Isolation and purification of a 316 Da preformed compound from strawberry (*Fragaria ananassa*) leaves active against plant pathogens

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Abstract An antibiotic called fragarin showing activities against bacterial and fungal plant pathogens was isolated and purified by FPLC chromatography from the soluble fraction of strawberry leaves. The molecular weight value determined by mass spectrometry is 316 Da. Fragarin remains fully active after protease treatment or alkaline hydrolysis at 100°C for 20 min. Biological and chemical analyses suggest that fragarin may be a new type of an antimicrobial preformed compound – phytoanticipin – and would constitute a primary non-specific barrier of strawberry defense.

Key words: Plant antibiotic; Fragarin; Phytoanticipin; Plant defense; Strawberry

1. Introduction

Plants growing in natural habitats are normally exposed to a broad range of microorganisms, many of which are pathogens or opportunists. However, they are able to resist microbial attack and depending on their tolerance toward a particular pathogen, they can continue to grow, overcoming the stress induced by the microbial action. The latter is possible, because plants have various physical and chemical strategies that allow them to defend themselves, the chemical mechanism being the most effective barrier against pathogens and herbivorous attacks [1]. These active compounds can be inducible or preformed, and they can be grouped into two major classes: (i) antimicrobial proteins (for review see [2]) and (ii) a wide variety of non-protein compounds or secondary metabolites. Their distribution is often tissue-specific [3,4] and they are usually found in cells located at the external layers of plant tissues, suggesting that these compounds would be the first line of defense against pathogen attacks. Although a great variety of preformed plant secondary metabolites have been reported (e.g. lactones, saponins, flavonoids, etc.), only few of them have been well characterized and studied in detail to determine their possible role in plant defense against fungal and bacterial pathogens [5].

In this communication we report the isolation, purification and partial characterization of an antimicrobial constitutive compound of 316 Da, present in the soluble fraction of strawberry (*Fragaria ananassa*) leaves. This compound shows in vitro activity against bacterial and fungal plant pathogens.

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2. Materials and methods

2.1. Plants

Strawberries (*Fragaria ananassa*) cultivar Chandler were grown for 20 days in sterilized substrate (3:2:1 soil/sand/perlome) at 25°C, 70% humidity and a photo-period of 16 h. Leaves were collected and immediately frozen in liquid nitrogen.

2.2. Microorganisms

Clavibacter michiganensis subsp. *sepedonicus*, strain C5, which is a tomato bacterial pathogen, was kindly provided by Dr. A. Molina from the ETSIA (Escuela Técnica Superior de Ingenieros Agrónomos) collection, Madrid (Spain). Pathogenic bacteria were obtained from I. Zarzoza of the Phytopathology Department of the Facultad de Agronomía y Zootecnia of the Universidad Nacional de Tucumán (UNT). *Pseudomonas corrugata* was isolated from tomatoes, *P. syringae* was isolated from onion, *Erwinia* spp. were isolated from rose leaves. Fungi of the genus *Colletotrichum* (*C. acutatum*, *C. fragariae* and *C. gloeosporioide*), which are agents associated with the antracnose disease, were isolated by our group (unpublished result) from strawberries cultivated in Tucumán (Argentina).

2.3. Extraction procedure and purification

Leaf homogenate was obtained from 100 g of fresh material that was previously frozen and ground in liquid nitrogen. The powder was extracted with Tris/EDTA buffer (0.1 M Tris-HCl, 10 mM EDTA), 20 mM β-mercaptoethanol, and 1.5% PVP (polyvinyl pyrrolidone), pH 7.5. The homogenate was centrifuged for 10 min at $16\,000\times g$. The supernatant proteins were precipitated with ammonium sulfate (80% saturation) during 60 min at 4°C and then centrifuged at $27\,000\times g$. The pellet was dissolved with 20 ml 0.1 M acetic acid, and dialyzed three times against 5 l of distilled water during 12 h, using benzoylated membrane (molecular weight cut-off: 2000). The dialyzate was concentrated 10-fold, dissolved in 3.0 ml of buffer A (2 mM Tris-HCl, 0.2 mM EDTA, pH 7.5) and filtered through a Sephadex G-75 column (90 cm×2 cm), equilibrated with buffer A. Fractions of 3 ml were collected and antibiotic activity monitored against *C. michiganensis* (Fig. 1). Pooled fractions of high molecular weight (H) and low molecular weight (L) were lyophilized and dissolved in water to continue purification procedures.

Fractions corresponding to the L peak of activity were subjected to FPLC chromatography using a reverse phase C1/C8 column (ProRPC HR 5/10, Pharmacia) equilibrated with water containing 0.1% TFA (trifluoroacetic acid). The elution was performed with a linear gradient of 0–100% of acetonitrile/water (v/v) containing also 0.1% of TFA during 30 min. Fractions of 0.5 ml were collected, freeze-dried and re-dissolved in water for antibiotic activity determinations. Fractions with antibiotic activity that eluted at 100% acetonitrile were pooled, freeze-dried, dissolved in water and re-chromatographed using a linear gradient of acetonitrile (90–100%) during 15 min with the same C1/C8 column. The active peak was analyzed by mass spectrometry using a V6 Quattro Spectrometer with an electrospray ionization sample preparation system.

Fractions corresponding to the H peak of activity were subjected to anion exchange chromatography on a DEAE cellulose Fast Flow column (10×1 cm, Sigma), equilibrated with buffer A and eluted with a linear gradient of 0–0.5 M NaCl in buffer A. Fractions showing activity against *C. michiganensis* were filtered through a regenerated cellulose membrane NMWL 1000 Da (Millipore) at 4°C. Filtered and retained fractions were tested for antibiotic activity. The active fraction was further subjected to the same purification steps as the L peak.

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2.4. Electrophoresis

Fractions L_1 and L_2 from FPLC chromatography (Fig. 2) showing antibiotic activity were pooled and analyzed by 16.5% SDS-PAGE electrophoresis [6]. Gels were prepared in duplicate. One of them was stained with Coomassie brilliant blue or silver following Bio-Rad protocols and the other gel was used for detecting the migration profile of the active compound by means of the growth inhibition area. For the visualization of growth inhibition bands, the gel was washed twice with sterile water and covered with a suspension of *C. michiganensis* in Luria broth (LB) soft agar (Sigma) at a concentration of 10^6 cfu/ml, and incubated for 12 h at 28°C. Thionine of type II, which is an antibiotic peptide of 5000 Da, was used as control [7]. Protein concentration was determined by the Bradford [8] and fluorescamine methods [9] using bovine serum albumin as standard.

2.5. Antibiotic activity determination

Antibiotic activity was routinely evaluated against *C. michiganensis* using the inhibition haloes method. 100 μl of each chromatography fraction was lyophilized, dissolved in 10 μl of bi-distilled water and assayed for biological activity. Fractions were laid on an LB plate, air-dried and covered with 3 ml of melted soft agar (40°C) containing a suspension of 10^6 cfu/ml bacteria. Plates were incubated for 12 h at 28°C.

EC_{50} values (μg/ml) of fragarin for bacterial and fungal plant pathogens were determined according to Fernandez de Caleya et al. [10]. Fragarin was dispensed into wells that contained sterile nutrient broth and serially diluted in the same medium prior to inoculation. The wells were inoculated with bacterial or conidial suspensions at a final concentration of 10^4 cfu/ml (final volumes of 150 μl/well). Fungal conidia were collected from 8-day-old cultures grown at 28°C on potato dextrose agar plates. After 12–24 h of incubation for bacteria and 12–48 h for fungi at 28°C, growth was evaluated by measuring absorbance at 490 nm in an ELISA plate reader.

2.6. Hemolytic activity assay

Hemolytic activity was determined according to Terras et al. [11] using human red blood cells.

2.7. Alkaline hydrolysis

Purified fragarin (100 μl, 1 mg/ml) was lyophilized, dissolved in 100 μl of 0.5 N NaOH and autoclaved at 120°C for 20 min. Treated samples were neutralized with 100 μl of 0.5 N HCl and used for testing antibiotic activity against *C. michiganensis*.

2.8. Protease treatment

The effect of protease type VIII, chymotrypsin and trypsin on the antibiotic activity of fragarin was tested. 100 μl samples of a fragarin suspension (1 mg/ml) were mixed separately with 40 μl of each protease (1 mg/ml, in buffer A) and incubated at 37°C for 24 h. Antibiotic activity was monitored against *C. michiganensis*.

3. Results

3.1. Chromatographic separation of antimicrobial activities on Sephadex

Extracts of strawberry leaves prepared as described in Section 2 were filtered through a Sephadex G75 column. Fig. 1 shows two peaks of activity, peak H at about 40 kDa, and peak L of low molecular weight (in the range of 3–5 kDa). Fractions exhibiting antimicrobial activity corresponding to the H and L peaks were pooled separately and lyophilized for further purification.

3.2. FPLC analysis of peak L

First we focussed our attention on the purification of active agent contained in peak L because it exhibited higher activity than peak H (Fig. 1). Pooled fractions corresponding to the L peak were analyzed by FPLC as described in Section 2. Two fractions with antibiotic activity were found (Fig. 2): a hydrophilic peak L_1 that exhibited no interaction with the column and a hydrophobic peak L_2 spread in approximately 10 ml fractions.

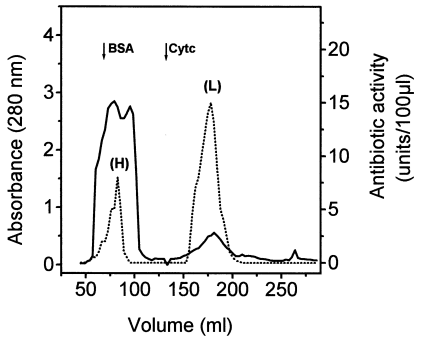

Fig. 1. Sephadex G-75 chromatographic profile of the extracts from strawberry leaves. H, high molecular weight fraction; L, low molecular weight fraction; BSA, bovine serum albumin; Cytc, cytochrome *c*. Solid line, absorbance at 280 nm; dotted line, antibiotic activity (see Section 2).

3.3. SDS-PAGE of peaks L_1 and L_2

Fractions containing peaks L_1 and L_2 were separately collected, pooled, concentrated and analyzed by SDS-PAGE. In Fig. 3A we can see that whereas peak L_1 contains few well-defined bands (lane 5), peak L_2 does not contain any visible protein bands (lane 4). The absence of protein in the fraction corresponding to peak L_2 was further confirmed by overloading the gel with 10-fold concentrated sample and silver staining.

3.4. Peak L_1 contains peak L_2

Results shown in Fig. 3B suggest that the active compound detected in peaks L_1 and L_2 may be the same. In order to test this hypothesis, peaks L_1 and L_2 were subjected to FPLC purification cycles using the same column and conditions used in Fig. 2. Results showed that whereas chromatographic and activity profiles of peak L_2 do not change throughout successive purification cycles, peak L_1 remains active and in each FPLC cycle releases a compound that elutes at the same time as peak L_2 (results not shown). The identity of L_2 and the active compound released from L_1 were further confirmed by fluorometric analysis (see below). Currently we are carrying out experiments to explore other active compounds in peak L_1.

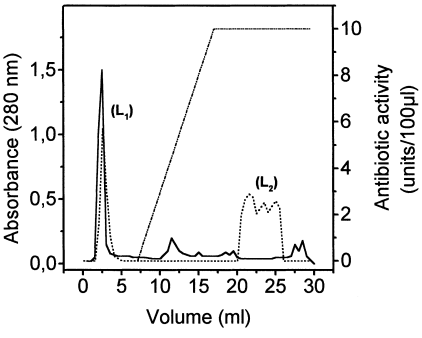

Fig. 2. FPLC chromatographic profile of the low molecular weight peak L (see Fig. 1). L_1, hydrophilic fraction; L_2, hydrophobic fraction; solid line, absorbance at 280 nm; dotted line, antibiotic activity; dot-dashed line, acetonitrile gradient (0–100%).

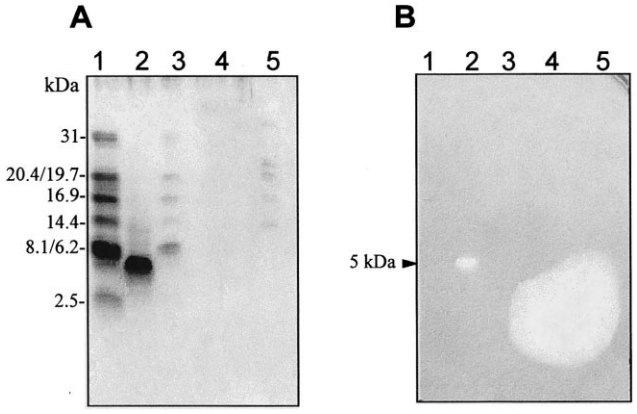

Fig. 3. SDS-PAGE of peaks L_1 and L_2 obtained from FPLC chromatography (see Fig. 2). A: Gel stained with Coomassie brilliant blue. B: Gel revealed for biological activity. Lanes 1 (5 μg) and 3 (0.5 μg), molecular weight markers (Promega); lane 2, thionine type II; lane 4, peak L_2; lane 5, peak L_1.

3.5. Purification of the active compound from fraction H

To characterize the active compound present in peak H eluted from the Sephadex G-75 column (Fig. 1), peak H was subjected to DEAE-cellulose chromatography. The activity was eluted as a single sharp peak at 200 mM NaCl. With the aim of comparing the size of this active compound with L_2, peak H was filtered through the NMWL 1000 Da membrane. The activity was detected in the filtrate. This suggests that the active compound present in fraction H and L_2 might be the same. To confirm this assumption, we decided to follow the same experimental procedure used for the purification of fraction L. Our outcome confirmed that the antimicrobial agent present in fraction H is identical to L_2. Fluorometric analyses also confirmed this result (see below). The fact that previous to DEAE chromatography the biological activity bound to peak H did not pass through the 1000 Da membrane filter could be explained by the association of the antimicrobial compound with molecules of larger molecular weight (i.e. protein ligands). This hypothesis would also be valid for the behavior of the active compound release from peak L_1.

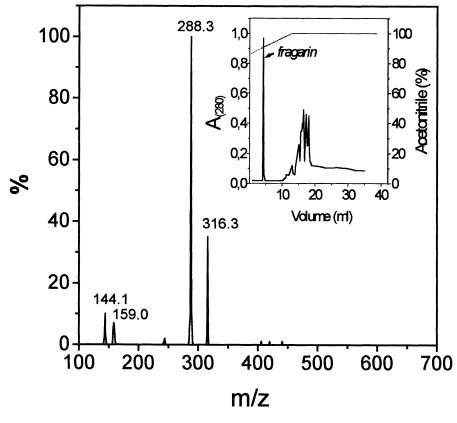

Fig. 4. Mass spectrum of fragarin obtained from an electrospray ionization V6 Quattro Spectrometer. Inset: FPLC chromatography from L_2.

Table 1
Inhibition of bacterial and fungal plant pathogens by fragarin

Pathogen	EC_{50} (μM)
Bacteria	
Clavibacter michiganensis	0.07
Pseudomonas siringae pv. *gladiolii*	0.25
Pseudomonas corrugata	0.29
Erwinia carotovora	0.95
Fungi	
Colletotrichum fragariae	8.92
Colletotrichum acutatum	7.91
Colletotrichum gloeosporioides	9.37

EC_{50}: effective concentration for 50% inhibition.

3.6. Determination of the molecular weight of fragarin

The peaks L_2 from different chromatography runs were pooled, freeze-dried, re-suspended in pure acetonitrile and re-chromatographed as in Fig. 2 but using a 90–100% acetonitrile gradient. Under these new experimental conditions peak L_2 showed a sharp unique and contaminant-free peak of activity eluting at 98% acetonitrile (see inset in Fig. 4). The molecular weight of the molecule called fragarin determined by mass spectrometry is 316 Da (Fig. 4).

3.7. Additional characterizations

Stability assays showed that fragarin remains fully active after protease treatment and alkaline hydrolysis at 120°C for 20 min. These outcomes clearly indicate that fragarin has no peptide-bound structure. However, fragarin has reactive primary amines in its structure because it displays a positive fluorescamine test. Fluorometric analyses shows that fragarin is a fluorescent compound with excitation/emission maxima at 320/380 nm. Fragarin did not show hemolytic activity, therefore, we ruled out that fragarin may be a saponin [5,12].

In order to determine whether purified fragarin is capable of inhibiting the growth of other plant pathogens, dose-response experiments were carried out with five pathogenic bacteria of different plants and three fungi of the genus *Colletotrichum*. From dose-response curves we determined the concentrations required for 50% inhibition of the growth of microbial pathogens (IC_{50}). The results are summarized in Table 1. It shows that the activity of fragarin was 10–100-fold higher against bacterial than fungal pathogens.

4. Discussion

In the present work we have isolated an amphipathic molecule of 316 Da (fragarin) that shows activity against both bacterial and fungal pathogens. Fragarin was 10–100 times more active against bacteria than fungi. The range of concentrations at which fragarin shows inhibitory properties led us to speculate that it may be related to defense compounds [12–14].

The fact that fragarin did not dialyze out of the benzoylated membrane (pore >2 kDa) in the first stage of the purification procedure could be explained by its association with molecules of larger molecular weight (i.e. protein ligands). These accompanying proteins may keep fragarin tightly bound during the first step of purification but release it during the rest of the procedure. Our results show that fragarin is also present in peak H (Fig. 1) and peak L_1 (Fig. 2).

For many years it was assumed that secondary metabolites were useless metabolic by-products, but now it has been demonstrated that these metabolites have many functions in plants and many of them would be important components involved in effective plant defense mechanisms against pathogens and herbivores [15]. Two categories for the secondary metabolites with antibiotic activity were proposed according to how they are produced: (i) inducible compounds, like phytoalexins, which are antimicrobials that are de novo synthesized in response to biotic or abiotic elicitors [16] and (ii) constitutive compounds, like phytoanticipins, which can be found in their biologically active form even in uninjured plants, or as inactive precursors that can be activated by means of specific enzymes [5]. Therefore, the fundamental difference between phytoalexins and phytoanticipins would be that, whereas the former are involved in an active plant response against a pathogen, phytoanticipins would be involved in a passive plant response in its interaction with pathogens [17]. Since fragarin was isolated from the cytosolic fraction of strawberry leaves that were not previously exposed to any pathogenic agent nor to any other biotic or abiotic stress, we also concluded that it may be a preformed soluble compound that is present in its active form. According to biological and chemical features shown by fragarin we speculate that it may be a new type of phytoanticipin related to secondary metabolites [5] and it would be involved in the defense of strawberry against pathogen attacks. Currently we are carrying out experiments to explore if fragarin is involved in plant defense and the possible existence of fragarin-protein ligand.

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