Fungicidal properties of two saponins from Capsicum frutescens and the relationship of structure and fungicidal activity

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Abstract: Two steroidal saponins have been purified from cayenne pepper (Capsicum frutescens). Both have the same steroidal moiety but differ in the number of glucose moieties: the first saponin has four glucose moieties (molecular mass 1081 Da) and the second contains three glucose moieties (molecular mass 919 Da). Solubility in aqueous solution is less for the saponin containing three glucose moieties than for the one containing four glucose moieties. The larger saponin was slightly fungicidal against the nongerminated and germinating conidia of Aspergillus flavus, A. niger, A. parasiticus, A. fumigatus, Fusarium oxysporum, F. moniliforme, and F. graminearum, whereas, the second saponin (molecular mass 919 Da) was inactive against these fungi. Results indicate that the absence of one glucose molecule affects the fungicidal and aqueous solubility properties of these similar molecules.

Key words: fungicidal, saponin, structure–function.

Résumé : Deux saponines stéroïdiques ont été purifiées du poivre de cayenne (Capsicum frutescens). Les deux avaient le même groupe stéroïdique mais différaient dans le nombre de groupes glucosé : la première saponine avait quatre groupes glucosés (masse moléculaire de 1081 Da) et la seconde contenait trois groupes glucosé (masse moléculaire de 919 Da). La solubilité en solution aqueuse était inférieure pour la saponine contenant trois glucoses comparativement à la molécule contenant quatre glucoses. La plus grosse saponine était légèrement fongicide contre des conidies non germinées et en germination de Aspergillus flavus, A. niger, A. parasiticus, A. fumigatus, Fusarium oxysporum, F. moniliforme et F. graminearum alors que la seconde saponine (masse moléculaire de 919 Da) était inactive contre ces champignons. Les résultats indiquent que l’absence d’une molécule de glucose affecte les propriétés fongicides et de solubilité aqueuse de ces molécules semblables.

Mots clés : fongicide, saponine, structure–fonction.

Introduction

Saponins are constitutive triterpenoid, steroid, or steroidal glycoalkaloid molecules having one or more sugar chains and are important in plant defense against microbial infection (Fenwick et al. 1991; Hostettmann and Marston 1995; Osbourn 1996a, 1996b). Saponins have detergent-like properties and are lethal to fungi because of their ability to complex with membrane sterols, resulting in the loss of membrane integrity (Fenwick et al. 1991; Price et al. 1987). Plant mutants defective in saponin biosynthesis have compromised resistance to a variety of fungal pathogens, and a number of lines of evidence suggest that this loss of resistance is a direct consequence of saponin deficiency (Papadopoulou et al. 1999).

Earlier work described the purification, chemical structure, and biological activity of CAY-1, a fungicidal, gitogenin-based, steroidal saponin present in the fruit of cayenne pepper and paprika, members of the Capsicum plant family (De Lucca et al. 2001; Yajima et al. 2000). CAY-1 is lethal to fungi: at concentrations between 3 and 20 µmol·L⁻¹ it produces a 90% lethal dose (LD₉₀) or greater to the germinating conidia of Aspergillus flavus, A. niger, A. parasiticus, and A. fumigatus. Minimal inhibition concentration (MIC) assays show that CAY-1 inhibits the growth of Candida albicans, C. glabrata, C. tropicalis, and C. parapsilosis at 4–31 µg·mL⁻¹, but Renault et al. (2003) showed that at 100 µg·mL⁻¹ it was not toxic to 55 mammalian cell lines. Purification of CAY-1 requires the use of high pressure liquid chromatography – mass spectrometry (HPLC–MS). During this step, CAY-1 is separated from two compounds having molecular masses that are 162 (compound 1, molecular mass 1081 Da) and 324 (compound 2, molecular mass 919 Da) mass units smaller than that of CAY-1 (molecular mass 1243 Da).

The purpose of this work is (i) to purify and confirm the chemical structure of compounds 1 (molecular mass 1081 Da)
and 2 (molecular mass 919 Da). (ii) to determine their fungicidal properties, and (iii) to determine whether compounds 1 and 2 could be breakdown products of CAY-1.

Materials and methods

Crude extraction and semipurification of compounds 1 and 2 from cayenne pepper

Cayenne (Capsicum frutescens) pepper was commercially obtained and extracted as described earlier (De Lucca et al. 2001). Basically, 500 g of ground cayenne pepper (Zatarain’s, New Orleans, Louisiana, USA) was extracted overnight (4 °C) in 2 L of 1% potato dextrose broth (Difco Laboratories, Detroit, Michigan, USA). The pepper slurry was centrifuged (8000g, 4 °C, 10 min) and the supernatant collected. This liquid was again centrifuged (20 000 g, 4 °C, 10 min) to remove fine particles not pelletted during the initial centrifugation. This second supernatant was filtered through a 0.2 µm filter ( Pall Supor AcroPak 200, Pall Corp., Ann Arbor, Michigan, USA) and freeze-dried. The freeze-dried material was dissolved in 140 mL of milliQ water and added to a 200 g C18 column (Waters Corp., Milford, Massachusetts, USA). The bound material was sequentially eluted from the column with a step gradient of methanol–water (at a volume fraction of 0%, 25%, 50%, 75%, and 100% MeOH). The compounds of interest were present in the 75% methanol eluate that was evaporated with nitrogen and freeze-dried.

Purification of compounds 1 and 2

The saponins containing three and four glycosidic moieties were isolated using preparative scale high pressure liquid chromatography (HPLC) and confirmed by electrospray mass spectrometry (MS). Acetonitrile (HPLC grade) was purchased from the Aldrich Chemical Company (Milwaukee, Wisconsin). Water treated with a Millipore system was used during sample preparation procedures and HPLC analyses. For both UV-photodiode array detection and MS, HPLC analyses were performed on a Waters 600E System Controller combined with a Waters UV-VIS 996 detector (200–400 nm). Separations were carried out using a Waters Radial Compression Bondapack C18 (25 mm × 100 mm; 10 µm particle size) reverse-phase column. Two column segments were combined using a PrepLC 25 mm extension kit (Waters). A guard column segment containing the same packing material was used. Elution was carried out at a flow rate of 8 mL·min⁻¹ with the following solvent system: 65 min, 5% B; followed by holding for 15 min at 100% B; wherein solvent A is 0.1% trifluoroacetic acid in water and solvent B is acetonitrile.

The mass spectrometer utilized was a Finnigan MAT LCQ ion trap (Finnigan, San Jose, California) equipped with an electrospray ionization interface. HPLC effluent was split with 0.5 mL·min⁻¹ introduced directly into the electrospray interface. Positive ion mode was used with a spray voltage of 3.5 kV and a capillary temperature of 200 °C. Full scan spectra from m/z 100 to 2000 were measured using 300 ms for collection time and three micro scans were summed. CAY-1 was identified by an intense (M+H)+ ion at m/z 1244 at 13.86 min. The ion (M+H-1glucosyl moiety)+ at m/z 1082 eluted at 15 min. The ion corresponding to (M+H-2glucosyl moieties)+ at m/z 920 eluted at 16.8 min.

Structural analysis of compounds 1 and 2

Nuclear magnetic resonance (NMR) data for CAY-1 (Fig. 1) dissolved in pyridine-d5 (Cambridge Isotope Laboratories, Andover, Massachusetts) were collected on a GE Omega PSG 500 spectrometer at 20 °C in a sample volume of 0.6 mL in 5 mm tube (Willmard 528-PP). The spectrometer was equipped with a 5 mm GE inverse and a broad-band obverse double resonance probe.

MS and MS/MS analyses

The mass spectrometer utilized was a Finnigan MAT LCQ (Finnigan) ion trap equipped with an electrospray ionization interface. Sample flow rate at 300 µL·min⁻¹ was introduced directly into the interface without splitting. Positive ion mode was used with a spray voltage of 4.5 kV. The capillary temperature was 210 °C. The full scan mass spectra of CAY-1 from m/z 500 to 1500 was measured for 400 ms (collection time) and three micro scans were summed. Tandem mass spectrometry was performed at a collision energy of 23% for MS/MS analyses. The MS/MS scan range was collected in full scan mode from m/z 500 to 1300 for 400 ms (collection time) with three micro scans summed.

Bioassays of purified compounds

The purified compounds (1 and 2) were tested for fungicidal activity as described previously (De Lucca et al. 2001). Cultures of A. flavus, A. fumigatus, A. niger, A. parasiticus, Fusarium moniliforme, and F. oxysporum were grown on potato dextrose agar (Difco) slants for 7 days at 30 °C and stored at 4 °C until needed. The fungicidal properties of each compound were determined by performing separate bioassays with nongerminated and germinating conidia (De Lucca et al. 2001). Sonication was required to solubilize the test compounds, particularly compound 2 (919 Da molecular mass). Four millilitres of potato dextrose broth (Difco) was...
aseptically added to the test fungal slant culture and the conidia suspended by agitation with the pipette tip. Stock suspensions of nongerminated conidia \([3 \times 10^4 \text{ mL}^{-1}]\) were prepared prior to, and used immediately in, bioassays. Nongerminated conidia (25 \(\mu\text{L}\)) were added to the test compound dissolved in 225 \(\mu\text{L}\) of 1\% (v/v) potato dextrose broth. Stock suspensions of germinating conidia (A. flavus, A. niger, A. parasiticus \([3 \times 10^5 \text{ mL}^{-1}];\) A. fumigatus, F. moniliforme, F. oxysporum \([3 \times 10^4 \text{ mL}^{-1}]\)) were prepared the morning of the experiment and incubated for 8 h at 30 °C. Afterwards, 25 \(\mu\text{L}\) of the germinating conidia were added to 225 \(\mu\text{L}\) of the test compound. Final test compound concentrations were 0–30 \(\mu\text{mol} \cdot \text{L}^{-1}\). Controls consisted of conidia (25 \(\mu\text{L}\)) and 1\% potato dextrose broth (225 \(\mu\text{L}\)). Controls and test mixtures were vortexed and incubated for 30 min (28 °C). Aliquots (50 \(\mu\text{L}\)) of the samples were aseptically spread on each of four potato dextrose agar plates that were then incubated for 24 h (28 °C). Developed colonies were enumerated. Each compound was tested against each test fungus on three separate occasions \((n = 12)\).

**Statistical analysis**

Data were analyzed statistically using SigmaPlot 3.0 ANOVA one-way analysis on ranks followed by multiple comparisons with Holm-Sidak’s or Tukey’s method \((p \leq 0.05)\).

**Results**

**Structural determination of compounds 1 and 2**

The structure of compound 1 (molecular mass 1081 Da) is shown in Fig. 1. This four-sugar saponin and the tetrasaccharide fragment of this molecule was identified by NMR spectroscopy to be 3-O-\(\beta\)-d-glucopyranosyl(1→2)-\(\beta\)-d-glucopyranosyl(1→3)-\(\beta\)-d-glucopyranosyl(1→4)-\(\beta\)-d-galactopyranoside-25\(\alpha\),5\(\alpha\)-spirostane-2\(\alpha\),3\(\beta\)-diol, a gitogenin-type saponin. Compound 2 (Fig. 1) isolated has a molecular mass of 919 Da and the trisaccharide fragment of this molecule was identified by NMR spectroscopy to be 3-O-\(\beta\)-d-glucopyranosyl(1→2)-\(\beta\)-d-glucopyranosyl(1→4)-\(\beta\)-d-galactopyranoside-25\(\alpha\),5\(\alpha\)-spirostane-2\(\alpha\),3\(\beta\)-diol, which is also a gitogenin-type steroidal saponin.

**Breakdown products of CAY-1**

In Fig. 2, the MS spectrum of CAY-1 shows an intense molecular ion \([\text{M+H}]^+\) at \(m/z\) 1244. Fragmentation of protonated CAY-1 ion leads to the loss of 162 Da, which is indicative of one hexose sugar, while the loss of a second hexose sugar leads to the \(m/z\) 920 ion displayed in Fig. 2. The breakdown of CAY-1 into compounds 1 and 2 is shown in Fig. 3.

**Fungicidal properties of compounds 1 and 2**

The fungicidal properties of compounds 1 and 2 for the
The tested fungi are listed in Table 1. Compound 1 (molecular mass 1081 Da) showed significant ($p \leq 0.05$) lethality only to the germinating conidia of *A. flavus* ($\geq 10 \, \mu\text{mol} \cdot \text{L}^{-1}$), *A. fumigatus* ($\geq 10 \, \mu\text{mol} \cdot \text{L}^{-1}$), *A. parasiticus* ($\geq 15 \, \mu\text{mol} \cdot \text{L}^{-1}$), and *A. niger* ($\geq 5 \, \mu\text{mol} \cdot \text{L}^{-1}$). Compound 1 was inactive against the nongerminated conidia of the tested *Aspergillus* species and both the nongerminated and germinating conidia of the tested *Fusarium* species. Compound 2 (molecular mass 919 Da) was not lethal against the nongerminated and germinating conidia of all the tested fungi.

**Discussion**

The NMR data indicate that the saponins (compounds 1 and 2, respectively) purified in this study have molecular masses of 1081 and 919 Da, respectively, (Fig. 1). They are similar in structure to CAY-1 except that compound 1 lacks the number four glucose moiety while compound 2 lacks the numbers three and four glucose moieties of the oligosaccharide chain of CAY-1. These saponins (compounds 1 and 2) with four and three glycosidic moieties (not the five-glycosidic saponin CAY-1) were found earlier in *Capsicum annum* var. *conoides* and *C. annum* var. *fasciculatum* (Izumitani et al. 1990). CAY-1 was later found in paprika and cayenne pepper (De Lucca et al. 2001; Yajima et al. 2000). CAY-1 and compounds 1 and 2 elute very closely together and can only be separated by HPLC–MS. These findings suggest that not all members of the *Capsicum* family produce CAY-1 and that a difference in the saponin-producing metabolic pathways exists among the different species of this plant genus.

Steroidal glycosides from *C. annum* have been studied extensively in the past (Izumitani et al. 1990; Yahara et al. 1991, 1994; Tschesche and Gutwinski 1975). The gitogenin glycosides 3S and 4S have been already isolated from *C. annum* and characterized (Yahara et al. 1994). However, we believe that this is the first report of these particular gitogenin-based steroidal saponins with the reported glycone structures being
present in *C. frutescens*. Data from the MS/MS analyses of CAY-1 breakdown products clearly show that compounds 1 ($m/z = 1082$) and 2 ($m/z = 920$) are progeny ions, lacking one and two glucose molecules, respectively, of the protonated CAY-1. Though the results show that compounds 1 and 2 are breakdown products of CAY-1, it is also possible to speculate that these molecules could also be precursors of CAY-1 should the appropriate enzymes be present.

Saponins are fungicidal by forming complexes with membrane sterols, which is similar to the mode of action of polyene antibiotics (Fenwick et al. 1991; Price et al. 1987). It is the combination of the aglycone and oligosaccharide moieties that contributes to membraneolytic properties of the intact saponin molecule (Fenwick et al. 1991; Osbourn 1996a). The complexing of the complete saponin with membrane sterols results in pore formation and loss of membrane integrity culminating in cell death (Osbourn 1996b). Therefore, the loss of any of the sugar molecules from the complete saponin should result in the loss of antifungal properties. A number of phytopathogenic fungi are known to detoxify saponins by enzymatically decoupling one or more sugars from the steroid or terpenoid moiety. As a group, these fungi...
produce specific glycosyl hydrolases that remove sugar moieties from the glycosyl chains at the C-3 carbon position, with the resulting molecule less toxic to fungal growth (Osbourn 1996a, 1996b; Schönbeck and Schlösser 1976; Crombie et al. 1986).

The relationship between the loss of saponin fungicidal activity and the loss of glycosidic groups was also observed in this study. The intact CAY-1 molecule contains five sugar moieties and is a potent fungicide in vitro (De Lucca et al. 2001; Renault et al. 2003). CAY-1 produced the following LD90 values for the germinating conidia of *A. flavus* (7.5 µmol·L⁻¹), *A. fumigatus* (4.0 µmol·L⁻¹), *A. parasiticus* (20.0 µmol·L⁻¹), and *A. niger* (10.0 µmol·L⁻¹). CAY-1 was also highly active against several human fungal pathogens, such as *Pneumocystis carinii* (IC50: 9.5 µmol·L⁻¹), *Candida albicans* (IC90: 6.2 µmol·L⁻¹), and *Cryptococcus neoformans* (IC90: 1.0 µg·mL⁻¹).

As noted earlier, compound 1 (molecular mass 1081 Da) with four glucose moieties has the same steroidal group as CAY-1 and the same saccharide group with one fewer glucose than the CAY-1 oligosaccharide. The former compound has greatly reduced fungicidal properties compared with those of CAY-1, published previously (De Lucca et al. 2001; Renault et al. 2003). Compound 2 (molecular mass 919 Da), which also shares the same steroidal group as that of CAY-1 and has the same glycone structure with two fewer glucose moieties than CAY-1, is not significantly active against any of the test fungi. Our findings that as the number of glucose moieties is reduced, compounds 1 and 2 loose fungicidal ability compared with that of CAY-1, appear to be based on the number of sugar moieties present in their structures.

Saponins interact with steroids present in the fungal membrane and cause a loss of cytoplasmic constituents, leading to cell death. That compound 1 is active against the germinating conidia of *A. fumigatus* and *A. niger* but not the others studied suggests a difference in the cell membranes of these species. The loss of one or more glucose moieties also affects solubility. Compounds 1 and 2 require sonication to be placed into solution into the same aqueous menstruum as CAY-1 at the same concentrations in which CAY-1 readily dissolves.

In conclusion, this is the first report of the presence of these smaller saponins (compounds 1 and 2) in cayenne pepper (*Capsicum frutescens*). These saponins differ from CAY-1, a fungicidal saponin, only in the number of sugar moieties. Their fungicidal and solubility properties differ significantly from that of CAY-1 and appear to be based on the number of sugar moieties present in their structures.

### References


