of recombinant drosomycin

10 ml of an overnight culture of the selected transformant were used to inoculate 500 ml of selective medium. After 48 h incubation at 30°C under vigorous shaking, the culture (OD_{600}: 9.5) was centrifuged (12000×*g*, 30 min, 4°C) and the supernatant subjected to 30 min heat treatment at 100°C and centrifuged again. The supernatant, containing the recombinant drosomycin, was acidified with acetic acid to pH 3 and subjected to solid-phase extraction on an open column filled with C_{18} reversed-phase (preparative C_{18} 125 Å, 6 g of phase/500 ml of supernatant) equilibrated with acidified water. Elution was performed stepwise with solutions of 15 and 30% acetonitrile in acidified water. The fraction eluted with 30% acetonitrile was lyophilized, reconstituted in water and applied onto a preparative reversed-phase column (Aquapore ODS 20 μm, 250×10 mm, Brownlee). Elution was performed with a linear gradient of 7–57% acetonitrile in acidified water over 90 min at a flow rate of 5 ml/min. After lyophilization, drosomycin was purified by ion-exchange chromatography on a cation column (Aquapore cation 20 μm, 250×10 mm, Brownlee) equilibrated in 50 mM ammonium acetate buffer pH 5.2. For elution, a gradient of 0–100% NaCl (1 M) in ammonium acetate buffer was used over 60 min at a flow rate of 4.5 ml/min. The column effluent was monitored by the absorbance at 225 nm. Finally, the fraction containing drosomycin was desalted by reversed-phase HPLC, lyophilised and kept as dry powder.

2.6. CZE and MALDI-TOF-MS

Capillary zone electrophoresis (CZE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) were performed as described in [10].

2.7. Antifungal assay

The antifungal assays were as described in [2] with the following fungal strains: *Alternaria brassicola* (MUCL 20297), *Fusarium culmorum* (IMI 180420), *Neurospora crassa* (CBS 327-54), and *Botrytis cinerea* (MUCL 30158), all gifts from W.F. Broekaert (Janssens Laboratory of Genetics, Leuven, Belgium) and *F. oxysporum*, *F. oxysporum meloni*, *Stemphylium*, *Verticillium*, *B. petunia* and *A. dauci* were from the Société Clause.

2.8. Assignment of the disulfide bridges

Drosomycin (100 μg) was incubated with a combination of endoproteinase Glu-C and endoproteinase Lys-C (Boehringer, Mannheim) at a peptide/enzyme ratio of 100:10 (w/w) and 100:5 (w/w), respectively. Digestion was performed in a mixture of two buffers: 25 mM Tris-HCl, pH 8.5, 1 mM EDTA and 25 mM ammonium carbonate, pH 7.8, for 16 h at 25°C. The digestion was stopped by acidification with 0.1% TFA. Separation of peptide fragments was performed onto an Aquapore RP300 C_8 reversed-phase column (220×4.6 mm, Brownlee) with a linear gradient of 2–80% acetonitrile in acidified water over 120 min at a flow rate of 1 ml/min at 40°C.

2.9. Microsequence analysis

Automated Edman degradation of the purified peptides and detection of the phenylthiohydantoin derivatives were performed on a pulse-liquid automatic sequenator (Applied Biosystems model 473A).

3. Results

To allow secretion of drosomycin by *S. cerevisiae*, the peptide coding sequence was fused with the preprosequence of the yeast mating factor MFα1 gene. Expression is directed by the strong MFα1 promoter and the fusion protein is expected to be C-terminally cleaved after the basic dipeptide Lys-Arg of the MFα1 spacer peptide by the Kex2 endopeptidase, releasing mature drosomycin into the culture medium [11,12]. We constructed a synthetic gene where 15 nucleotides corresponding to the triplets 81–85 of the MFα1 prosequence were added 5′ to the mature drosomycin coding sequence, generating a *Hin*dIII site necessary to obtain an in-frame fusion with the prosequence. The drosomycin expression block was cloned into pTG3828, yielding pJM576. Yeasts were transformed either by the drosomycin expression vector pJM576 or by pTG3828 as a control. The cell-free culture supernatant was subjected to solid-phase extraction and the fraction eluted with 30% acetonitrile was analyzed by reversed-phase HPLC. An absorption peak, eluting at 26% acetonitrile, was detected in yeast cultures carrying pJM576, but was absent in the control experiment. The material present in this absorption peak exhibited strong antifungal activity against *N. crassa* and *B. cinerea* and was subjected to CZE. The electropherogram revealed the existence of four expression products, in unequal amounts (data not shown). This fraction subjected to MALDI-TOF mass spectrometry analysis yielded four distinct masses corresponding to correctly matured drosomycin (4889.7 Da) accounting for 25% of the total, plus three N-terminally extended expression products consisting of drosomycin with either a 14-residue N-terminal extension (6373.0 Da), a 9-residue extension (5903.4 Da) or a 7-residue extension (5645.1 Da). All these residues correspond to the C-terminal amino acids of the MFα1 prosequence. Their presence in this peptide obviously results from incomplete maturation of recombinant drosomycin (see Section 4).

To improve this yield of mature drosomycin (25%), we co-expressed drosomycin with the Kex2 endoprotease by inserting the previously described expression cassette from M13JM575 into pTG4812, which carries a full length copy of the *KEX2* gene. The resulting drosomycin/*KEX2* expression

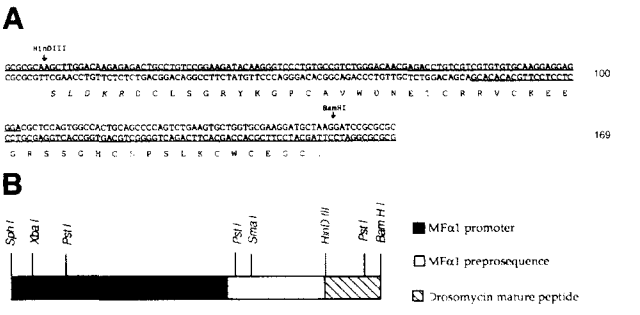

Fig. 1. (A) Sequence of the synthetic gene encoding the 44 residues of mature drosomycin. The gene was fused to the last five amino acids of the MFα1 proregion (italics). The sequences of the two oligonucleotides (DRO-1, sense; DRO-2, antisense) used for the construction are underlined. The 169 bp sequence obtained after Klenow elongation of these two annealed oligonucleotides (see Section 2) harbours a *Hin*dIII restriction site which enables in-frame fusion with the MFa1 proregion in the expression cassette. The *Bam*HI site located after the stop codon is also mentioned. (B) Structure and restriction map of the 1.4 kb drosomycin expression cassette.

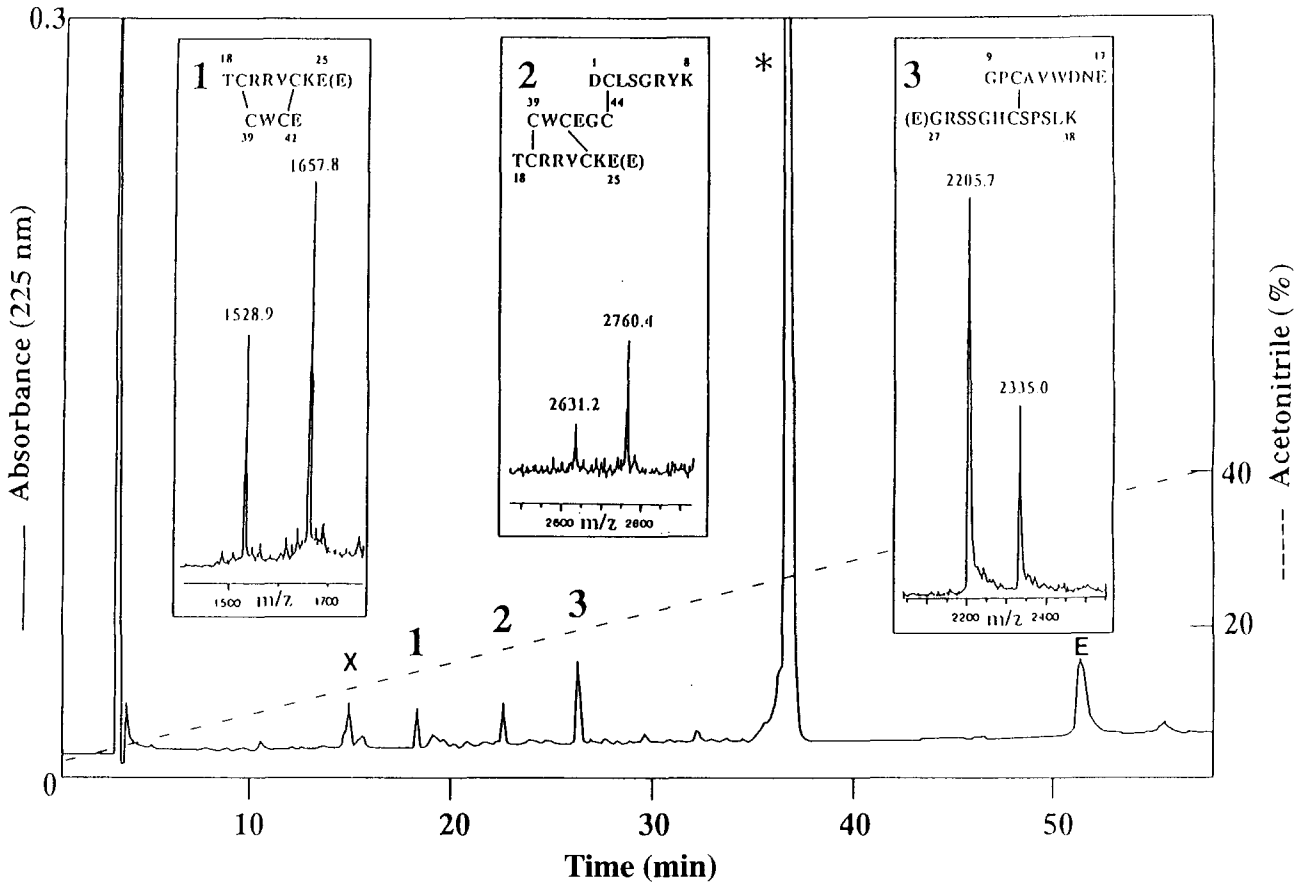

Fig. 2. Analysis of the recombinant drosomycin proteolysis products. Recombinant drosomycin digested by a combination of endoproteinases Glu-C and Lys-C was submitted to reversed-phase HPLC. Elution was performed with a linear gradient of 2–80% acetonitrile in acidified water (dotted line) and absorbance was monitored at 225 nm (full line). Peaks 1–3 were analyzed MALDI-TOF mass spectrometry and sequenced by Edman degradation. Each inset shows the *m/z* values obtained and the sequence of the corresponding fragments. X labels the elution peak containing a mixture of masses which could not be associated to any fragment. E denotes the elution peak containing the enzymes. * designates the elution peak containing undigested drosomycin.

plasmid is pJM600. The cell-free culture media of yeasts transformed with pJM600, or pTG4812 as control, were submitted to the same treatment as above. Again, the strong absorption peak eluting at 26% acetonitrile exhibited antifungal activity. It was shown by CZE and mass spectrometry analysis to contain 90% of mature drosomycin (4890.0 Da) and 10% of a N-terminally extended peptide (5645.1 Da).

After solid-phase extraction and an initial purification step of the 30% acetonitrile fraction by preparative reversed-phase chromatography, the partially purified antifungal material was applied on a preparative cation exchange column (see Section 2). The mature drosomycin fraction, eluting at approx. 250 mM NaCl, was desalted by reversed-phase HPLC and lyophilised. CZE confirmed that the mature peptide had been purified to homogeneity. The identity of drosomycin was ascertained by mass spectrometry analysis. The specific antifungal activities of recombinant and native drosomycin were compared by measuring the minimal inhibitory concentration (MIC) on *N. crassa*, *A. brassicola*, *F. culmorum* and *B. cinerea*. The MIC values were found to be 0.3–0.6, 0.6–1.2, 0.6–1.2 and 1.2–2.5 μM, respectively, and were identical for both molecules. We extended the analysis of the activity spectrum to several other fungal strains. Drosomycin exhibits antifungal activity against five of six fungal strains tested, with MIC values ranging from 0.3–0.6 μM against *B. petunia* and *Verticillium*, 0.6–1.2 μM against *Stemphylium*, 1.2–2.5 μM against *F. oxysporum* L. to 2.5–5 μM against *F. oxysporum meloni*. In contrast, low activity was found against *A. dauci* (MIC > 20 μM).

Knowing that drosomycin shares sequence similarities with *Raphanus sativus* defensin [2], the disulfide array of which was determined by NMR studies [13], we assumed that the position of the disulfide bridges was identical in drosomycin and plant defensin, and selected endoproteinase Glu-C and endoproteinase Lys-C to digest 100 μg of recombinant drosomycin. The digestion mixture was subjected to HPLC purification. It

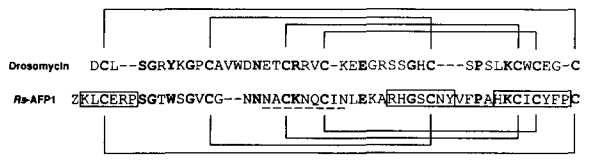

Fig. 3. Amino-acid sequences of drosomycin and *Rs*-AFP1 (*Raphanus sativus* antifungal peptide) and schematic representation of the elements of the 3D structure of *Rs*-AFP1. Gaps introduced for optimal alignment are indicated by dashes; boxed sequences represent β-strands. The β-turn is underlined and the dashed line corresponds to the α-helix. Identical amino acids and conservative replacements are in bold-face. Disulfide bridges are indicated by the lines connecting Cys pairs. Z represents the N-terminal pyroglutamate.

is apparent from the chromatogram (Fig. 2) that the bulk of drosomycin remained undigested, in keeping with our earlier results on the remarkable resistance of drosomycin to protease treatment [2]. Three minor peaks (referred to as 1–3 in Fig. 2) corresponded to digestion products (approximate yield of digestion: 1%). Mass spectrometry measurements of peak 1 yielded two masses of 1527.9 and 1657.8 Da corresponding to two potential cleavage fragments of drosomycin each containing four cysteines engaged in two disulfide bridges. The difference of 129 Da between the two masses can be explained by partial cleavage by endoproteinase Glu-C between the two C-terminal Glu residues. Peak 1 was submitted to sequencing by Edman degradation to confirm position of the disulfide bridges. The results were as follows: Thr at cycle 1, cystine (Cys-Cys) and Trp at cycle 2, Arg at cycle 3, Arg and Glu at cycle 4, Val at cycle 5, cystine at cycle 6, Lys and Glu, respectively, at cycles 7 and 8. In the case of the higher mass fragment we detected a Glu residue at cycle 9. These data are in agreement with the sequence proposed in Fig. 2 (inset 1) and indicate that the disulfide bonds were Cys^{19}(3)–Cys^{39}(6) and Cys^{23}(4)–Cys^{41}(7), knowing that in Edman degradation of peptide fragments linked by disulfide bridges, cysteine residues are only set free as doublets. Peak 2 yielded two masses differing by 129 Da, i.e. the mass of the Glu residue. As presented in Fig. 2 (inset 2), these masses can be explained by admitting the connection of the fragment containing residues 1–8 of drosomycin to the peptide identified in peak 1 via a Cys^{2}(1) to Cys^{44}(8) link. Again, the enzyme endoproteinase Glu-C imperfectly digested this compound as no cleavage occurred between residues Glu^{12} and Gly^{13}. Peak 3 also yielded two masses differing by 129 Da, i.e. the mass of the Glu residue. The higher mass is in perfect agreement with the cleavage product proposed in Fig. 2 (inset 3). Taken together, these data are compatible with the following disulfide array: Cys^{2}(1)–Cys^{44}(8), Cys^{11}(2)–Cys^{33}(5), Cys^{19}(3)–Cys^{39}(6) and Cys^{23}(4)–Cys^{41}(7) which is identical to that determined for *Raphanus* defensin (Fig. 3). This array was confirmed on 80 μg of native drosomycin extracted from 2000 immune-challenged adults of *Drosophila*.

4. Discussion

The aim of the present study was to establish an expression system for large-scale production of drosomycin for further investigations on its 3D structure, activity spectrum, and mode of action.

Over the last decade, yeast has become the preferred eukaryotic host organism for the production of heterologous proteins: they can indeed be targeted into the secretion pathway, which is particularly important when disulfide bound formation is required to generate the correct 3D structure of a recombinant protein. Several peptides containing multiple disulfide bridges have been successfully produced using this system, namely peptides containing three disulfide bridges, such as leech hirudin [14] and defensin A from *Phormia terranovae* [6] and peptides with four disulfide bridges, e.g. the plant antifungal peptide *Rs*-AFP2 [15] and scorpion neurotoxin AαHIT$_1$ [16]. Native drosomycin contains four disulfide bridges and, although it has potent activity against filamentous fungi, it is inactive against *S. cerevisiae*, even at concentrations as high as 100 μM.

We decided to use this strategy and fused a synthetic drosomycin gene in frame with the preprocoding sequence of the *MFα1* gene. The fusion gene led to the expression and secretion of four peptides: 25% of mature drosomycin, and three other molecules containing various N-terminal extensions corresponding to the C-terminal part of the α-factor prosequence. This indicates that the cleavage after the basic Lys-Arg dipeptide by the Kex2 endoprotease was rate-limiting. To test whether this could result from overloading of the secretion pathway, we decided to overexpress the *KEX2* gene concomitantly with the drosomycin gene. The yield of fully processed drosomycin was then increased from 25 to 90%. This clearly indicates that the amount of Kex2 endoprotease present in the yeast strain carrying pJM576 was insufficient to process the amount of drosomycin being made. The presence of 10% of an N-terminally extended form still recovered in spite of the overexpression of *KEX2* may result either from the poor accessibility of the cleavage site between the α-factor proregion and the mature drosomycin or from endoproteolytic cleavage of the precursor, the product of which might no longer be recognized by the Kex2 endoprotease.

With this system, the overall yield of expression and purification of drosomycin was of 2 mg of pure active peptide per l of culture medium (i.e. about 2×10^{11} cells). Using the recombinant drosomycin, we extended the activity spectrum studies of the molecule and showed that it exhibits a potent activity against fungal strains which are of major agronomic interest, such as the wilt pathogen *F. oxysporum*. We also determined the disulfide bond array of the molecule, reinforcing the similarities between drosomycin and the plant defensin *Rs*-AFP1, an antifungal peptide found in radish seeds [17,18]. The two molecules are aligned in Fig. 3 and show 38% sequence identity following introduction of several minor gaps. Here we show that they share a common array of disulfide bridges (Cys^{1}–Cys^{8}, Cys^{2}–Cys^{5}, Cys^{3}–Cys^{6} and Cys^{4}–Cys^{7}), extending the similarities from primary to secondary structure. The 3D structure of the plant defensin *Rs*-AFP1 from radish seeds has been determined by NMR studies: it is constituted by a triple-stranded antiparallel β-sheet and an α-helix parallel to the β-sheet [18]. The determination of the tertiary structure of drosomycin is currently in progress, since the expression system described here provided sufficient amounts of pure peptide to undertake NMR studies. This will reveal whether the 3D structure of drosomycin is identical to that of the plant defensin *Rs*-AFP1.

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References

[1] Hoffmann, J.A., Reichhart, J.-M. and Hetru, C. (1996) Curr. Opin. Immunol. 8, 8–13.
[2] Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C. and Hoffmann, J.A. (1994) J. Biol. Chem. 269, 33159–33163.
[3] Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A. and Osborn, R.W. (1995) Plant Physiol. 108, 1353–1358.
[4] Lepage, P., Bitsch, F., Roecklin, D., Keppi, E., Dimarcq, J.-L.,

Reichhart, J.-M., Hoffmann, J.A., Roitsch, C. and Van Dorsselaer, A. (1991) Eur. J. Biochem. 196, 735–742.
[5] Mer, G., Kellenberger, C., Koehl, P., Stote, R., Sorokine, O., Van Dorsselaer, A., Luu, B., Hietter, H. and Lefèvre, J.-F. (1994) Biochemistry 33, 15397–15407.
[6] Reichhart, J.-M., Petit, I., Legrain, M., Dimarcq, J.-L., Keppi, E., Lecocq, J.-P., Hoffmann, J.A. and Achstetter, T. (1992) Invert. Reprod. Dev. 21, 15–24.
[7] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] Achstetter, T., Nguyen-Juilleret, M., Findeli, A,. Merkamm, M. and Lemoine, Y. (1992) Gene 110, 25–31.
[10] Bulet, P., Urge, L., Ohresser, S., Hetru, C. and Otvos, L. Jr. (1996) Eur. J. Biochem. 238, 64–69.
[11] Julius, D., Schekman, R. and Thorner, J. (1984) Cell 36, 309–318.
[12] Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. (1984) Cell 37, 1075–1089.
[13] Fant, F., Santos, L., Vranken, W., Boulez, K., Martins, J.C. and Borremans, F.A.M. (1994) in: Proceedings of the 12th European Experimental NMR Conference.
[14] Riehl-Bellon, N., Carvallo, D., Acker, M., Van Dorsselaer, A., Marquet, M., Loison, G., Lemoine Y., Brown, S.W., Courtney, M. and Roitsch, C. (1989) Biochemistry 28, 2941–2949.
[15] Vilas-Alves, A.L., De Samblanx, G.W., Terras, F.R.G., Cammue, B.P.A. and Broekaert, W.F. (1994) FEBS Lett. 348, 228–232.
[16] Martin-Eauclaire, M.-F., Sogaard, M., Ramos, C., Cestele, S., Bougis, P.E. and Svensson, B. (1994) Eur. J. Biochem. 223, 637–645.
[17] Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1992) J. Biol. Chem. 267, 15301–15309.
[18] Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1995) Plant Cell 7, 573–588.