

Chapter 2

Ginsenosides: Phytoanticipins or Host Recognition Factors?

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2.1 Introduction

Plants produce a wide range of natural products, also referred to as secondary metabolites, including alkaloids, isoprenoids, and phenolics, many of which possess potent biological activity. While it is often difficult to distinguish between “primary” and “secondary” metabolism, a functional definition describes secondary metabolites as those molecules that are involved in the interaction between plants and their environment [1]. From a human perspective, the biological activity of plant natural products has been exploited by their use as medicinal compounds (either directly or as starting point for the development of synthetic analogs); several examples are provided within this volume. However, plants do not make natural products for the benefit of humans, but instead as protective measures against the myriad challenges (both biotic and abiotic) that they face in their environment. The evolutionary development of natural products has been driven by these challenges [2]. In this chapter, we provide a summary of our work investigating the potential role of ginsenosides (a subclass of triterpene saponins produced by *Panax* spp.) in the ecology of *Panax quinquefolius* (American ginseng). In doing so, we draw upon the vast literature on saponins in general and provide our perspective on the ecological relevance of ginsenosides in the ginseng-*P. irregulare* pathosystem.

2.2 Saponins

2.2.1 Chemical Structure and Diversity

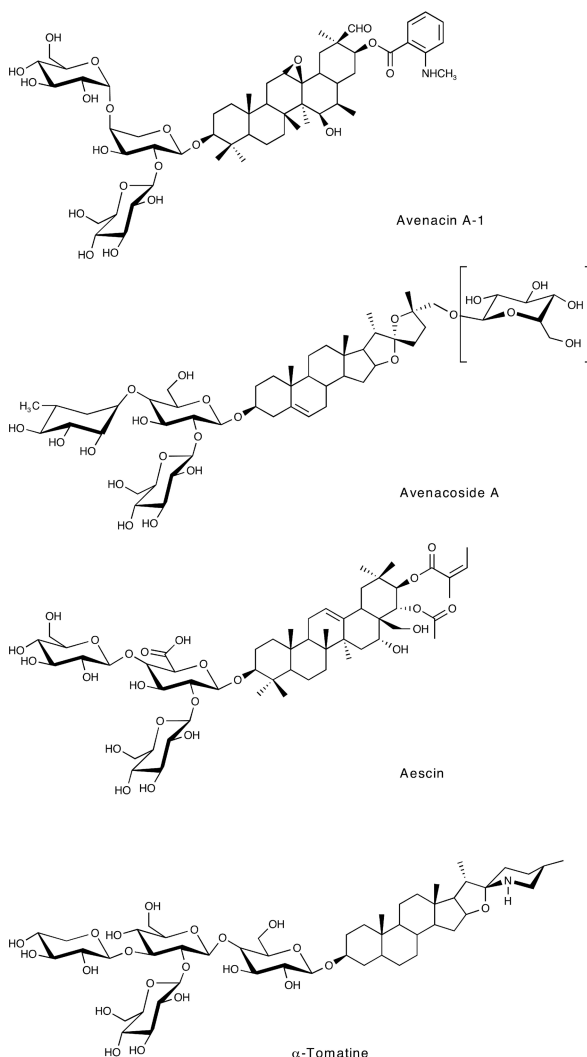
Saponins are glycosylated natural products with soap-like properties [3]. The aglycones (sapogenins) are either triterpenoid- or steroid-derived. Dicotyledonous

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Fig. 2.1 Structures of some common saponins. The structures of some of the saponins described in the text are shown, including aescin from horse chesnut, avenacin A-1 and avenacoside A from oat, and α -tomatine from tomato. The glucose molecule enclosed in square brackets in the structure of avenacoside A highlights the glucose moiety that is cleaved off by hydrolysis by glycosidases in disrupted oat leaf tissue, leading to the fungitoxic 26-desglucosyl avenacoside A. Redrawn from [94]



plants, for example, have pentacyclic (e.g., α - and β -amyrin) or tetracyclic (e.g., the dammaranes) triterpenoid sapogenins, although in the Solanaceae, steroidal glycoalkaloid saponins (nitrogen analogs of steroidal saponins) are also present. In monocotyledonous plants, steroidal saponins are common. Examples of several common saponins are shown in Fig. 2.1.

The structural diversity of saponins is further enhanced by the number and placement of sugars around the aglycone. That is, saponins can be either mono- or bidesmosidic, with the latter having sugar moieties attached at either end of the parent carbon skeleton. Monodesmosidic saponins are generally glycosylated at the C-3 hydroxyl group; bidesmosidic saponins are generally glycosylated at the C-3

hydroxyl group as well as elsewhere in the molecule. Glucose, arabinose, galactose, rhamnose, and xylose, as well as sugar acids, may be present, either as monosaccharides or as higher order oligomers. Subtle differences in the sugar substitution pattern of a common carbon skeleton result in large numbers of compounds with closely related structures and properties.

2.2.2 Involvement of Saponins in Host–Pathogen Interactions

Saponins are biologically active phytochemicals that act upon diverse organisms, including fungi [4, 5]. For example, alfalfa, clover, oat, soybean, potato, ginseng, tomato, and pepper all contain either triterpenoid, steroid, or glycoalkaloid saponins, and many of these saponins possess fungitoxic activity. However, assessment of fungitoxicity through the use of in vitro bioassays has limited use when the goal is to elucidate host-pathogen interactions. The Osbourn group in the United Kingdom has used a variety of approaches over the past 15 years to clearly demonstrate that saponins can act as constitutive plant defenses (i.e., phytoanticipins) [6]. This study complements earlier avenacin bioassay observations [7]. Taken as a whole, the avenacin model appears to have confirmed the long-held assumption that the ecological function of saponins is to defend the plant against fungal pathogens, and this concept has taken hold within the literature [8, 9]. With acknowledgment that the “saponins as defense” story has been told by many groups using different pathosystems, the most complete story is represented by avenacin A-1 from oat and α -tomatine from tomato, which is described in the sections that follow.

2.2.2.1 Avenacin A-1 and α -Tomatine as Models for Plant Defense

Oat produces structurally diverse triterpenoid saponins in different plant organs. Bidesmosidic saponins (avenacosides) are produced in oat leaves and monodesmosidic saponins (avenacins) are produced in oat roots [6]. Avenacin A-1 is considered the main oat root saponin and is represented by a pentacyclic oleanane skeleton with a trisaccharide (arabinose and two glucose moieties) at C-3 (Fig. 2.1). Research with saponin-deficient oat mutants clearly demonstrated the link between in planta saponin content and defense against fungal pathogens. Avenacin fluorescence was used to screen for saponin-deficient (sad) mutants of a wild oat, *Avena strigosa*. Saponin-deficient mutants were found to have an increased susceptibility to the fungus *Gaeumannomyces graminis* var *tritici* (which is usually only pathogenic to wheat) as well as pathogenic species of *Fusarium* [10].

Glycoalkaloid saponins are found mainly in the Solanaceae. In tomato, for example, the glycoalkaloid saponin, α -tomatine, is found in relatively high concentrations in unripe fruits and is fungitoxic [11–13]. However, the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* can degrade tomatine via the tomatinase enzyme Tom1. Targeted disruption and overexpression studies of the *Tom1* gene demonstrated that this enzyme is required for the pathogen to reach full virulence on this plant [14]. Heterologous expression of a tomatinase in yeast resulted in improved yeast saponin

resistance [15] and provides further evidence that this glycoalkaloid saponin likely functions in plant defense.

2.2.2.2 Mode of Action Through Membrane Disruption

Avenacin A-1, α -tomatine, and other saponins are toxic to fungi because saponins form complexes with sterols [16–18] and thereby disrupt the integrity of biological membranes. Formation of pores and leakage of cell contents have been observed in membranes treated with avenacin and tomatine alike [11, 12, 16, 18]. For example, incubating fungal mycelium with α -tomatine caused loss of electrolytes from different fungal isolates. Of particular note, three pathogens of tomato were more resistant to the effects of α -tomatine than four nonpathogens [11]. It is presumed that saponins exert their effect in fungi by binding to ergosterol, the major membrane sterol of higher fungi [19].

The structure of saponins, especially the position of the sugar moieties at one (monodesmosidic) or two (bidesmosidic) sites on the sapogenin, has a great influence on fungitoxicity. Monodesmosidic, amphiphilic saponins are more fungitoxic than bidesmosidic saponins, and removal of one, some, or all of the sugars of the monodesmoside generally results in a reduction in toxicity [9, 12, 20]. A recent report [12] demonstrated that some sapogenins exerted fungitoxic effects on yeast, but it was concluded that the mode of action was not through the disruption of membranes. In stark contrast, oomycete pathogens are reported to be unaffected by saponins in vitro. It is probably the lack of ergosterol in these species that allows them to avoid saponin toxicity and provides “innate resistance” [21].

2.3 Ginsenosides Are Bidesmosidic Saponins with Mild Fungitoxicity

2.3.1 Structure, Nomenclature, and Biosynthesis of Ginseng Saponins

Although numerous secondary metabolites can be found in ginseng, many of the pharmacological virtues of this plant are generally thought to be due to triterpenoid saponins called ginsenosides [22]. Both American (*P. quinquefolius*) and Asian species of ginseng (e.g., *Panax ginseng*, *P. notoginseng*, *P. vietnamensis*) contain numerous, and often overlapping, types of ginsenosides. While there are >25 known ginsenosides [23] in American ginseng, the most common are ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc, and Rd (named on the basis of their original chromatographic elution order). All ginsenosides are based on a tetracyclic dammarane skeleton [24] (Fig. 2.2), and since they have saccharides attached at both C-3 (or C-6) and C-20, they are bidesmosidic. They are further classified into two groups, according to the hydroxylation pattern of the parent aglycone and the attachment position of the various saccharide molecules. For example, ginsenosides Rb₁, Rb₂, Rc, and Rd

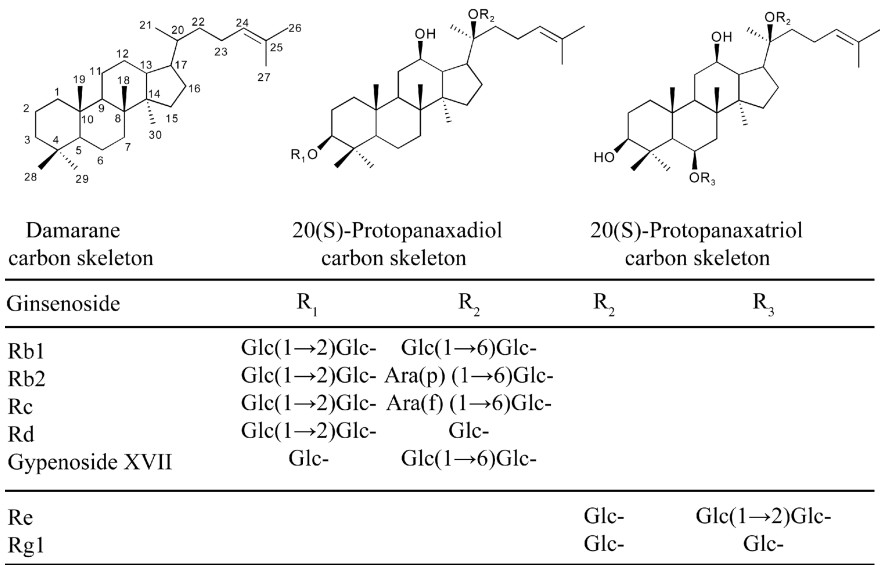


Fig. 2.2 Structures of common ginsenosides. The core damarane structure common to ginsenosides is shown along with the carbon skeletons of the 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol ginsenosides. The sugar decorations of the most common ginsenosides are indicated in the table below the structures. Legend: Ara(*f*), arabinose (furanose form); Ara(*p*), arabinose (pyranose form); Glc, glucose

are based on a 3,12,20-trihydroxylated-20(*S*)-protopanaxadiol aglycone, with sugars attached to the hydroxyl groups at positions 3 and 20. Ginsenosides Rg₁ and Re, on the other hand, are based on a 3,6,12,20-tetrahydroxylated-20(*S*)-protopanaxatriol aglycone, with sugars attached to the hydroxyl groups at positions 6 and 20.

Biosynthetically, ginsenosides and sterols share a common intermediate precursor, oxidosqualene. Whereas cycloartenol synthase catalyzes the first committed step in sterol production, the enzyme dammarenediol synthase catalyzes the alternative folding and cyclization of 2,3-oxidosqualene into dammarenediol, the precursor of the damarane skeleton of the ginsenosides [25, 26]. Unlike other classes of terpenoids, such as the chloroplast-derived monoterpenoids and diterpenoids, triterpenoids are a product of the cytosolic mevalonate pathway [27]. Newer results from the overexpression of squalene synthase [28], as well as interference on dammarenediol synthase [29] and cycloartenol synthase [30], have improved our understanding of the regulation of ginsenoside biosynthesis and demonstrated the feasibility of increasing ginsenoside content in planta using biotechnological approaches.

2.3.2 Distribution of Ginsenosides Within the Plant

American ginseng (*P. quinquefolius* L.) is a native North American member of the Araliaceae, a family whose more than 800 species are found mostly in the tropics

[31]. *P. quinquefolius* is a perennial understory herb that is associated with deciduous forests [32, 33] and ranges from Ontario and Quebec, south to northern Florida, and west to Minnesota [22]. The aboveground tissues senesce at the end of each growing season and estimates of the maximum age of this plant are from 23 to 30 years [33] to greater than 50 years [34]. However, occurrences of wild populations of American ginseng are becoming increasingly rare, and virtually all American ginseng products currently on the market are derived from cultivated sources. American ginseng has a long history of cultivation in North America. For example, Proctor and Bailey [35] estimate that this plant has been commercially grown in Canada since the late nineteenth century under artificial shade or in woodlands. The main commercial product from both cultivated and wild ginseng alike is the root system, which is usually harvested after 3–5 years and is sought for its medicinal properties.

Ginsenosides have been demonstrated to occur throughout the plant including within the main roots, secondary roots, stem, leaves, flowers, and fruit [36, 37]. Although much of the research and medicinal uses have traditionally focused on ginseng roots, the foliage also contains high amounts of ginsenosides, sometimes at concentrations higher than those found in the roots [36, 37]. In American ginseng, the six main ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc, and Rd represent between 3 and 5% of the dry weight of the roots. With analyses that include a more complete profile of ginsenosides, this value can reach over 5% [37]. The distribution of the ginsenosides in different plant organs is not uniform. For example, the highest concentrations of ginsenoside Rb₁ and Rd are found in the roots and leaves, respectively, whereas ginsenoside Re is found in relatively equal amounts throughout the plant [36–38].

2.3.3 Fungitoxicity of Ginsenosides

Even though American ginseng accumulates large amounts of structurally different saponins, this plant is attacked in commercial gardens by a diverse array of fungi. These include the foliar pathogens *Alternaria panax*, *Alternaria alternata*, and *Botrytis cinerea* and the root pathogens *Cylindrocarpon destructans*, *Rhizoctonia solani*, *Phytophthora cactorum*, *P. irregulare*, *P. ultimum*, and several species of *Fusarium*, including *Fusarium oxysporum* and *Fusarium solani* [39–43]. Although the pathogens in the family Pythiaceae (i.e., *Phytophthora cactorum*, *P. irregulare*, and *P. ultimum*) are superficially similar to and share the same nutritional mode as the other pathogens, these organisms are not true fungi. The Oomycota, a phylum that contains water molds and phytopathogens that cause downy mildews, is more related to golden algae, brown algae, and diatoms and belongs collectively with these organisms in the Stramenopila. Most of the other ginseng pathogens, as well as *Trichoderma* spp. that may act as agonists of the pathogens in the soil, are true fungi belonging to the ascomycetes.

The fungitoxic activity of ginsenosides on many of these phytopathogens and agonists have recently been completed [44, 45] and represent the most comprehensive research in this area to date. Ginsenosides were characterized as mildly

fungitoxic, mainly based on a comparison to aescin, a mixture of saponins from horse chestnut. A clear selectivity in fungitoxicity was observed when ginsenosides were tested against true fungi *in vitro*. The growth of *Trichoderma* spp., as well as the leaf pathogen *A. panax*, was inhibited to a greater degree than the growth of the root pathogens *Fusarium* spp. and *C. destructans* [44]. For example, the fungus that exhibited the greatest growth inhibition was *Trichoderma harzianum* (over 26% less growth than control), whereas the fungus that exhibited the least growth inhibition was *C. destructans* (over 7% more growth than control) at concentrations of 1 mg/mL crude ginsenoside fraction [44]. In addition, similar results were obtained when the tests were repeated under different temperature and nutrient regimes (R.W. Nicol, PhD Thesis, The University of Western Ontario, 2003). Unexpected growth stimulation was observed when the oomycotan pathogens were exposed to ginsenosides. The colony weights of both *P. cactorum* and *P. irregulare* grown with 5 mg/mL crude ginsenoside fraction were found to be significantly higher than controls [45]. Thus, both the fungal and oomycete root pathogens of American ginseng exhibited greater resistance to the chemical defenses of this plant. Moreover, the growth of *P. irregulare* was clearly stimulated by ginsenosides in a concentration-dependent manner [45].

2.3.4 Ginsenosides in the Rhizosphere

Although plant roots are known to influence the composition of soil microbes [46, 47], the exact mechanisms remain unknown. Several groups [45, 48, 49] have since suggested that secondary metabolites exuded from plant tissue can influence the composition of rhizosphere microbes. For example, our initial investigations into the exudation of ginsenosides from American ginseng revealed that these secondary compounds are present in ginseng garden rhizosphere soil as well as in root exudates collected from plants grown in a greenhouse. LC-MS and HPLC analyses of rhizosphere soil demonstrated that ginsenosides were present in this material at a concentration of 0.06% [45]. Moreover, when this low and ecologically relevant concentration of ginsenosides was used in bioassays, the growth of *P. irregulare* was found to be significantly greater than control [45].

2.4 Some Pathogens Can Degrade/Detoxify Saponins

2.4.1 Overview of “Saponinases” from Plant Pathogens

“Saponinases” are pathogen-produced glycosidases able to hydrolyze plant saponins, often leading to their detoxification/degradation. Numerous pathogenic fungi produce saponinases and some of these enzymes have been shown to determine host specificity [50, 51]. The most studied is the cereal-infecting fungus *G. graminis* var. *avenae*. While this species normally produces an extracellular

“avenacinase,” which detoxifies the oat triterpenoid saponin avenacin A-1 [50–52], mutants of the same species that do not produce the enzyme were unable to infect the saponin-containing host [50].

Septoria avenae and *Stagonospora avenae* are other examples of pathogens that are able to detoxify oat saponins. However, these two pathogens infect the leaves, and unlike *G. graminis* (which encounters avenacins when infecting the roots), they encounter a different family of antifungal compounds: the 26-desglucoavenacosides [20]. Both species have been shown to metabolize these saponins via extracellular glycosidases; while *Septoria avenae* secretes an avenacosidase with dual activity (α -L-rhamnosidase and β -D-glucosidase) [53], *Stagonospora avenae* secretes an α -rhamnosidase and two β -glucosidases [54]. Even though *Stagonospora avenae* α -rhamnosidase and β -glucosidases are expressed during infection [54], and *Septoria* isolates unable to deglycosylate the 26-DGAs are nonpathogenic to oats [53], neither of the *Stagonospora* β -glucosidases or *Septoria* avenacosidase has been proven to be either required for resistance to 26-desglucoavenacosides or pathogenicity to oats [53, 54].

The tomato leaf spot fungus *Septoria lycopersici* produces an extracellular tomatinase capable of converting α -tomatine into β 2-tomatine, which is much less toxic to fungi [55–58]. Targeted mutation of the tomatinase gene in *S. lycopersici* resulted in the loss of the pathogen’s ability to degrade α -tomatine and an increase in its sensitivity to the saponin [56, 58]. The ability of *S. lycopersici* to infect *Nicotiana benthamiana* (a solanaceous species that produces saponins) was shown to require the presence of tomatinase [59]. Further, the hydrolysis products were found to suppress the induced defense responses in plants by interfering with signal transduction processes associated with disease resistance [59]. The expression of tomatinase in *Nectria haematococca*, which resulted in its ability to detoxify α -tomatine and infect green tomato fruits [58, 60], also confirmed the involvement of this saponinase in pathogenicity.

Although the avenacinase of *G. graminis* var. *avenae* and the tomatinase of *S. lycopersici* are specific for their respective host saponins, both enzymes hydrolyze terminal β -1,2-linked D-glucose molecules and have similar mechanisms of action [55]. Sequence analysis of tomatinase has established that this enzyme belongs to the same family of β -glucosyl hydrolases as *G. graminis* avenacinase [55]. Other tomato-infecting fungi such as *Alternaria solani* [61], *Verticillium albo-atrum* [62], *F. oxysporum* f. p. *lycopersici* [63–65], *F. solani* [66], and *B. cinerea* [67] have also been reported to produce saponinases that deglycosylate the glycoalkaloid α -tomatine. Moreover, the causal agent of gray mold disease in many crops and vegetables, *B. cinerea*, is believed to possess at least three distinct saponin-specific glycosidases (a xylosidase and two different glucosidases), enabling it to detoxify avenacin, avenacosides, and digitonin [67, 68].

Although tomatinases from different fungal sources share the same substrate, they cleave different sugar moieties from α -tomatine. For example, tomatinases of *S. lycopersici* [55] and *V. albo-atrum* [62] remove the terminal β -1,2-linked glucose molecule, while that from *B. cinerea* removes the terminal β -1,3-linked xylose [67]. On the other hand, *F. oxysporum* sp. *lycopersici* [63, 64], *F. solani* [66], and

A. solani [61] remove the β -1-linked galactose and release the aglycone tomatidine along with the tetrasaccharide β -lycotetraose (*F. oxysporum* and *F. solani*), or the four individual monosaccharides (*A. solani*).

Gibberella pulicaris, a fungus that causes potato dry rot, is also able to detoxify saponins, particularly α -chaconine and α -solanine from potato, by removing the α -1, 2-L-rhamnose moieties of these molecules [69]. A chaconinase from this fungus has been purified, and although it was shown to be induced by its substrate, the enzyme's importance to virulence was not yet determined [70]. *G. pulicaris* was also shown to deglycosylate α -tomatine, by removing the β -lycotetraose as described for *F. oxysporum* f. sp. *lycopersici* [20, 71].

More recently, saponinases that are able to hydrolyze soybean saponins have been purified from the soybean pathogen *Neocosmospora vasinfecta* [72] and the saprotrophs *Aspergillus oryzae* and *Eupenicillium brefeldianum* [73]. While the saponinase of *N. vasinfecta* is considered to be required for detoxification of soybean saponins, the saponin hydrolases from *A. oryzae* and *E. brefeldianum* do not induce defense responses in plant cells [73]. Consequently, while saponin deglycosylation may be a component of the infection process of some organisms, it may not be sufficient in and of itself for pathogenicity. Even though the *N. vasinfecta*, *A. oryzae*, and *E. brefeldianum* saponin hydrolases belong to the same glycosidase family, they possess different specific activities, reflecting the organism's adaptation to different environments [73].

The majority of the fungal saponin-detoxifying glycosidases that have been characterized to date belong to family 3 of glycosyl hydrolases, along with cellobiose-degrading enzymes from yeasts and *Trichoderma* species [74]. Still other tomatinases, such as those secreted by *F. oxysporum* f. sp. *lycopersici* and *G. pulicaris*, belong to family 10 of glycosyl hydrolases, among many fungal xylanases [20, 60].

Unlike fungal saponinases, little is known about hydrolytic enzymes secreted by phytopathogenic oomycetes or their role in pathogenicity [75, 76]. Although innate resistance is believed to be the main mechanism by which oomycetes avoid the toxicity of saponins (see above), some oomycetes have been found to produce saponin hydrolases [77], as well as other glycosyl hydrolases [76].

2.4.2 The Metabolism of Ginsenosides by Pathogens of Ginseng

As noted above, ginsenosides are mildly fungitoxic against leaf pathogens and non-pathogenic fungi, but appear to promote the growth of more virulent root pathogens such as *P. irregulare* and *C. destructans*. Since the fungitoxic properties of some saponins are reduced through enzymatic degradation by pathogen-produced glycosidases, we explored the possibility that pathogens of ginseng produce glycosidases that degrade ginsenosides (R.W. Nicol, PhD Thesis, The University of Western Ontario, 2003) [77]. Accordingly, we cultured pathogenic (*P. irregulare*, *C. destructans*, *P. cactorum*) and nonpathogenic (*Trichoderma hamatum*) organisms in the

presence of ginsenosides *in vitro*, and after several days of growth, profiled the ginsenosides that could be recovered from the spent medium. While minor alterations in the profile of ginsenosides recovered from the nonpathogenic fungus *T. hamatum* (e.g., decrease in Rb₁ and gypenoside XVII and an increase in Rd and F2) were observed, the overall profile was nearly identical to that of the profile of ginsenosides added to the medium at the start of the incubation (Fig. 2.3a,b). By contrast, the profile of ginsenosides recovered from cultures of *P. irregulare* (Fig. 2.3c–e), *C. destructans*, and *P. cactorum* (R.W. Nicol, PhD Thesis, The University of Western Ontario, 2003) were both quantitatively and qualitatively different from the initial ginsenoside mixture added to the culture medium. These alterations in ginsenoside profiles suggested either the degradation of ginsenosides or the presence of ginsenoside-hydrolyzing enzymes in the culture medium. Interestingly, the ability of different *P. irregulare* isolates to alter ginsenoside profiles was different (Fig. 2.3c–e). Two isolates (BR 598 and BR 1068), originally obtained from cultivated ginseng plants, nearly completely degraded all the 20(*S*)-protopanaxadiol ginsenosides (Fig. 2.3d, e), while a third isolate (BR 486; obtained from bean) showed limited activity (Fig. 2.3c).

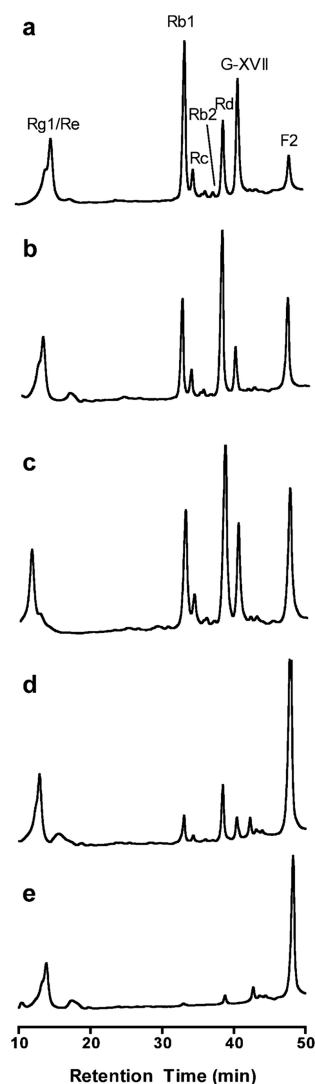
2.4.3 *P. irregulare* Specifically Deglycosylates 20(*S*)-Protopanaxadiol Ginsenosides into Ginsenoside F2

One striking feature of the ginsenoside profiles obtained from the spent medium of *P. irregulare* cultures incubated with a crude mixture of ginsenosides is the apparent selectivity toward 20(*S*)-protopanaxadiol ginsenosides (Fig. 2.3). That is, in all profiles, the amount of 20(*S*)-protopanaxatriols (Rg₁, Re) remained unchanged, whereas significant quantitative changes were noted for all 20(*S*)-protopanaxadiol ginsenosides. While the amounts of most 20(*S*)-protopanaxadiol ginsenosides were depleted, one compound became enhanced in the profiles (Fig. 2.3d, e). The identity of this compound was confirmed as the 3,20-*bis*(*O*-β-D-glucose)-substituted 20(*S*)-protopanaxadiol ginsenoside F2, by a combination of LC-MS, ¹H-, and ¹³C-NMR as well as negative ion FAB-MS [77]. Examination of the structures of the major 20(*S*)-protopanaxadiol ginsenosides reveals that F2 represents a structural core that could arise via simple deglycosylation of the 20(*S*)-protopanaxadiol ginsenosides Rb₁, Rb₂, Rc, and Rd (Fig. 2.4). Based on the existence of saponinases in other organisms that degrade saponins (see above), we concluded that *P. irregulare* also produces saponinases, which include specific ginsenoside-degrading ones; we termed these ginsenosidases.

2.4.4 *P. irregulare* Ginsenosidases Are Induced by Exposure to Ginsenosides In Vitro

Ginsenosidases of *P. irregulare* appear to be induced by their substrate *in vitro*. For example, when *P. irregulare* is incubated in the presence or absence of

Fig. 2.3 HPLC analysis of ginsenosides recovered from the spent medium of *Trichoderma hamatum* and *Pythium irregulare* isolates. A ginsenoside mixture extracted from 3-year-old ginseng roots was added to the culture medium of both *T. hamatum* and *Pythium irregulare*, recovered after several days of incubation and analyzed by HPLC. The profile of ginsenosides added to the culture medium (a) included ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc, Rd, and F2, as well as gypenoside XVII (G-XVII). (b–e) Profiles of ginsenosides recovered from *T. hamatum* isolate 3–323 (b), and *Pythium irregulare* isolates BR 486 (c), BR 598 (d), and BR 1068 (e). Adapted from M.A. Neculai, MSc Thesis, The University of Western Ontario, 2008



ginsenosides and screened for ginsenosidase activity the cultures pre-exposed to the ginsenosides show higher ginsenosidase activity (L.F. Yousef, MSc Thesis, The University of Western Ontario, 2005) [78]. Interestingly, both pre-exposed cultures and those that had not been exposed to ginsenosides (termed naïve) secreted proteins with *glycosidase* activity (measured with the artificial substrate *p*-nitrophenyl- β -D-glucoside, pNPG); however, the former secreted two to three times more proteins and ten times more glycosidase activity than naïve cultures [78]. Although in this case the two cultures had very similar glycosidase profiles, only glycosidases from cultures pre-exposed to ginsenosides readily deglycosylated the ginseng saponins

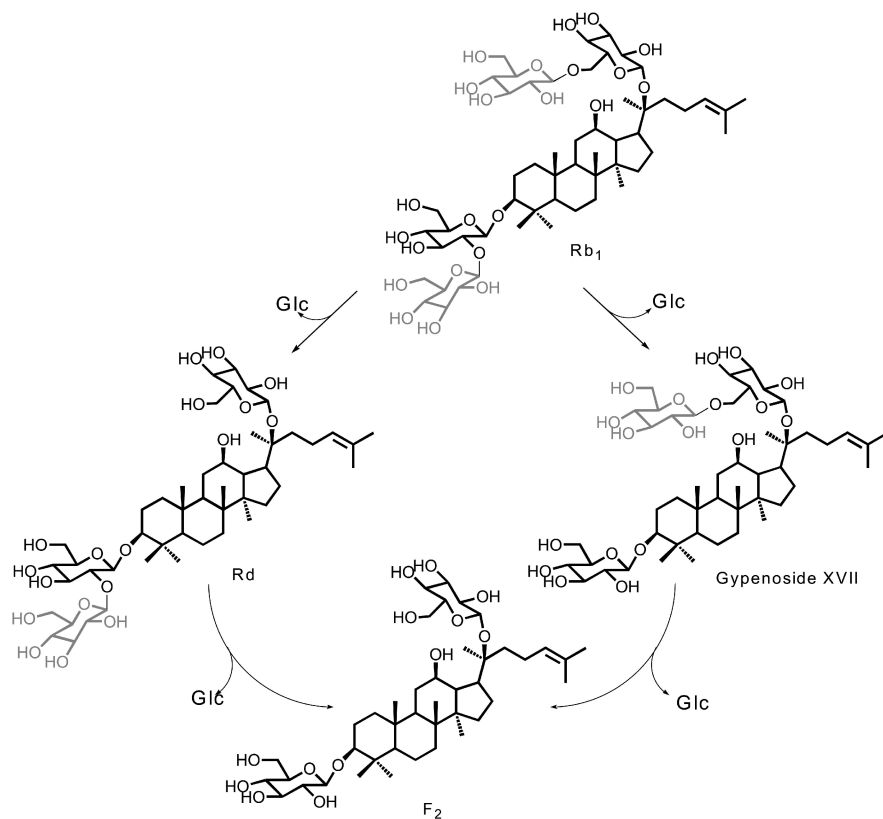


Fig. 2.4 Structural relationship between common 20(*S*)-protopanaxadiol ginsenosides and ginsenoside F2. The common core structure of ginsenoside F2 is highlighted in bold (*black*) within the structures of ginsenosides Rb₁, Rd, and gypenoside XVII. The sugar molecules that are cleaved off by hydrolysis by extracellular glycosidases are shaded in *gray*. In order to convert common 20(*S*)-protopanaxadiol ginsenosides into the common ginsenoside F2 product, the combined activity of both $\beta(1\rightarrow2)$ and $\beta(1\rightarrow6)$ glycosidases is required. Two additional 20(*S*)-protopanaxadiol ginsenosides, Rb₂ and Rc (data not shown), which contain an arabinose-glucose disaccharide at position C-20, could also feed into the production of ginsenoside F2; however, it is not clear at this time whether the same glycosidase that converts Rb₁ into Rd can also hydrolyze the arabinose $\beta(1\rightarrow6)$ glucose linkages of Rb₂ and Rc

[78]. In a manner similar to that reported for glycosidases of *G. pulicaris* [70] and *F. oxysporum* f. sp. *lycopersici* [64], we conclude that the secretion of ginsenosidases by *P. irregulare* is induced by the presence of the substrate. In other words, little or no saponinase activity was present in the filtrates of cultures pre-exposed to ginsenosides for 3–6 days, even though glycosidases were present. After that, ginsenosidases were found in relative abundance [78], suggesting an inductive process. Although these data may indicate involvement of ginsenosidases in the pathogenicity of *P. irregulare* toward American ginseng, further studies involving purified enzymes had to be undertaken.

2.4.5 Purification and Characterization of Ginsenosidases from *P. irregulare*

Three ginsenoside-deglycosylating enzymes were purified 13- to 25-fold from *P. irregulare* cultures grown in the presence of ginsenosides, by employing acetone precipitation (50% v/v), chromatofocusing between pH 7 and 4, gel filtration on Sephacryl S-200, and ion-exchange chromatography on Q Sepharose Fast Flow [78]. Chromatofocusing indicated the presence of more than one glycosidase in the sample or of different isoforms of the same glycosidase, similar to the tomatinase isolated from *F. oxysporum* f. sp. *lycopersici* [64], and predicted the isoelectric points of the enzymes to be 4.5–5.0. Gel filtration separated two distinct glycosidase activities, designated G1 and G2. Ion-exchange chromatography further purified the two glycosidase activities and electrophoretic analysis of the final ion-exchange fractions identified three enzymes: G1, which had eluted in the first peak with glycosidase activity after gel filtration (i.e., the G1 fractions), and G2/3, two enzymes similar in size, which had both eluted in the second peak with glycosidase activity from gel filtration (i.e., the G2 fractions) [78].

The molecular weights of *P. irregulare* ginsenosidases were estimated by gel filtration and SDS-PAGE. While gel filtration estimated 161 kDa for G1 and 46 kDa for G2/3, SDS-PAGE revealed a 78 kDa protein for G1, and two proteins of 61 and 57 kDa, respectively, for G2 and G3. It was concluded that G1 is a homodimer with two subunits of 78 kDa, and G2/3 are two monomeric enzymes [78]. Overall, the molecular weights and pI values of *P. irregulare* glycosidases were found to be similar to those of other characterized ginsenosidases such as the β -glucosidase secreted by *Panax ginseng* [79], the arabinopyranosidase and arabinofuranosidase of *Bifidobacterium breve* [80], and the β -glucosidase from *Fusobacterium* [81]. Moreover, these properties were also shared by some of the fungal saponinases (e.g., the tomatinase and avenacinase of *B. cinerea* [67, 68], the tomatinase of *F. oxysporum* sp. *lycopersici* [65]) previously characterized.

Although both purified glycosidase fractions (i.e., G1 and G2/3 obtained after ion-exchange chromatography) were found to deglycosylate ginsenosides, differences in the catalytic mechanisms were observed. While G1 showed $\beta(1\rightarrow6)$ hydrolytic activity, cleaving the terminal glucose from the disaccharide at C-20 of ginsenosides Rb₁ and G-XVII (to form Rd and F2, respectively), G2/3 showed $\beta(1\rightarrow2)$ hydrolytic activity, cleaving the terminal glucose from the disaccharide at C-3 of ginsenosides Rb₁ and Rd, with accumulation of G-XVII and F2, respectively [78]. These findings were confirmed by incubating partially purified enzyme preparations (i.e., culture filtrates subjected only to gel filtration) with a crude mixture of ginsenosides, but also with pure Rb₁ and Rd [78]. In comparison to G1 and G2/3 isolated from *P. irregulare*, the β -glucosidase of *Fusobacterium* showed both β -1 \rightarrow 2 and β -1 \rightarrow 6 activities able to deglycosylate ginsenoside Rb₁ to F2, and further transforming ginsenoside F2 into compound K (CK) [81]. Similar to G1, the β -glucosidase isolated from *P. bainier* was found to be able to convert ginsenoside Rb₁ to Rd, showing β -1 \rightarrow 6 activity [82].

De novo sequence analysis of purified *P. irregulare* ginsenosidases and subsequent comparison of the generated peptide sequences against the NCBI non-redundant protein sequence database revealed a number of interesting findings. Firstly, G2 (61 kDa) and G3 (57 kDa), the enzymes that eluted in the same fractions after gel filtration, were found to be different enzymes, sharing only six peptide sequences out of the 150 and 130, respectively, generated by PEAKS [78]. Moreover, both G2 and G3 were found to share sequence similarities with β -glucosidases from a variety of organisms, but exhibited the highest homology to enzymes from plants and bacteria, all classified in the glycosyl hydrolase family-1 [78]. On the other hand, only 20 peptide sequences were generated for G1 (78 kDa) and none of them showed similarities with known glycosidases [78].

2.5 The Involvement of Ginsenosides and Ginsenosidases in the Ginseng-*P. irregulare* Pathosystem

P. irregulare glucosidases are induced in the presence of ginsenosides and these enzymes catalyze the conversion of 20(*S*)-protopanaxadiol ginsenosides to ginsenoside F2, a metabolite with monosaccharides (glucose) at C-3 and C-20 of the sapogenin [78]. It is still unclear how (or if) these reactions contribute to the growth stimulation of *P. irregulare* observed in vitro [44]; however, we are working under the assumption that enzymatic conversion of ginsenosides into a common metabolite F2 could increase pathogen growth in a manner similar to that observed in vitro for ergosterol (on *P. irregulare*) [77] or soybean sterols (*Phytophthora sojae*) [83]. In addition, glucosidases converting ginseng saponins to ginsenoside F2 may be a part of the pathogen's host recognition processes. Both of these scenarios, elaborated on below, would have important implications for the severity of disease in ginseng.

Generally, oomycetes are unable to synthesize sterols because of deficiencies at key points in the sterol pathway [84, 85] and they are therefore dependent upon exogenous sterols for growth and development. These organisms acquire sterols from the environment through the action of the sterol-carrying proteins, known as elicitins [86–88]. Ginsenosides are structurally and biosynthetically similar to sterols; therefore it is possible that the sterol-binding (elicitin-like) proteins of *Pythium* spp. [89, 90] transport ginsenoside metabolites into the hyphae of the pathogen. The action of elicitin-like proteins, coupled with specific glycosidase activity, and the recovery of ginsenoside F2 from the hyphae of *P. irregulare* [77] could account for the growth stimulation of *P. irregulare* observed in vitro [44]. The induction of G1 and G2/3 in cultures supplemented with ginsenosides, but not in naïve cultures, although the latter also produce glycosidases [78] supports the idea that the formation of ginsenosidases by *P. irregulare* may represent an important step in the recognition of host roots by this pathogen. This is further supported by the observation that *P. irregulare* isolates obtained from different plant sources appear to have different levels of ginsenosidase activity in vitro (see Section 2.3.2).

the same gardens as previous ones [91]. Regardless, further experimental evidence is required to explicitly address this hypothesis.

2.6 Summary and Future Directions

The question posed in the title of this chapter remains to be answered. Are ginsenosides phytoanticipins? Or are they host recognition factors? Arguably, they are both. Since they are preformed antifungal compounds, they are by definition phytoanticipins. Their effectiveness is tempered by the fact that some organisms, but especially the oomycetes, are unaffected by their fungitoxicity, either via innate resistance or through active degradation. While such broad generalizations require further experimental validation, it has been shown that the ginseng pathogen *P. irregulare* can selectively deglycosylate the 20(*S*)-protopanaxadiol ginsenosides via extracellular glycosidases. We hypothesize that *P. irregulare* uses ginsenoside-specific glucosidases to help find its host and/or obtain nutrients/growth factors from its environment [78]. This hypothesis is supported by two unrelated observations: (1) different isolates of *P. irregulare* have been shown to have significant differences in pathogenicity between hosts [92] and (2) different isolates of *P. irregulare* have different levels of ginsenosidase activity in vitro (M.A. Neculai, MSc Thesis, The University of Western Ontario, 2008) (Fig. 2.3). If these observations are related, then the virulence of *P. irregulare* isolates on ginseng should correlate with ginsenosidase activity.

If the correlation between virulence and ginsenosidase activity exists, it may be feasible to generate avirulent strains of *P. irregulare* by knocking down these ginsenosidases in vitro. The corollary may also be true: if exposure to ginsenosides induces ginsenosidase activity (as suggested by in vitro data; [78]) and ginsenosidase activity is correlated with pathogenicity, then one might expect to increase a given isolate's pathogenicity toward ginseng (i.e., alter its host selectivity) by pre-exposing it to ginsenosides. This is supported by the observation that *P. irregulare* isolates are not necessarily more pathogenic on their host of origin but rather more selective in their pathogenicity toward different hosts [92]. This same trend of selective host pathogenicity based on genotypic relation has also been reported for *Pythium sylvaticum* and *P. ultimum* [93]. Establishing the selective host pathogenicity of *P. irregulare* will be vital in understanding the infection cycle of this oomycete and how exactly it is related to the metabolism of ginsenosides. This could inevitably lead to the establishment of better pathogen protection for ginseng growers whose crops are heavily affected by this pathogen.

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