Pergamon

PII: S0031-9422(97)00388-9

Phytochemistry, Vol. 46, No. 6, pp. 1005–1009, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

METABOLISM OF THE POTATO SAPONINS α-CHACONINE AND α-SOLANINE BY *GIBBERELLA PILICARIS*

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(*Received in revised form* 1 *April* 1997)

Key Word Index—*Gibberella pulicaris*; *Fusarium*; fungi; metabolism; saponins; steroid alkaloids; potato.

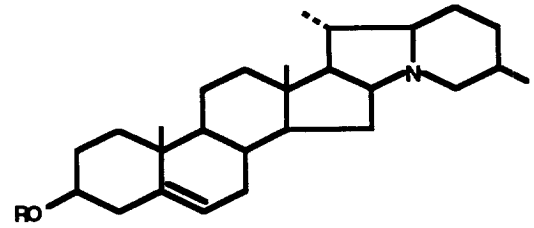
Abstract—Potato tubers accumulate varying amounts of several saponins preferentially in the peel. These compounds are toxic to living cells containing sterols in their plasma membrane and are therefore thought to be preformed chemical defence compounds. Two strains of the potato pathogen *Gibberella pulicaris* (*Fusarium sambucinum*), R-6380 and R-7843, were analysed for their ability to metabolize the most predominant saponins found in tubers, α-chaconine and α-solanine. The first compound is degraded by both strains via removal of α-1,2-L-rhamnose leading to β_2-chaconine. This product is converted to the aglycone, solanidine, which is further metabolized to unknown products. The release of α-1,2-L-rhamnose is also the first step in the break down of α-solanine by strain R-6380, followed by the removal of the β-1,3-bound glucose molecule leading to γ-solanine, which is not metabolized any further. Strain R-7843 is not able to metabolize α-solanine. Crude protein extracts of the culture fluid of both strains contained enzymes able to convert α-chaconine to β_2-chaconine, but with no α-solanine metabolic activity. This result indicates that *G. pulicaris* excretes enzymes specific for different saponins. © 1997 Elsevier Science Ltd

INTRODUCTION

Saponins are found in over 100 plant species [1]. They consist of an aglycone with a steroidal or terpenoid structure and one or more sugar residues. Saponins can form complexes with the 3β-OH group of plasma membrane sterols leading to membrane disruption and electrolyte leakage [2, 3], and thus are potentially toxic to all organisms carrying these membrane constituents [4, 5].

Potatoes (*Solanum tuberosum*), like other solanaceous plants, produce glycoalkaloids. The main saponins of potato are α-chaconine (**1**) and α-solanine (**2**) [6]. They are found in all tissues and their concentration can vary significantly under different environmental conditions and among cultivars. In tubers both glycoalkaloids are located largely in the peel below the primary periderm and upon mechanical injury their concentration can increase markedly [7, 8]. Because of their fungitoxic potential and their presence at possible sites of entry of micro-organisms, the saponins have long been regarded as plant defence compounds or phytoanticipins [7, 9].

G. pulicaris, one of the causal agents of potato dry rot, infects potato tubers through wounds and is, therefore, expected to be exposed to **1** and **2**. There are two known mechanisms enabling a phytopathogenic fungus to be resistant to these compounds. One is to have few or no sterols in the membrane like the *Oomycetes* [2, 3]. The other mechanism involves the production of enzymes, which remove one or more sugar residues from the saponins leading to a major reduction of toxicity of these compounds [5]. Examples for such detoxifications are the metabolism of



α-L-rha(1->4)
α-L-rha(1->2)
β-D-glu(1->) = R α-chaconine (1)

β-D-glu(1->3)
α-L-rha(1->2)
β-D-gal(1->) = R α-solanine (2)

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the oat saponins avenacin A-1 by *Gaeumannomyces graminis* var. *avenae* [10], and the avenacosides A and B by *Drechslera avenae* [11], and *Septoria avenae* [12]. Another well studied example is the tomato saponin α-tomatine, which is either degraded by complete or sequential release of all sugars to the aglycone tomatidine by *Fusarium oxysporum* f. sp. *lycoperisci* [13, 14], *Botrytis cinerea* [15], and *Alternaria* species [16], or by release of single sugars like β-D-glucose by *Septoria lycopersici* [17], or L-xylose by *B. cinerea* [18]. These examples demonstrate that fungi obviously have evolved different strategies to break down a given saponin.

The tomatinases of *S. lycopersici* [19] and *F. oxysporum* f. sp. *lycoperisci* [14], as well as the avenacinase of *G. graminis* [20, 21] and the avenacosidase of *S. avenae* [12] have been isolated. The enzymes of *S. lycopersici* and *G. graminis* show clear preferences for their natural substrates with 2% or less relative activity towards other substrates [21]. The specificity of these enzymes indicates that the metabolism of saponins is catalysed by enzymes which might be specifically designed for the conversion of these plant defence compounds.

Avenacinase-minus mutants of *G. graminis* var. *avenae* generated by targeted gene disruption were no longer able to infect oat but retained full pathogenicity on wheat, which does not produce avenacin. This experiment and the substrate specificity of the saponinases indicate that the ability of a phytopathogenic fungus to detoxify a saponin is likely to be essential for colonization of the host plant and therefore in determining the host range of the fungus [22].

As part of our investigation of traits required for pathogenicity by *G. pulicaris* on potato tubers we analysed the metabolism of **2** and **1** by two strains of this fungus. The virulent strain R-6380 was isolated from potato and the avirulent strain R-7843 from carnation (*Dianthus*) [21]. The strains were compared for their ability to metabolize the potato saponins.

RESULTS AND DISCUSSION

Kinetics of saponin metabolism

The time course of metabolism of **1** and **2** by *G. pulicaris* strains R-6380 and R-7843 was studied by TLC of extracts of 1 ml aliquots taken after 0, 1, 2, 4, 8, 12, 24 and 48 hr of incubation in saponin supplemented McIlvain buffer. Of the two strains only R-6380 metabolized **2** (R_f-value of 0.15) very slowly within 24 hr to a product with an R_f-value of 0.35. No further change was observed with incubations of even up to 72 hr. In experiments where the concentration of **2** was raised to 100 μg ml^{-1} the transient accumulation of a product with an R_f-value of 0.2 was detectable after 4 to 8 hr. This substance disappeared later and was replaced by the R_f 0.35 end product. No product was detectable after 48 hr incubation of **2** with crude protein extracts of culture filtrates of both strains.

Compound **1** (R_f-value of 0.26) was metabolized by both strains within 2 hr to a product with a R_f-value of 0.36. This compound accumulated only transiently in the case of strain R-6380, where it was no longer detectable after 12 hr. This strain degraded the first product to a compound with the same R_f-value of 0.61 and the same yellow colour under UV-light as the aglycone solanidine. It was detectable between 4 and 12 hr and was metabolized further to at least two products with R_f-values of 0.38 and 0.43, and which stained blue with 50% H_2SO_4 without heating the TLC plate. Strain R-7843 was not able to completely convert the first product to solanidine. Only trace amounts of the aglycone were detectable after 24 hr. Incubation of **1** with crude protein extracts of culture filtrate of both strains lead to the complete conversion of the substrate to the first product with the R_f-value of 0.36 within 24 hr. No aglycone was detectable with any of the extracts.

Structure of metabolites of compound **1**

The TLC analysis of the metabolism of **1** indicated that the substrate was degraded to the aglycone with the transient accumulation of only one product. This compound had an R_f-value only slightly greater than that of the substrate, suggesting the loss of only one sugar residue. Since two rhamnoses were bound to the glucose attached to the aglycone solanidine, it was impossible to use MALDI-TOF-MS for structure elucidation of the product. We therefore applied a chemical analysis of the metabolite employing carbohydrate constituent analysis, LSIMS of permethylated compounds and GC as well as electron impact and chemical ionization GC/MS analyses.

Constituent analysis (data not shown) and the pseudomolecular ions observed at *m/z* 965 and 791 in the LSIMS spectra of permethylated compounds revealed the loss of only one rhamnose residue. Methylation analysis further proved that *G. pulicaris* specifically removed the α-1,2-L-rhamnose from **1**, resulting in a conversion of the 2,4-disubstituted glucose into a 4-monosubstituted glucosyl residue. There was no indication that the fungus was able to split off the α-1,4-L-rhamnose. This suggests that the aglycone was produced by removing the α-1,4-rhamnose and the glucose as a disaccharide. The structures of the products produced from the aglycone remain to be elucidated.

The specific removal of the α-1,2-L-rhamnose as the first step in the break down of **1** was remarkable, because our data clearly provide no evidence for any release of the adjacent α-1,4-bound rhamnose leading to β_1-chaconine. Since the produced β_2-chaconine was not purified before the chemical analysis a possible loss of β_1-chaconine during preparation can be excluded. This result, and the lack of γ-chaconine, indicate that the β_2-chaconinase had no side activity

for the α-1,4 bond and that the fungus might have no enzyme for the hydrolysis of this bond. The detected aglycone therefore appeared to be produced by removal of the remaining sugars of β_2-chaconine as a disaccharide, which was likely to be catalyzed by another glycosidase. On the other hand, without purification of the β_2-chaconinase it cannot be excluded that the release of the disaccharide was not catalysed by a side activity of the proposed β_2-chaconinase, because dual activities for saponin-hydrolysing enzymes have been reported before [12, 24].

Structure of metabolites of compound **2**

The R_f-value of metabolites of **2** indicated that *G. pulicaris* successively removed the α-1,2-L-rhamnose and the β-1,3-D-glucose leaving the galactose still bound to the aglycone. In order to examine which of the sugar molecules was split off first, the molecular masses of the products were determined. In the case of **2** this analysis should clearly distinguish between both possibilities, because of the distinct mass difference between a rhamnose and a glucose molecule. Since the first metabolite of **2** accumulated only for a short time and in small amounts, we used MALDI-TOF-MS for this analysis. This method allowed the measurement of the molecular mass of each component in a mixture of several compounds. The sample for the MALDI-TOF-MS analysis contained only traces of the substrate and both products at a ratio of about 1:10 for the first and the second product. Analysis of the starting material led to a pseudomolecular ion at *m/z* 868.1, whereas signals at *m/z* 722.4 and 560.5 were obtained in the case of the metabolites of **2** clearly demonstrating that *G. pulicaris* first removed the α-1,2-L-rhamnose and in a second step the β-1,3-D-glucose moiety. No signal was observed at *m/z* 706 corresponding to a solanidine-galacto-rhamnoside, which would be detectable if the glucose were removed first.

The metabolism of **2** did not lead to the aglycone solanidine suggesting that the fungus lacks the necessary galactosidase. The sequential removal of the other two sugar residues was most likely catalysed by two enzymes, because the fact that we could not detect any solanine lacking the β-1,3-glucose in the MALDI-TOF-MS analysis indicated that the glucosidase catalysing the removal of the β-1,3-glucose molecule was not able to accept **2** as a substrate. The obvious conclusion is that two enzymes, an α-1,2-rhamnosidase and a β-1,3-glucosidase were sequentially involved in the metabolism of **2**. Again, this proposal has to be verified by incubation of **2** with the purified enzyme(s), because the avenacosidase of *S. avenae* demonstrates both an α-L-rhamnosidase and a β-D-glucosidase activity [12].

A very interesting question is whether the first step in the degradation of both saponins is catalysed by the same rhamnosidase? The strongest argument against this speculation was the lack of metabolism of **2** by strain R-7843, because this strain has the rhamnosidase degrading **1** to β_2-chaconine. This means that the β_2-chaconinase converting **1** is specific for this substrate, which would be remarkable, because both saponins share the same aglycone and differ only in the third sugar residue. We are in the process of isolating the β_2-chaconinase specific for **1**. The purified enzyme will enable us to answer the question of whether *G. pulicaris* has two α-rhamnosidases specific enough to distinguish between such structurally similar saponins like **1** and **2**. A positive answer to this question would support the hypothesis that phytopathogenic fungi have evolved specific enzymes for the detoxification of preformed defence compounds of their host plants.

EXPERIMENTAL

Strains and media. G. pulicaris strains R-6380 isolated from potato and R-7843 isolated from carnation were obtained from Dr A. Desjardins [23]. For long term storage and to produce spores the fungus was grown on V-8 juice agar [25]. Liquid cultures were grown by inoculating spores from a 7 to 10 day old agar plate culture into 100 ml Mantle$^-$ medium containing 10 g of glucose instead of 100 g sucrose [26] and incubating for 3 days at 28° on a rotary shaker.

Compounds **1**, **2** and solanidine were purchased from Sigma. β_2-chaconine was a gift from Dr Erik A. J. Keukens. For metabolism assays the substrates were dissolved in 10% HOAc in concn of 100 μg ml^{-1} and added to 20 mM McIlvain buffer (NaH_2PO_4, Na-citrate, pH 5.0) to a final concn of 50 μg ml^{-1}.

Metabolism assay. Mycelium from liquid culture was sepd from the culture fluid by filtration through a nylon membrane and washed × 2 with 20 mM McIlvain buffer. The time course of metabolism of saponins was measured by incubating 1 g mycelium in 10 ml McIlvain buffer containing 50 μg ml^{-1} substrate at 26° on a rotary shaker and taking 1 ml samples at various time points. After making the samples alkaline with NH_4OH, the remaining substrate and the products were extracted with EtOAc and applied to a TLC plate, which was developed in the solvent system EtOAc–HOAc–MeOH–H_2O (30:20:10:1). The saponins were visualized under UV-light (365 nm) after spraying the TLC plate with 50% H_2SO_4 and incubation at 110° for 5 to 10 min.

Crude protein extract. A 2-day old culture of 500 ml of Mantle$^-$ medium inoculated with spores of strain R-6380 or R-7843 was filtered to remove the mycelium, frozen in liquid N_2 and lyophilized. The residue was resuspended in 30 ml 20 mM McIlvain buffer and centrifuged. The supernatant was dialysed against H_2O over night, lyophilized again, and resuspended in 1.5 ml McIlvain buffer. After incubation of this crude extract with the substrates in McIlvain buffer the products were extracted with EtOAc and analysed by TLC.

Large scale isolation of products. Degradation prod-

ucts of **2** were obtained by two incubations of 10 g (fr. wt) mycelium in 100 ml MacIlvain buffer with 5 mg substrate for 15 hr at 26° on a rotary shaker. After filtration to remove the mycelium, the culture filtrate was alkalized with NH_4OH and extracted three times with EtOAc. The combined organic phases were evapd to dryness. The oily residue was redissolved in MeOH at 80° and the products were ppt. by adding a few drops of 2% NH_4OH and incubation at 4° over night. The white ppt. was washed with 2% NH_4OH, vacuum dried and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Degradation products of **1** were produced by incubating 2.5 mg of the substrate in 5 ml McIlvain buffer with 1.5 ml crude protein extract at 30° over night. Since the reaction was not completed within this period, the sample was incubated again with fresh protein extracted from 500 ml culture medium. After making the product alkaline it was extracted with EtOAc, dried under a stream of N_2 and subjected to carbohydrate constituent and methylation analysis as well as liquid secondary-ion mass spectrometry (LSIMS).

Carbohydrate constituent analysis. Carbohydrate constituents were released by acid hydrolysis, converted into corresponding alditol acetates and analysed by capillary GC as detailed elsewhere [27].

Methylation analysis. To determine the linkage positions of the monosaccharide constituents present, **1**, β_2-chaconine and metabolites of **1** (50–500 nmol) were permethylated [28] and hydrolysed. Partially methylated alditol acetates obtained after $NaBH_4$ reduction and peracetylation were analysed by GC/MS using the instrumentation and microtechniques described earlier [29, 30].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For molecular mass determination of **2** and metabolites, a Vision 2000 instrument (Finnigan MAT, Bremen, Germany) was used in the positive-ion reflector mode. Pseudomolecular ions were formed by irradiation with a pulsed N_2 laser (emission wavelength 337 nm, laser power density approximately 10^6 W cm^{-2}) and accelerated at a potential of 5 kV. The matrix soln contained 9 mg ml^{-1} 2,5-dihydroxybenzoic acid and 1 mg ml^{-1} 2-hydroxy-5-methoxybenzoic acid in 0.1% aq. CF_3COOH–MeCN (2:1) [31]. To 1 μl matrix soln, 1 μl of the sample (*ca* 10 pmol in 0.1% aq. CF_3COOH–MeCN) was added on the stainless steel target. Analyte and matrix were dried in a cold air stream before introduction into the mass spectrometer. For calibration of the mass spectra, human angiotensin and bovine insulin were used as external standards. About 2–10 single spectra were accumulated.

Liquid secondary-ion mass spectrometry (LSIMS). LSIMS was carried out with a MAT 900 mass spectrometer (Finnigan MAT) equipped with a caesium gun, which was operated at an emission current of 2–3 μA. Mass spectra were recorded at an acceleration potential of 5 kV with a resolution of *ca* 2000 and were acquired using a DEC 2100 data system. Spectra of the permethylated samples were recorded in the positive-ion mode using 3-nitrobenzyl alcohol (Aldrich, Germany) as matrix.

Acknowledgements—We thank Werner Mink for carbohydrate constituent analysis and GC of partially methylated alditol acetates, Peter Kaese for GC/MS analyses, Siegfried Kühnhardt for MALDI-TOF-MS and LSIMS measurements and Vivian Miao for reviewing the manuscript. This study was financially supported by Deutsche Forschungsgemeinschaft (R.G.: Sonderforschungsbereich 535).

REFERENCES

1. Price, K. R. and Fenwick, G. R., *CRC Critical Reviews in Food Science and Nutrition*, Vol. 26, ed. T. E. Furia. CRC Press, Inc., Boca Raton, FL, 1987, p. 27.
2. Keukens, E. A. J., de Vrije, T., Fabrie, C. H. J. P., Demel, R. A., Jongen, W. M. F. and de Kruijff, B. E., *Biochimica et Biophysica Acta*, 1992, **1110**, 127.
3. Keukens, E. A. J., de Vrije, T., van den Boom, C., de Waard, P., Plasman, H. H., Thiel, F., Chupin, V., Jongen, W. M. F. and de Kruijff, B., *Biochimica et Biophysica Acta*, 1995, **1240**, 216.
4. Arneson, P. A. and Durbin, R. D., *Phytopathology*, 1968a, **58**, 536.
5. Steel, C. S. and Drysdale, R. B., *Phytochemistry*, 1988, **27**, 1025.
6. Kuc, J., in *Phytoalexins*, ed. J. A. Bailey and J. W. Mansfield. Blackie and Son Ltd., Glasgow, 1982, p. 81.
7. Allen, E. and Kuc, J., *Phytopathology*, 1968, **58**, 776.
8. McKee, R., *Annals of Applied Biology*, 1955, **43**, 147.
9. VanEtten, H. D., Sandrock, R. W., Wasmann, C. C., Soby, S. D., McCluskey, K. and Wang, P., *Canadian Journal of Botany*, 1995, **73** (Suppl.), 518.
10. Crombie, W. M. L., Crombie, L., Green, J. B. and Lucas, J. A., *Phytochemistry*, 1988, **25**, 2075.
11. Lüning, H. U. and Schlösser, E., *Journal of Plant Disease and Protection*, 1976, **83**, 317.
12. Wubben, J. P., Price, K. R., Daniels, M. J. and Osbourn, A. E., *Phytopathology*, 1996, **86**, 986.
13. Ford, J. E., McCance, D. J. and Drysdale, R. B., *Phytochemistry*, 1977, **16**, 545.
14. Lairini, K., Perez-Espinosa, A., Pineda, M. and Ruiz-Rubio, M., *Applied Environmental Microbiology*, 1996, **62**, 1604.
15. Verhoeff, K. and Liem, J. I., *Phytopathologische Zeitschrift*, 1975, **83**, 333.
16. Schlösser, E., *Acta Phytopathologica*, 1975, **10**, 77.

17. Arneson, P. A. and Durbin, R. D., *Phytopathology*, 1967, **57**, 1358.
18. Quidde, T. and Tudzynski, P., *Fungal Genetics Newsletter*, 1996, **43B**, 40.
19. Sandrock, R. W., DellaPenna, D. and VanEtten, H. D., *Molecular Plant–Microbe Interactions*, 1995, **8**, 960.
20. Osbourn, A. E., Clarke, B. R., Dow, J. M. and Daniels, M. J., *Physiological and Molecular Plant Pathology*, 1991, **38**, 301.
21. Osbourn, A. E., Bowyer, P., Lunness, P., Clarke, B. and Daniels, M., *Molecular Plant–Microbe Interactions*, 1995, **8**, 971.
22. Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M. J. and Osbourn, A. E., *Science*, 1995, **267**, 371.
23. Beremand, M. N., Desjardins, A. E., Hohn, T. M. and VanMiddlesworth, F. L., *Phytopathology*, 1991, **81**, 1452.
24. Segal, R. and Schlösser, E., *Archives of Microbiology*, 1975, **104**, 147.
25. Stevens, R. B., *Mycology Guidebook*. University of Washington Press, Seattle, 1974, p. 703.
26. Mantle, P. G. and Nisbet, L. J., *Journal of General Microbiology*, 1976, **93**, 321.
27. Geyer, R., Geyer, H., Kühnhardt, S., Mink, W. and Stirm, S., *Analytical Biochemistry*, 1982, **121**, 263.
28. Paz-Parente, J., Cardon, P., Leroy, Y., Montreuil, J., Fournet, B. and Ricart, G., *Carbohydrate Research*, 1985, **141**, 41.
29. Geyer, R., Geyer, H., Kühnhardt, S., Mink, W. and Stirm, S., *Analytical Biochemistry*, 1983, **133**, 197.
30. Geyer, R. and Geyer, H., *Methods in Enzymology*, 1994, **230**, 86.
31. Karas, M., Ehring, H., Nordhoff, E., Stahl, B., Strupat, K., Hillenkamp, F., Grehl, M. and Krebs, B., *Organic Mass Spectrometry*, 1993, **28**, 1476.