A Novel Family of Small Cysteine-rich Antimicrobial Peptides from Seed of Impatiens balsamina Is Derived from a Single Precursor Protein*

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Four closely related peptides were isolated from seed of Impatiens balsamina and were shown to be inhibitory to the growth of a range of fungi and bacteria, while not being cytotoxic to cultured human cells. The peptides, designated Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, are 20 amino acids long and are the smallest plant-derived antimicrobial peptides isolated to date. The Ib-AMPs (I. balsamina antimicrobial peptides) are highly basic and contain four cysteine residues which form two intramolecular disulfide bonds. Searches of protein data bases have failed to identify any proteins with significant homology to the peptides described here. Characterization of isolated cDNAs reveals that all four peptides are encoded within a single transcript. The predicted Ib-AMP precursor protein consists of a prepeptide followed by 6 mature peptide domains, each flanked by propeptide domains ranging from 16 to 35 amino acids in length. Such a primary structure with repeated alternating basic mature peptide domains and acidic propeptide domains has, to date, not been reported in plants.

An increasing number of cysteine-rich antifungal and antimicrobial peptides have been isolated from plants and in particular from plant seed. These peptides may have an important role to play in the protection of plants from microbial infection, and they could prove to be useful tools for the genetic engineering of fungal resistance in transgenic plants (1). Based on amino acid sequence homology, these peptides fall into at least six different classes. They include peptides isolated from seed of Mirabilis jalapa (1), Amaranthus caudatus (3), and Zea mays (4), members of the thionin family of peptides (5), members of the lipid transfer proteins (6–8), and members of the plant defensins (9–14).

From extracts of seed of Impatiens balsamina, we have isolated four small peptides the amino acid sequences of which are very closely related to each other but that do not resemble any peptides previously characterized from plants or other organisms. This paper describes the purification of the peptides and reports on their antimicrobial properties, in particular with respect to the inhibition of the growth of plant pathogenic fungi. Furthermore, a single class of cDNA has been identified that encodes all four members of this family of peptides as part of a preproprotein. Details of the characterization of the unusual structure of this cDNA and its products, as well as of their expression patterns, are presented.

EXPERIMENTAL PROCEDURES

Biological Materials—Seeds of I. balsamina were purchased from Sandeman Seeds (Pulborough, United Kingdom). Fungi and bacteria were grown and maintained as described previously (14, 15). The following fungal strains were used: Alternaria longipes (CBS62083); Botrytis cinerea (K1147); Cladosporium sphaerospermum (K0791); Colletotrichum gloeosporioides (SR24BTA); Fusarium culmorum (K0311); Gloeosporium pomigena (field isolate; T. Sutton); Gloeosporium solani (CBS19432); Nectria galligena (MUC12B8); Penicillium digitatum (K0879); Phalalohora malorum (field isolate; D. Sugar); Sclerotinia sclerotiorum (SES A); Trichoderma viride (K1127); and Verticillium alboatrum (K0937). The following Gram-negative bacterial strains were used: Bacillus subtilis (JHCC 55331); Micrococcus luteus (ATCC 9341); Staphylococcus aureus (ATCC 25923); Streptococcus faecalis (ATCC 29212); and the following Gram-negative bacterial strains: Erwinia amylovora (CFBP1430); Escherichia coli (HB101); Proteus vulgaris (JHCC 558711); Pseudomonas solanacearum (RGA4A); Xanthomonas campestris pathovar pelargonii (INRA 10342); and Xanthomonas oryzae (ETH 698).

Extraction of Peptides—The purification of antimicrobial peptides from the basic protein fraction of I. balsamina seed was essentially as described previously (10). One-kilogram amounts of seed were ground in a coffee mill, and protein was extracted by stirring overnight at 4 °C in extraction buffer (10 mM Na2HPO4, 1 M NaH2PO4, 100 mM KCl, 2 mM EDTA, pH 7). Ammonium sulfate was added to 80% relative saturation, and precipitated proteins were collected by centrifugation, resuspended in distilled water, and extensively dialyzed against distilled water using 2000-Da cutoff dialysis tubing (Sigma). The extract was adjusted to 50 mM NH4Ac (pH 9) and passed over a Q-Sepharose Fast Flow column (12 × 5 cm, Pharmacia) equilibrated in 50 mM NH4Ac (pH 9). The unbound fraction represents the basic protein fraction, and this was adjusted to pH 6 with acetic acid and passed over a S-Sepharose Fast Flow column (10 × 2.6 cm, Pharmacia) equilibrated in 50 mM NH4Ac (pH 6). Bound proteins were eluted with a linear gradient of 50 mM–1.5 M NaH2PO4 over 325 min at a flow rate of 3 ml/min. Proteins were monitored by the on-line measurement of the absorbance at 280 nm. Fractions with the highest antifungal activity were pooled for each peak and further purified by RP-HPLC1 on a Pep-S column (C18 silica, 25 × 0.93 cm, Pharmacia). Peptides were eluted with linear gradients of 0.1% (v/v) trifluoroacetic acid to 99.9% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over 100 min at a flow rate of 3 ml/min. Elution of peptides was monitored by absorbance at 210 nm.

Electrophoresis and Amino Acid Sequencing—The purified peptides were analyzed by SDS-PAGE on precast high density gels (PhastSys-

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1 The abbreviations used are: RP-HPLC, reversed phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis; AMP, antimicrobial peptide; Ib, Impatiens balsamina; SSPE, saline/sodium/phosphate/EDTA.
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**RESULTS**

**Purification and Primary Structure Determination**—The basic proteins from *I. balsamina* seed were fractionated by cation exchange chromatography, and fractions assayed for antifungal activity against *F. culmorum* spores. Following chromatography, the extract yielded four peaks of activity eluting between 400 mM and 700 mM NH₄Ac (Fig. 1, arrowheaded). Fractions from each peak showed the highest levels of activity were pooled and further purified by preparative RP-HPLC. Each pooled fraction yielded a single peak of absorbance at 210 nm which exactly matched the antifungal activity eluting from the column (data not shown). These active peaks were designated Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, respectively, according to the order of their elution from the cation exchange column.

The purified active fractions were further analyzed by SDS-
with 1 mM CaCl$_2$ and 50 mM KCl (medium B).

Initial attempts to sequence the Ib-AMPs by automated Edman degradation indicated that all four peptides were N-terminally blocked. To obtain their sequences, each peptide was digested with either trypsin or chymotrypsin following modification of cysteine residues with 4-vinylpyridine, and the resulting fragments were purified by RP-HPLC prior to sequencing. The following partial amino acid sequences were generated for peptides Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, respectively: GRRCCGWGPGRRYCVRW.

The following partial amino acid sequences were generated for peptides Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4, respectively: GRRCCGWGPGRRYCVRW, GRRCCGWGPGRRYCVRW, GRRCCGWGPGRRYCVRW, and GRRCCGWGPGRRYCVRW. Residue 7 of Ib-AMP2 could not be unambiguously identified as one of the common amino acids. Clearly, all four peptides are very close homologues of each other, with as little as one, and no more than five, amino acid differences between any pairwise alignment of the above sequences.

Electrospray Mass Spectrometry—The molecular mass of each of the purified Ib-AMP peptides was experimentally determined by electrospray mass spectrometry as 2464.6, 2527.4, 2536.6, and 2522.6 Da for Ib-AMP1, -2, -3, and -4, respectively (data not shown). These molecular mass determinations are consistent with the estimation based on SDS-PAGE.

Antifungal Activity of the Ib-AMPs—The antifungal activity of the purified peptides was assessed on 13 fungal strains, many of which are plant pathogens of significant importance to agriculture, using a standard antifungal activity assay (14). In medium A, all four peptides showed similar levels of broad spectrum activity (Table I, medium A). For the majority of the assays in this medium, the IC$_{50}$ values were <10 µg/ml. The antifungal activity of the peptides is, however, sensitive to the ionic strength of the assay medium and in the same medium supplemented with 1 mM CaCl$_2$ and 50 mM KCl, the activity of Ib-AMP1, Ib-AMP2, and Ib-AMP3 is severely reduced (Table I, medium B). Only Ib-AMP4 maintains any significant inhibitory activity even though its activity is also markedly reduced. On some fungi, notably F. culmorum, the Ib-AMPs cause a very distinct swelling and hyperbranching in the spore germination assay at subinhibitory rates (Fig. 3A). The Ib-AMPs also inhibit the growth of germlings, and in the case of Ib-AMP4 this is also apparent in medium B (Table I). On germlings, the Ib-AMPs cause swelling and branching along the length of hyphae and at the hyphal tip (Fig. 3B).

Antibacterial Assays—In addition to their broad spectrum antifungal activity, the Ib-AMPs are also inhibitory to the growth of a range of bacteria, especially Gram-positive bacteria (Table II). On the Gram-positive bacteria tested, the IC$_{50}$ values of Ib-AMP4 are lower than those obtained with the antibiotic peptide magainin I (18).

Human Cell Integrity Assays—The effect of Ib-AMP2 and Ib-AMP4 on human erythrocytes and cell cultures of human

![Fig. 2. SDS-PAGE analysis of the purified Ib-AMPs. 200 ng of each peptide were dissolved in reducing sample buffer and separated on a High Density Phastgel (Pharmacia). Peptides were transferred to nitrocellulose by capillary blotting and visualized by silver staining. Lanes 1 and 6, myoglobin fragments with molecular masses in kDa as indicated; lane 2, Ib-AMP1; lane 3, Ib-AMP2; lane 4, Ib-AMP3; lane 5, Ib-AMP4.](image)

**TABLE I**

| Fungus                        | IC$_{50}$ (µg/ml) |
|-------------------------------|------------------|
|                               | Ib-AMP1 | Ib-AMP2 | Ib-AMP3 | Ib-AMP4 |
| **Medium A, spores**          |          |          |          |          |
| Alternaria longipes           | 3        | 12       | 6        | 3        |
| Botrytis cinerea              | 12       | 25       | 6        | 6        |
| Cladosporum                   | 1        | 6        | 3        | 1        |
| sphaerospermum                |          |          |          |          |
| F. culmorum                   | 1        | 6        | 6        | 1        |
| Penicillium digitatum         | 3        | 6        | 3        | 3        |
| T. viride                     | 6        | 12       | 12       | 6        |
| V. albostrum                  | 3        | 12       | 6        | 6        |
| **Medium B, spores**          |          |          |          |          |
| Alternaria longipes           | 50       | >200     | >200     | 12       |
| Botrytis cinerea              | >200     | >200     | >200     | 200      |
| Cladosporum                   | 50       | >200     | 100      | 6        |
| sphaerospermum                |          |          |          |          |
| F. culmorum                   | 50       | >200     | 100      | 6        |
| Penicillium digitatum         | 200      | >200     | 100      | 25       |
| T. viride                     | >200     | >200     | >200     | 150      |
| V. albostrum                  | >200     | >200     | >200     | 50       |
| **Medium B, germlings**       |          |          |          |          |
| Colletotrichum gloeosporioides| ND*      | ND       | ND       | 25       |
| Gloeosporium pomigena          | ND       | ND       | ND       | >100     |
| Gloeosporium solani           | ND       | ND       | ND       | >100     |
| Nectria galligena             | ND       | ND       | ND       | 6        |
| Phialophora micorum           | ND       | ND       | ND       | 6        |
| Sclerotinia sclerotiorum      | ND       | ND       | ND       | 25       |

* ND, not determined.
skin fibroblasts was investigated. At a concentration of 200 μg/ml, these peptides did not cause lysis of erythrocytes nor did they perturb membrane integrity of the cultured fibroblasts (data not shown).

cDNA Isolation and Characterization—A total of 12 cDNA clones were isolated from the dry seed cDNA library by hybridization.

**TABLE II**

| Bacteria                  | IC₅₀ |
|---------------------------|-----|
|                           | Ib-AMP1 | Ib-AMP4 | Magainin I |
| Gram-positive bacteria    |       |        |            |
| *Bacillus subtilis*       | 10    | 5      | 20         |
| *Micrococcus luteus*      | 10    | 5      | 20         |
| *Staphylococcus aureus*   | 30    | 20     | 30         |
| *Streptococcus faecalis*  | 5     | 6      | 20         |
| Gram-negative bacteria    |       |        |            |
| *Erwinia amylovora*       | ND²  | >100   | ND         |
| *Escherichia coli*        | >50   | >50    | ND         |
| *Proteus vulgaris*        | >50   | >50    | ND         |
| *Pseudomonas solanacearum*| >50   | >100   | ND         |
| *X. campestris*           | ND    | 6      | ND         |
| *X. oryzae*               | ND    | 15     | ND         |

² ND, not determined.

**FIG. 3.** Morphological changes induced by Ib-AMP1 on partially inhibited *F. culmorum*. Fungal spores were either germinated and grown in medium A in the presence of the peptides and observed after 24 h of incubation (A) or pregerminated for 24 h prior to the addition of the peptide and observed 8 h later (B). The concentration of Ib-AMP1 was 0.5 μg/ml in A and 5 μg/ml in B. Control incubations were carried out with the addition of water.

**FIG. 4.** Complete nucleotide sequence of cDNA clone Ib22 insert and the predicted translation product. Amino acid residues comprising the domains representing the mature Ib-AMP peptides are in **bold** and **boxed**; those comprising the predicted signal sequence are **underlined**. The predicted termination codon is **double underlined**. The DNA sequences immediately upstream of poly(A) tails in otherwise identical cDNA clones are shown. The number of independently isolated cDNA clones exhibiting these different sites of polyadenylation is indicated in parentheses. The annealing positions of the named degenerate oligonucleotide PCR primers used to generate the initial hybridization probe are indicated by **double arrows** underneath the first of the Ib-AMP1 repeat regions.

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A general gene structure and alignment of peptide sequences. A, the generalized structure of the predicted 333 amino acid primary translation product in which the individual 20-amino acid domains representing the mature Ib-AMP peptides are shaded, with the Ib-AMP member indicated immediately above. The propeptide regions are unshaded, the predicted prepeptide (signal sequence) region is hatched, and the number of amino acids comprising each of these regions is indicated. B, amino acid sequence alignment of the predicted translation products of each mature domain. A consensus sequence for the Ib-AMPs appears below in which upper case letters represent invariant residues, a an aromatic residue, b a basic residue, and x a nonconserved residue. Cysteine residues are in bold text.

FIG. 5. General gene structure and alignment of peptide sequences. A, the generalized structure of the predicted 333 amino acid primary translation product in which the individual 20-amino acid domains representing the mature Ib-AMP peptides are shaded, with the Ib-AMP member indicated immediately above. The propeptide regions are unshaded, the predicted prepeptide (signal sequence) region is hatched, and the number of amino acids comprising each of these regions is indicated. B, amino acid sequence alignment of the predicted translation products of each mature domain. A consensus sequence for the Ib-AMPs appears below in which upper case letters represent invariant residues, a an aromatic residue, b a basic residue, and x a nonconserved residue. Cysteine residues are in bold text.

B

Clone Ib22 is considerably larger than would be anticipated for a cDNA encoding a single IbAMP peptide. In fact it contains an open reading frame encoding a predicted protein comprising 333 amino acids, with a molecular mass of 37,262 Da. Analysis of this predicted translation product reveals within it the presence of domains that correspond exactly in amino acid sequence to the four antimicrobial peptides described above. Ib-AMP1 is represented three times in consecutive repeats, the other Ib-AMPs once each. The six boxed regions in Fig. 4 represent, consecutively, Ib-AMP3, Ib-AMP1, Ib-AMP1, Ib-AMP1, Ib-AMP2, and Ib-AMP4. Hydrophyt plots and sequence analysis (not shown) of the predicted translation product deriving from clone Ib22 predict a 23-amino acid N-terminal signal sequence, consistent with the fact that many plant antifungal peptides are extracellularly located (1). The general structure of the predicted translation product and an amino acid alignment of those regions assumed to encode the individual mature peptides are presented in Fig. 5.

The proprotein domains flanking each Ib-AMP peptide domain vary in length between 16 and 35 amino acids but display some degree of homology with each other, each containing at least five negatively charged amino acids, generally organized as doublets in the vicinity of the presumed cleavage sites (see Fig. 4). This composition is in contrast to the highly basic Ib-AMP domains, which are separated by these proprotein regions. Although there is no clear homology to other peptide sequences or processing sites in the data bases, it is assumed that these regions contain information required for the correct processing of the preproprotein into constituent mature peptides.

Analysis of Expression—To investigate the accumulation of Ib-AMP-related transcripts and protein during seed development, both Northern and Western blot analysis was performed on material isolated from developing, dry, and germinating seed. The hybridization pattern resulting from Northern blots probed with the entire insert of clone Ib22 is shown in Fig. 6. There appears to be hybridization to a single class of transcript of ~1200 nucleotides, which is in accordance with the size predicted from the cDNA sequence analysis of clone Ib22. The pattern of hybridization indicates that the highest accumulation of related transcript is found in dry seed (Fig. 6, lane E) and in the stage of development immediately prior to this (Fig. 6, lane D).

Western blots using Ib-AMP1 antibody indicate that immunoreactive material is most abundant in dry seed, from which the Ib-AMPs were originally isolated, and in seed undergoing the early stages of germination (Fig. 7, lanes E, F, and G). There appear to be significant quantities of such material also present in the developmental stage immediately preceding seed dry-down (Fig. 7, lane D). Immunoreactive material migrating at a higher position on the gel (Fig. 7, lane C) may represent unprocessed or incompletely processed precursor protein. An investigation into the processes involved in maturation of the precursor protein is currently under way.

DISCUSSION

Four closely related, small, basic, cysteine-rich peptides have been purified from seed of I. balsamina and shown to be active in vitro against a range of fungal and bacterial species. The majority of the amino acid sequence of the four peptides could be determined experimentally, although complete assignment was prevented because the N terminus of each was blocked. The molecular mass of Ib-AMP1 as determined by electrospray mass spectrometry indicated that the full-length Ib-AMP1 sequence was only 2 amino acids longer at its N terminus than the 18 amino acids assigned by direct amino acid sequencing. It can be predicted that the amino acid residue N-terminally adjacent to the sequenced region must be either a tryptophan or a tyrosine for chymotrypsin to have cleaved Ib-AMP1 in that position. Furthermore, based on the molecular mass estimation and the fact that the peptide is N-terminally blocked, it can be considered likely that the unassigned N-terminal residue is a cyclized glutamine. Both these speculations were indeed confirmed for all four Ib-AMP peptides by the subsequent analysis.
of filamentous fungi when assayed in medium A. When this medium is supplemented with CaCl$_2$ and KCl, only Ib-AMP4 retains any significant inhibitory activity, even though it differs from the more sensitive Ib-AMP1 by only a single amino acid residue substitution. A reduction in antifungal activity in media of increased ionic strength is a common feature of most of the small cationic peptides isolated to date and probably reflects the weakening of electrostatic interactions with the target rather than any alteration of the structure of the peptide by the binding of ions from the medium (11). This is supported by the fact that the degree of reduction in antifungal activity is dependent on the test fungus (Table I). Moreover, the finding that the only one of the four Ib-AMP peptides that remains active in medium B is the most basic homologue would also support this. On the whole, the antifungal activity of the Ib-AMPs compares favorably with the more active antifungal and antimicrobial peptides purified to date from plants (2–4, 8, 10, 11).

In addition to their antifungal activity, the Ib-AMPs were also inhibitory to the growth of the four Gram-positive bacteria that were tested and to the growth of two Gram-negative Xanthomonas species. In these assays, their activity was compared with the antibiotic peptide magainin I (18), and on all four of the Gram-positive bacteria tested the Ib-AMPs were equally active as or more active than magainin I. Few of the other antifungal peptides isolated to date from plants show significant levels of activity on bacteria (2, 14).

From the literature, it is apparent that peptides with very different functions can share a common structure. The $\alpha$-conotoxins, which have been purified from marine snails, have a similar cysteine arrangement to the Ib-AMPs, although the spacing between the two C-terminal cysteines is different (23). The solution structure for $\alpha$-conotoxin G1 has been determined using two-dimensional NMR and shows that the two disulfide bonds stabilize a nonrandom coil structure with two $\beta$-turns (24). Preliminary work involving the analysis of purified products released from unreduced Ib-AMP1 following digestion by trypsin showed that Cys$_{15}$ could not be connected to Cys$_{20}$ but rather that the C-terminal cysteine was connected to either Cys$_{6}$ or Cys$_{7}$ (data not shown). The two remaining possible combinations of pairwise connectivities (i.e. Cys$_{6}$-Cys$_{15}$ and Cys$_{7}$-Cys$_{20}$ or Cys$_{6}$-Cys$_{20}$ and Cys$_{7}$-Cys$_{15}$) could not be resolved by protease digestion methods. However, NMR has since been used to determine a solution structure for Ib-AMP1, enabling a comparison with that of the $\alpha$-conotoxins, and will be reported elsewhere.$^2$

The amino acid sequence of each of the four Ib-AMP peptides isolated from dry seed can be identified within the predicted translation product of a single class of cDNA obtained from RNA also isolated from dry seed. One of the peptides is represented three times, the other three once. The fact that 12 individual cDNAs have essentially identical DNA sequences suggests that, at least in seed, only a single gene encoding such peptides is expressed. Southern blotting of genomic DNA suggests that there is only one gene (data not shown).

cDNAs encoding antibacterial peptides processed from a mature, proline-rich, 18-amino acid apidaecin peptides from bees are encoded by a family of cDNAs which contain up to 12 peptide repeats separated by well conserved “processing” regions (25). The cDNA sequence coding for Xenopus prepmagainins also has a multipeptide structure (26). In plants, there are two published examples of cDNAs encoding multipeptide precursors. The first well known example is that

$^2$ S. Patel and J. Thornton, personal communication.
The size of the mature peptide (number of amino acids) and spacing of cysteine residues within its sequence is presented for Ib-AMP1 and representative members of other reported plant antifungal peptide families. Figures in the final column represent the number of amino acid residues flanking the cysteine residues indicated.

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### TABLE III

**Comparison of plant antifungal peptides**

| Peptide family | Representative member | Ref. | Size | Spacing of cysteine residues |
|---------------|-----------------------|------|------|-----------------------------|
| Plant defensins | Re-AMP2 | 1, 10 | 51 | 3-C-10-C-3-C-2-C-2-C-2-C-2-
| Knottin-type | Mj-AMP1 | 39 | 1-C-6-C-4-3-C-2-C-2-C-2-C-2-
| Lipid transfer protein | Ac-AMP1 | 8 | 3-C-3-C-2-CC-1-C-1-C-3-C-1-C-3-C-|
| Hevein-type | Ac-AMP2 | 3 | C-2-C-2-C-2-C-2-|
| Macadamia | Mi-AMP1 | 20 | 76 | 1-C-9-C-1-C-25-C-14-C-11-C|
| Maize basic protein | MBP-1 | 4 | 33 | 6-C-3-C-13-C-3-C-4|
| Thionin (8-Cys type) | α-Purothionin | 21 | 45 | 2-CC-7-C-3-C-8-C-3-C-1-C-8-C-6|
| Impatiens | Ib-AMP1 | This paper | 20 | 5-CC-8-C-3-C|

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