Characteristics and antifungal activity of a chitin binding protein from *Ginkgo biloba*

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Abstract An antifungal peptide from leaves of *Ginkgo biloba*, designated GAFP, has been isolated. Its molecular mass of 4244.0 Da was determined by mass spectrometry. The complete amino acid sequence was obtained from automated Edman degradation. GAFP exhibited antifungal activity towards *Pellicularia sasakii* Ito, *Alternaria alternata* (Fries) Keissler, *Fusarium graminearum* Schw. and *Fusarium moniliforme*. Its activities differed among various fungi. GAFP could also cause increased hyphal membrane permeabilization and a rapid alkalization of the medium when applied at 100 μg/ml to *Pellicularia sasakii* Ito hyphae. The amino acid sequence of GAFP shows characteristics of the cysteine/glycine-rich chitin binding domain of many chitin binding proteins. The cysteine residues are well conserved.

Key words: Antifungal peptide; Chitin; Membrane permeabilization; Chitinase; *Ginkgo biloba*

1. Introduction

Plants use a variety of cysteine/glycine-rich small antimicrobial proteins to protect themselves from pathogen invasion. Thionines [1–3], plant defensins [4–6], antimicrobial peptides from *Amaranthus caudatus* (Ac-AMPs) [7,8], hevein-like [8–10] and knottin-like peptides [11] belong to these classes of antimicrobial proteins. They are all small (29–54 residues) and cysteine-rich peptides, but they are highly divergent in primary structure and exhibit quite different antimicrobial activities. Among them, hevein-like peptides and Ac-AMPs are also chitin binding peptides. They contain either a hevein domain or a homologous sequence.

At present, it is not known how most antimicrobial peptides exhibit antifungal properties and/or antibacterial activities at the molecular level. Van Parijs et al. [9] speculated that the antifungal properties of hevein and *Urtica dioica* agglutinin are somewhat related to the size of protein molecules. These proteins are small enough to penetrate through the fungal cell wall and to reach the plasma membrane, where they may have an effect on active sites of cell wall morphogenesis. Thionines are believed to make pores in the membranes, which then results in leakage of the membrane and leads to the death of the microorganism [2].

This paper reports the isolation, structural determination, and the mode of action of a novel antifungal peptide (GAFP) from leaves of *Ginkgo biloba*.

2. Materials and methods

2.1. Materials

The leaves of *G. biloba* were obtained from the garden of our institute in September. All plant pathogens, including *Pellicularia sasakii* Ito, *Alternaria alternata* (Fries) Keissler, *Fusarium graminearum* Schw., *Fusarium moniliforme* and *Phytophthora boehmeriae*, were provided by the Jiangsu Institute of Agriculture. Regenerated chitin was freshly prepared from 3.5 g of chitosan according to the procedure of Molano et al. [12], autoclaved, equilibrated with 20 mM $NaHCO_3$, and filled in a chromatography column (2.6 cm in diameter). The resulting bed volume was about 46 ml. The other reagents were of the highest grade commercially available.

2.2. Purification of antifungal peptide

Unless otherwise stated, all purification steps were carried out at 4°C. The leaves of *G. biloba* were fully homogenized with 2–3 volumes of 30 mM NaPi–citrate buffer, pH 5.0 (1 mM phenylmethylsulfonyl fluoride). The homogenate was filtered on three layers of gauze and the filtrate was centrifuged at $10\,000\times g$ for 30 min. Solid ammonium sulfate was added to the supernatant to 80% saturation. After stirring overnight, the crude proteins were collected by centrifugation ($38\,000\times g$, 30 min), resuspended in 30 ml of 20 mM $NaHCO_3$ and dialyzed extensively against 20 mM $NaHCO_3$, with a 3.5 kDa dialysis membrane. The dialyzed solution was then loaded on the regenerated chitin column at a flow rate of 0.5 ml/min. The first washing was performed with 20 mM $NaHCO_3$ until the eluate was free of proteins. A second washing with 20 mM sodium acetate–acetic acid buffer, pH 5.6, followed for 90 min. Finally the proteins were released from the chitin matrix with 20 mM acetic acid, pH 3.3, and immediately dialyzed against distilled water, with a 3.5 kDa dialysis membrane. After dialysis, the protein solution was dried in a vacuum concentrator. Then the samples were dissolved in 200 mM $NaHCO_3$ and subjected to FPLC (Pharmacia Biotech FPLC system) on a Superose 12 column (Pharmacia Biotech) equilibrated with 200 mM $NaHCO_3$. The samples were eluted with 200 mM $NaHCO_3$ at a flow rate of 0.4 ml/min within 70 min. The eluate was monitored for protein by measurement of the absorbance at 280 nm. The molecular mass of each fraction was determined by SDS–PAGE [13]. Antifungal activity was also monitored in each fraction. One active fraction which eluted at 19 min was then loaded on a reverse-phase C_{18} column (ABI) in equilibrium with 0.1% trifluoroacetic acid (TFA) for further purification. The column was eluted at 1 ml/min with the following gradient (solvent B is acetonitrile containing 0.1% TFA): 0–50 min, 0–50% B. The eluate was monitored for protein by measurement of the absorbance at 214 nm. Chromatography was performed on a HP 1090 HPLC station.

2.3. Mass spectrometry analysis

Mass spectrometry analysis of peptides was performed on a Finnigan LCQ-MS, an instrument that essentially consists of an atmospheric pressure electrospray positive ion source, attached to a triple quadrupole mass analyzer. The purified peptide (100 pmol) was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a peptide concentration of 5 μmol/l, and then applied on the MS instrument.

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2.4. Protein concentration determination and amino acid sequencing

Protein concentration was determined by the method of Bradford [14], with bovine serum albumin as the standard.

After the purified peptide was reduced and alkylated according to the method described by Fullmer [15], automated Edman degradation of the pyridylethylated peptide and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems model 491). The amino acid sequence of residues was obtained from N-terminal sequencing.

2.5. Assay of antifungal activity

Antifungal activity was determined under sterile condition using a hyphal extension–inhibition assay as described by Roberts and Selitrennikoff [16]. Fungal mycelia were harvested from actively growing fungal plates and placed in the center of Petri dishes containing potato dextrose agar used for maintenance of the fungus under test. After incubation of these dishes for 48 h at 28°C to allow for mycelial growth, sterile filter paper disks were placed on the agar surface in front of the advancing fungal mycelium and then 20 μl of the protein solution in 10 mM sodium acetate buffer, pH 5.4, was applied to each disk. The plates were incubated at 28°C for 24 h and then photographed. In this manner, if the material being tested was antifungal, a crescent-shaped zone of inhibition was observed around the disk.

Percent growth inhibition was assayed as described by Broekaert et al. [7]. Briefly, fungal spores or hyphal pieces were collected as described by Duvick et al. [17] and standard methods [18]. 90 μl of PD liquid medium containing fungal spores or hyphal pieces was incubated for 24 h. After this period, 10 μl aliquots of various concentrations of peptide were added. Growth was recorded after 48 h of further incubation at 28°C. The absorption at 595 nm served as a measure for fungal growth [5].

2.6. Hyphal membrane permeabilization assay and external pH measurements

P. sasakii Ito was grown at an inoculum density of 3×10^5 spores/ml at 28°C in PD liquid medium. After 30 h of incubation, [^{14}C]leucine (2 μCi/ml) was added. After an additional incubation of 3 h to allow loading of the hyphae with [^{14}C]leucine, 250 μl samples were taken and transferred to 0.5 ml Eppendorf tubes. The samples were centrifuged at $3000\times g$ for 5 min at 20°C. After the supernatant was discarded, hyphae were washed three times with PD liquid medium. The washed hyphae were then resuspended in 250 μl of PD liquid medium containing antifungal proteins (control suspension contained no antifungal protein). After 3 h of incubation in the presence of the protein, mycelial suspensions were centrifuged at $5000\times g$ for 5 min at 20°C, and supernatants were collected into a 96 well microtiter plate and counted for ^{14}C.

Mycelial suspensions of *P. sasakii* Ito, 24 h old, grown in PD liquid medium at an inoculum density of 3×10^5 spores/ml, were centrifuged at $3000\times g$ for 5 min at 20°C and resuspended after centrifugation in PD liquid medium containing 100 μg/ml antifungal protein. At different times the pH of the suspension was measured using a pH meter. Control suspension contained no antifungal protein.

3. Results

3.1. Purification of antifungal peptide

GAFP was purified from the leaves of *G. biloba*. Purification of GAFP entailed the following four steps: ammonium sulfate fractionation, affinity chromatography on regenerated chitin, FPLC on a Superose 12 column, and C_{18} reverse-phase HPLC. Fractions or eluates from the second to the last steps were assayed for growth inhibition activity against the fungus, *P. sasakii* Ito. At the second step, proteins, which could bind chitin especially, were released from chitin matrix with 20 mM acetic acid, pH 3.3. The whole protein peak contained a 30 kDa protein and peptides. By the method of Trudel et al. [19], the band of the 30 kDa protein in SDS–PAGE gels was detected to have chitinase activity (data not shown). The whole protein peak was then subjected to FPLC. A fraction that eluted at 19 min showed strong antifungal activity. SDS–PAGE analysis showed that this fraction only contained peptides. This fraction was then subjected to C_{18} reverse-phase HPLC. Two separate protein peaks were eluted (Fig. 1). The second peak showed strong antifungal activity while very low activity was detected in proteins of the other peak (Figs. 1 and 2). From 500 g of *G. biloba* leaves, approximately 4 mg of the purified GAFP was obtained for analysis.

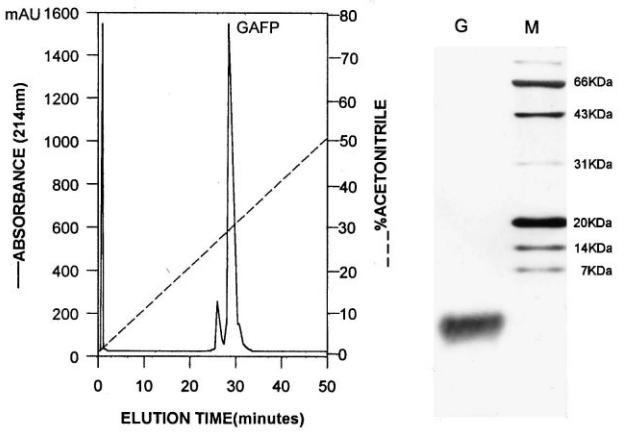

Fig. 1. Purification of GAFP by HPLC. 1 mg amounts of the FPLC GAFP peak were loaded on a reverse-phase C_{18} column (ABI) in equilibrium with 0.1% TFA. The column was eluted at 1 ml/min with the following gradient (solvent B is acetonitrile containing 0.1% TFA): 0–50 min, 0–50% B. The eluate was monitored for protein by measurement of the absorbance at 214 nm. Chromatography was performed on a HP 1090 HPLC station. The molecular mass of the active fraction was determined by 15% SDS–PAGE (right panel). Lane M: molecular mass standards; lane G: active fraction after C_{18} reverse-phase HPLC.

3.2. Amino acid sequence and mass spectrometry analysis

The amino acid sequence of 38 residues was obtained from N-terminal sequencing (Fig. 3), thereafter the sequencer response dropped abruptly. According to the mass spectrometry analysis, the sequence of 38 residues must be the complete sequence of GAFP. The molecular mass obtained from the mass spectrometry analysis was 4244.0 Da, and the mass calculated from the 38 residues determined by sequencing was 4250.7 Da, i.e. 6 Da in excess of that measured by mass spectrometry. This difference of 6 Da is attributable to the arrangement of six cysteine residues into three intramolecular disulfide bridges, a process that eliminates six hydrogen atoms. The first residue of the sequence is not methionine, therefore GAFP is probably synthesized as part of a larger precursor.

The amino acid sequence of GAFP contains characteristics of the cysteine/glycine-rich chitin binding domain of many chitin binding proteins as seen in Fig. 3. The cysteine residues are well conserved.

3.3. Assay of antifungal activity

Antifungal activities of GAFP were tested against five phytopathogenic fungi. The fungi examined in this study were *P. sasakii* Ito, *A. alternata* (Fries) Keissler, *F. graminearum* Schw., *F. moniliforme* and *P. boehmeriae*. GAFP showed potent antifungal activities against the agronomically important pathogen *P. sasakii* Ito with as little as 100 ng of GAFP per disk (Fig. 2). It also inhibited the growth of other plant patho-

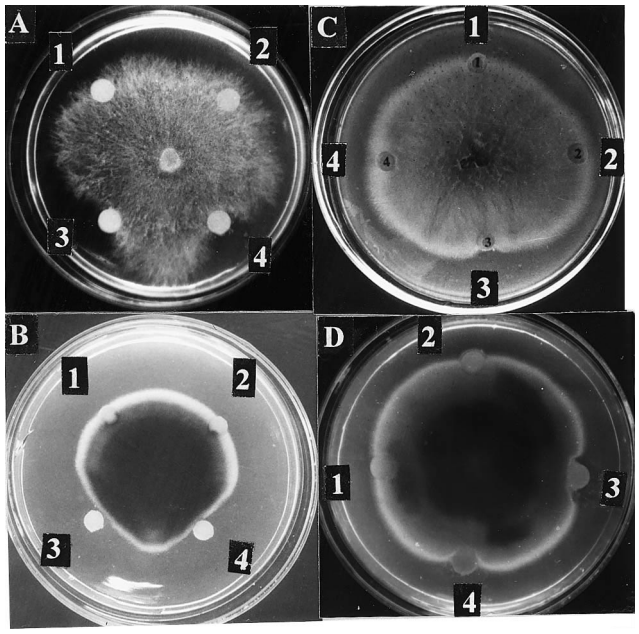

Fig. 2. Antifungal activities in vitro of the purified GAFP against *P. sasakii* Ito (A), *A. alternata* (Fries) Keissler (B), *F. graminearum* Schw. (C), and *F. moniliforme* (D). Approximately 2 and 1 μg of the purified GAFP in 20 μl of 10 mM sodium acetate buffer, pH 5.4, were applied on the disks numbered 3 and 4 respectively. For control, 20 μl of the water and bovine serum albumin were tested on the disks numbered 1 and 2 respectively.

gens, including *A. alternata* (Fries) Keissler, *F. graminearum* Schw. and *F. moniliforme* (Fig. 2). The purified GAFP had no effect on the growth of the pathogen *P. boehmeriae* at 15 μg of GAFP per disk.

The percent growth inhibition of GAFP against *P. sasakii* Ito was assayed (Fig. 4). At concentrations ranging from 0 to 10 μg/ml GAFP did not show evident antifungal activity. GAFP showed increased growth inhibition activity to *P. sasakii* Ito as the concentration rose from 20 to 100 μg/ml. Then the activity did not rise rapidly any more.

3.4. Hyphal membrane permeabilization assay and external pH measurements

To test the effect of GAFP on permeabilization of the hyphal membrane, *P. sasakii* Ito hyphae were incubated in the

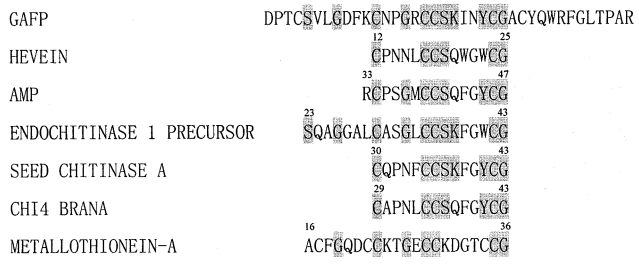

Fig. 3. Amino acid sequences of GAFP and comparison of the sequence with related proteins. Amino acids identical to GAFP are indicated by gray boxes. HEVEIN: rubber latex hevein [21]. AMP: two antimicrobial peptides from *A. caudatus* (accession number P27275). ENDOCHITINASE 1 PRECURSOR: endochitinase 1 precursor (accession number P52403). SEED CHITINASE A: seed chitinase A (accession number P29022). CHI4 BRANA: basic endochitinase CHB4 precursor (accession number Q06209). METALLOTHIONEIN-A: metallothionein A (accession number P04734).

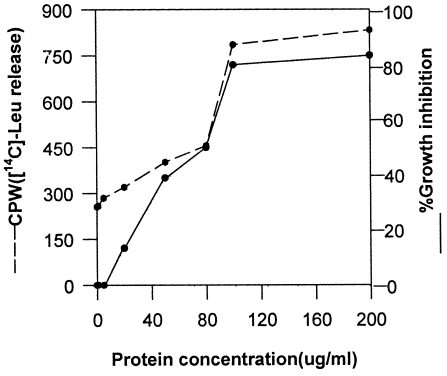

Fig. 4. The effect of GAFP on growth inhibition and [^{14}C]leucine release in *P. sasakii* Ito hyphae. Dose–response curves of growth inhibition (solid line) and [^{14}C]leucine release (dashed line) in *P. sasakii* Ito hyphae treated with GAFP. Values are expressed as the means of three replicates.

presence of [^{14}C]leucine. After uptake of this compound, the hyphae were washed and treated with GAFP, and then release of [^{14}C]leucine was measured. As shown in Fig. 4, GAFP caused a 3–4-fold increase in the release of the amino acid when the concentration of GAFP rose from 20 to 100 μg/ml. The minimal concentration required for evident growth inhibition effect was about 20 μg/ml. Above the concentration tested (100 μg/ml), GAFP could not cause more release of the amino acid.

When applied at 100 μg/ml to *P. sasakii* Ito hyphae, GAFP caused a rapid alkalization of the medium. The pH of the external medium rose from 6.01 to 6.30 within 5 min. After 5 min, the pH of the external medium rose slowly from 6.30 to 6.41. At last the pH of the external medium did not rise any more and kept at about pH 6.41.

4. Discussion

Hevein is believed to be small enough to penetrate through the fungal cell wall and to reach the plasma membrane, where it may have an effect on active sites of cell wall morphogenesis [9]. In this paper we found that GAFP could cause increased hyphal membrane permeabilization, as measured by increased [^{14}C]leucine release from preloaded hyphae, at a concentration of 20 μg/ml, which is equal to the minimal concentration required for an evident growth inhibition effect. But compared with thionine which affects growth of filamentous fungi mainly by causing membrane permeabilization [20], GAFP only caused a 3–4-fold increase in the release of the amino acid at the concentration tested (100 μg/ml). At this concentration (100 μg/ml), thionine could cause a 20-fold increase in the release of the amino acid [20]. Hence, for GAFP, membrane permeabilization appears to be a secondary effect, but not the primary cause of hyphal growth inhibition. When applied at 100 μg/ml to *P. sasakii* Ito hyphae, GAFP caused a rapid alkalization of the medium within 5 min. It seems that GAFP can cause a change in ion flux on the fungal membrane. Meanwhile, as seen in Fig. 4, the hyphal growth inhibition action of GAFP corresponds to the release of the amino acid. According to these data, we speculate that GAFP exhibits its antifungal activity by interaction with the fungal membrane.

Many proteins exist in a precursor form. The first residue of

GAFP is not methionine, so we think there must exist another large protein that contains GAFP in vivo. Some unknown signal may cleave the large protein to produce GAFP, which is small enough to penetrate through the fungal cell wall.

As a kind of effective fungistat, GAFP showed potent antifungal activities against four agronomically important pathogens in vitro. Moreover, its primary structure is very simple. All this will benefit its application in agriculture.

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