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The formation of sugar chains in triterpenoid saponins and glycoalkaloids

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Abstract

Triterpenoid saponins and structurally related steroidal glycoalkaloids are a large and diverse family of plant glycosides. The importance of these compounds for chemical protection of plants against microbial pathogens and/or herbivores is now well-documented. Moreover, these compounds have a variety of commercial applications, e.g. as drugs or raw materials for pharmaceutical industry. Until recently there were only sparse data on the biosynthesis of saponins and glycoalkaloids, especially at the enzyme level. Substantial progress has recently been made, however, in our understanding of biosynthetic routes leading to the formation of the diverse array of aglycone skeletons found in these compounds as well as mechanisms of synthesis of their sugar moieties. This review highlights some of the advances made over past two decades in our understanding of the formation and modification of sugar moieties in triterpenoid saponins and glycoalkaloids.

Abbreviations: ESTs – expressed sequence tags; GalTase – galactosyltransferase; GTase – glycosyltransferase; GlcTase – glucosyltransferase; GlcUATase – glucuronosyltransferase; PSPG – putative secondary product glycosyltransferase; UDPGal – uridine 5′-α-D-galactopyranosyl diphosphate; UDPGlc – uridine 5′-α-D-glucopyranosyl diphosphate; UDPGlcUA – uridine 5′-α-D-glucopyranosuronic acid diphosphate

Introduction

Vascular plants can synthesize a vast array of secondary metabolites – very often in the glycoside form. Triterpenoid glycosides, i.e. true triterpene and steroidal glycosides, referred collectively as saponins, as well as steroidal glycoalkaloids, which are closely related to steroidal saponins from both structural and biogenetic point of view, are synthesized in numerous species, including many economically important crop plants (Fenwick et al., 1991; Maga, 1994; Hostettmann and Marston, 1995; Mahato and Garai, 1998; Osbourn, 2003). For a long time these glycosides have attracted much attention due to their toxicity for animals and humans (Friedman and McDonald, 1997, 1999) as well as their considerable potential as drugs or raw materials for pharmaceutical industry (Balandrin, 1996). Many saponins and glycoalkaloids, isolated from a variety of sources, have very interesting pharmacological properties, i.e. anti-inflammatory, hepatoprotective, hypocholesterolemic, immunomodulatory, anti-carcinogenic, hypoglycaemic, anticoagulant and antioxidant activities (Balandrin, 1996; Rao and Gurfinkel, 2000; Francis et al., 2002; Sparg et al., 2004). Some saponins are used as sweeteners, flavor enhancers, emulsifiers or surfactants in food and cosmetics industry (Tanaka et al., 1996). Physiological functions of triterpenoid saponins and glycoalkaloids in plants as resistance factors against pathogenic fungi, nematodes, molluscs and insects are now well-documented (Roddick, 1987; Fewell et al., 1994; Osbourn, 1996; Valkonen et al.,

1996; Fewell and Roddick, 1997; Morrissey and Osbourn, 1999; Papadopoulou et al., 1999; Bouarab et al., 2002). Moreover, there is evidence that saponins may play a specific role in the interrelations between various plant species, i.e. to act as allelopathic substances (Hoagland et al., 1996).

There is currently considerable interest in bioengineering commercially important plant species for better resistance against pathogens and herbivores or for improved production of commercially valuable plant metabolites, such as pharmaceuticals or raw materials for industrial purposes. In this context, triterpenoid glycosides are one of the most interesting groups of secondary plant products. In some cases an increased ability for the synthesis of a particular glycoside or glycosides may be desirable, e.g. for medicinal use, drug production or increased resistance against pathogens. On the other hand, in plants containing triterpenoid glycosides with toxic or anti-nutritional properties, a reduction of the synthesis of these compounds may be beneficial, at least in these plant parts which are used for food production or as feeding stuffs for domestic animals.

At present, there are many potential strategies for bioengineering the production of specific plant secondary metabolites based on techniques of modern genetics and molecular biology. However, practical applications of these strategies require a comprehensive knowledge of the biosynthetic pathways leading to the biosynthesis of various categories of secondary plant products. Particularly important is detailed characterization of the metabolic intermediates, the enzymology and regulation of these biosynthetic pathways. An understanding of the metabolic pathways, enzymes and regulatory mechanisms involved in the biosynthesis of triterpenoid glycosides is essential for the development of plants with altered content of these compounds using genetic engineering techniques and may be also of great importance for the elaboration of methods enabling commercial production of some valuable glycosides using large-scale plant cell or tissue cultures. Over the last decade, substantial progress has been made in our understanding of mechanisms of triterpenoid glycosides formation in plants. This paper will summarize the present state of knowledge on the biosynthesis of glycosidic derivatives of plant triterpenoids. Particular attention will be paid to the enzymology of sugar chains formation.

It is believed that glycosylation regulates many properties of triterpenoid aglycones, including their bioactivity, their solubility and their transport within the plant cell or throughout the whole plant (Ross et al., 2001; Bowles et al., 2005). There is also good evidence that structures of sugar moieties present in triterpenoid saponins and glycoalkaloids are crucial for their numerous biological activities (Hostettmann and Marston, 1995; Mahato and Garai, 1998, Takechi et al., 2003).

Biosynthesis of triterpene and triterpenoid aglycones

There is strong reason now to believe that isopentenyl pyrophosphate (IPP) is an universal donor of C_5-units for the biosynthesis of all terpenoids found in the Nature. IPP is synthesized in higher plants by one of two independent routes: the well-characterized mevalonate pathway operating in the cytoplasm or a recently discovered non-mevalonate pathway, i.e. 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, located in plastids (Rohdich et al., 2001; Rohmer, 2003). At the present moment it is generally assumed that the 'classical' mevalonate pathway is involved in the synthesis of IPP used for the synthesis of C_{30} hydrocarbon – squalene. Subsequently, squalene is oxidized by squalene epoxidase (squalene monooxygenase, EC 1.14.99.7) to squalene 2,3-epoxide. The latter compound is then transformed by a family of 2,3-oxidosqualene cyclases (triterpene synthases, EC 5.4.99.x) into four- or pentacyclic triterpenes, immediate precursors of triterpene and steroidal sapogenins as well as steroidal alkaloids (see Figure 1). For the synthesis of 'true', C_{30} triterpene sapogenins, squalene 2,3-epoxide is cyclized to one of several penta- or tetracyclic compounds such as β-amyrin, α-amyrin, lupeol, isomultiflorenol or dammaradienol. Genes of a number of triterpene synthases have been cloned, expressed in yeast and catalytic functions of their products confirmed by enzymological studies; examples are: β-amyrin synthase from *Panax ginseng* (Kushiro et al., 1998), α-amyrin synthase from garden pea, *Pisum sativum* (Morita et al., 2000), lupeol synthase from common dandelion, *Taraxacum officinale* (Shibuya et al., 1999) or isomultiflorenol synthase from loofah, *Luffa cylindrica* (Hayashi et al., 2001). It is noteworthy that, besides the above mentioned specific

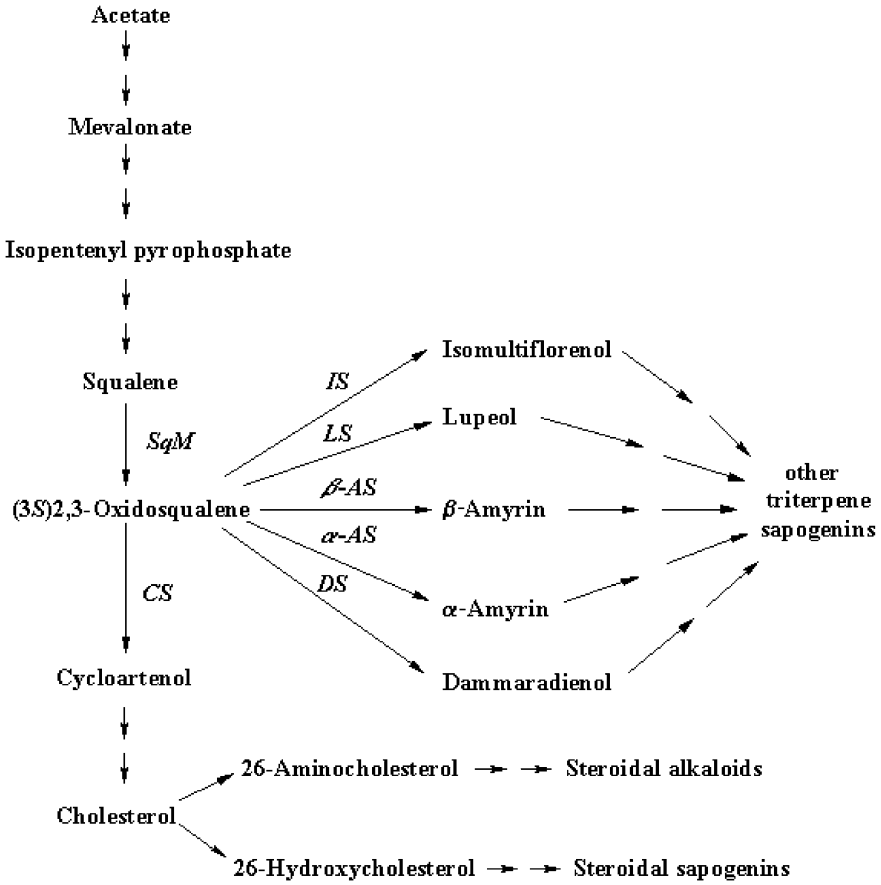

Figure 1. Outline of the biosynthetic routes leading to various types of triterpenoid aglycones: SqM – squalene monooxygenase; CS – cycloartenol synthase; β-AS – β-amyrin synthase; α-AS – α-amyrin synthase; LS – lupeol synthase; DS – dammaradienol synthase; IS – isomultiflorenol synthase.

enzymes, multifunctional triterpene synthases have been found in some plants. An example is the multifunctional enzyme from mouse-ear cress (*Arabidopsis thaliana*) which can catalyze cyclization of squalene 2,3-epoxide to β-amyrin, α-amyrin and lupeol in the ratio 55:30:15 (Husselstein-Muller et al., 2001). More detailed information about plant triterpene synthases can be found in a recent review (Haralampidis et al., 2002).

The subsequent conversions of the primary cyclization products, i.e. α-amyrin, β-amyrin, lupeol, dammaradienol, isomultiflorenol, etc., lead to the great variety of aglycones of triterpene saponins occurring in plants. It has been reported, for example, that in pot marigold (*Calendula officinalis*) shoots, the synthesis of oleanolic acid, one of the most wide-spread aglycones of triterpene saponins, involves a stepwise oxidation of the angular methyl group present in β-amyrin at C-17 with transient formation of erythrodiol and oleanolic aldehyde (Kasprzyk and Wojciechowski, 1969). Similarly, in cell cultures of watermelon (*Citrullus lanatus*), bryonolic acid (3β-hydroxy-D:c-friedoolean-8-en-29-oic acid) is synthesized from isomultiflorenol, via bryonolol and bryonolal which are alcohol and aldehyde analogues of bryonolic acid, i.e. by stepwise oxidations of the methyl group attached to C-20 of the isomultiflorenol molecule (Cho et al., 1993). This was further confirmed by studies with the use of crude extracts prepared from 10-day-old cell culture of loofah. Administration of [2–^{14}C]mevalonate to these crude enzyme preparations revealed labeling, in addition to the radioactive bryonolic acid, a

series of its presumable intermediates: squalene, squalene 2,3-epoxide, isomultiflorenol as well as bryonolol and bryonolal (Tabata et al., 1993). There is also evidence that β-amyrin is a precursor of the pentacyclic triterpene aglycone of antifungal saponins (avenacins) synthesized in oat (*Avena sativa*) roots (Haralampidis et al., 2001). Recent studies (Flores-Sanchez et al., 2002) on biosynthesis of triterpene sapogenins in cell suspension cultures of cat's claw (*Uncaria tomentosa*) furnished some evidence that α-amyrin is a precursor of ursolic and quinovic acids which are, respectively, mono- and dicarboxylic acids containing the α-amyrin skeleton. It should be stressed, however, that very little is known about enzymes and genes involved in the above described modification processes. Most probably, introductions of additional oxygen functions into triterpene skeletons are catalyzed by cytochrome P450-dependent monooxygenases. Such an enzyme, catalyzing hydroxylation of 18β-glycyrrhetinic acid to its 24-hydroxy-derivative, was isolated from the microsomal fraction of liquorice (*Glycyrrhiza glabra*) cell suspension cultures and characterized (Hayashi et al., 1993).

The synthesis of steroidal sapogenins and alkaloids involves cyclization of squalene 2,3-epoxide, mediated by cycloartenol synthase, with the formation of tetracyclic, C_{30}-compound – cycloartenol (Brown, 1998). Cycloartenol is then converted into plant sterols, among others into C_{27}-sterol, cholesterol. The latter compound, which is usually a minor sterol component of plant tissues, is believed to be a precursor of steroidal sapogenins and alkaloids (Heftmann, 1983; Bergenstrahle et al., 1996; Friedman and McDonald, 1997; Arnqvist et al., 2003). 26-Hydroxy- and 26-amino-derivatives of cholesterol have been identified as early intermediates on biosynthetic routes leading to the synthesis of steroidal sapogenins and alkaloids, respectively – for a recent review see Brown (1998).

Formation of sugar chains

Triterpene saponins

A great variety of triterpene saponins have been isolated which differ in the structure of both the aglycone and carbohydrate moieties, containing sometimes 10 or more monosaccharide units, often in the form of branched chains. Some triterpene saponins are bi- or tridesmosidic, i.e. they contain, besides of sugar chain usually present at C-3 hydroxyl group of the aglycone, one or even two additional carbohydrate moieties attached to hydroxyl or carboxyl groups located in some other positions. It is generally believed that these sugar chains are synthesized by sequential additions of single monosaccharide moieties to the aglycone. There are only a few reports on the occurrence of specific glycosyltransferases (GTases) catalyzing the formation of monosaccharide derivatives of various triterpene aglycones (see Table 1). In all cases when subcellular compartmentation of these enzymes was studied, they were found to be tightly membrane-bound and were located in the microsomal fraction. The use of detergents such as Triton X-100 (Wojciechowski, 1975) or CHAPS (Shiraiwa and Kurosawa, 2001; Kurosawa et al., 2002) was necessary for solubilization of glucuronosyltransferases (GlcUATases) present, respectively, in pot marigold (*Calendula officinalis*)

Table 1. Plant triterpene glycosyltransferases catalyzing the formation of 3-*O*-monoglycosides.

Plant species	Sugar acceptor	Sugar donor	Localization	References
Calendula officinalis (seedlings)	Oleanolic acid	UDPGlcUA	Microsomal	Wojciechowski (1975, 1983)
Pisum sativum (seedling axis)	β-Amyrin	UDPGlc	Microsomal	Baisted (1979)
Glycyrrhiza glabra (cell suspension culture)	24-Hydroxy-glycyrrhetinic acid	UDPGlcUA	Microsomal	Hayashi et al. (1993, 1996)
Gypsophila paniculata (root *in vitro* culture)	β-Glycyrrhetinic acid	UDPGlcUA	?	Herold and Henry (2001)
Glycine max (germinating seeds)	Soyasapogenols (B > E > A)	UDPGlcUA	Microsomal	Shiraiwa and Kurosawa (2001), Kurosawa et al. (2002)

seedlings and germinating soybean (*Glycine max*) seeds. The latter GlcUATase was purified 205-fold and SDS–PAGE analysis suggested that its activity coincided with 66-kDa and/or 50-kDa proteins. Always when the enzyme specificity was determined, natural sapogenins present in a given plant were preferred substrates (chemical structures of these sapogenins are given in Figure 2). The UDPGlcUA-dependent enzyme from pot marigold which efficiently catalyzed the formation of oleanolic acid 3-*O*-*β*-D-glucuronopyranoside was completely inactive with several structurally related triterpenes such as *β*-amyrin, erythrodiol or methyl oleanolate. Similar GlcUATase from soybean, highly active with natural aglycones of soybean saponins, i.e. soyasapogenols A, B and E, and converting them to the corresponding 3-*O*-*β*-D-glucuronopyranosides, was inactive towards some other oleanane-type triterpenes, e.g. *β*-amyrin, sophoradiol or glycyrrhetinic acid, as well as flavonoids. On the other hand, GlcUATase from liquorice cell suspension culture readily synthesized glucuronosides of 24-hydroxyoleanane derivatives: 24-hydroxyglycyrrhetinic acid and soyasapogenol B but not of glycyrrhetinic acid, *β*-amyrin or sophoradiol which are also oleanane-type compounds, however, lacking the 24-hydroxyl group (Hayashi et al., 1993, 1996).

Very little is known about the biosynthetic steps leading to the formation of saponins containing more extended carbohydrate moieties and bidesmosidic saponins. Flowers and shoots of pot marigold contain six structurally related saponins (Kasprzyk and Wojciechowski, 1967; Kintia et al., 1974; Vidal-Ollivier et al., 1989) which are mono- or bidesmosidic derivatives of oleanolic acid containing up to four monosaccharide moieties (Figure 3a). Our *in vivo* studies of the dynamics of labeling of the individual saponins with [2–^{14}C]mevalonate (Kintia et al., 1974) indicated that their synthesis proceeds, most probably, by the stepwise elongation of carbohydrate moiety, i.e. by subsequent additions of single monosaccharides. This was confirmed by further studies (Wojciechowski, 1975; 1983) with cell-free enzyme preparations obtained from young seedlings (see Figure 3b). The first step in this process is the above mentioned enzymatic synthesis of oleanolic acid 3-*O*-*β*-D-glucuronopyranoside (glycoside F) from free oleanolic acid and UDPGlcUA, catalyzed by microsomal fraction obtained from pot marigold seedlings. However, in the presence of labeled UDPGlc and glycoside F the formation of bidesmosidic glycoside D_2 was catalyzed, in contrast to the former reaction, by a soluble (cytosolic?) enzyme. The glucosyltransferase (GlcTase)

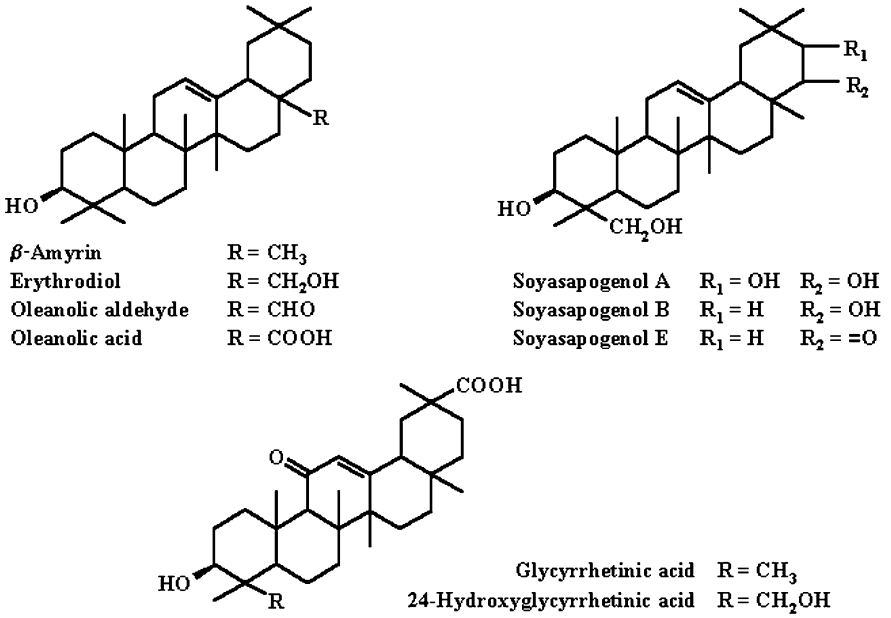

Figure 2. Chemical formulas of some triterpene sapogenins mentioned in the text.

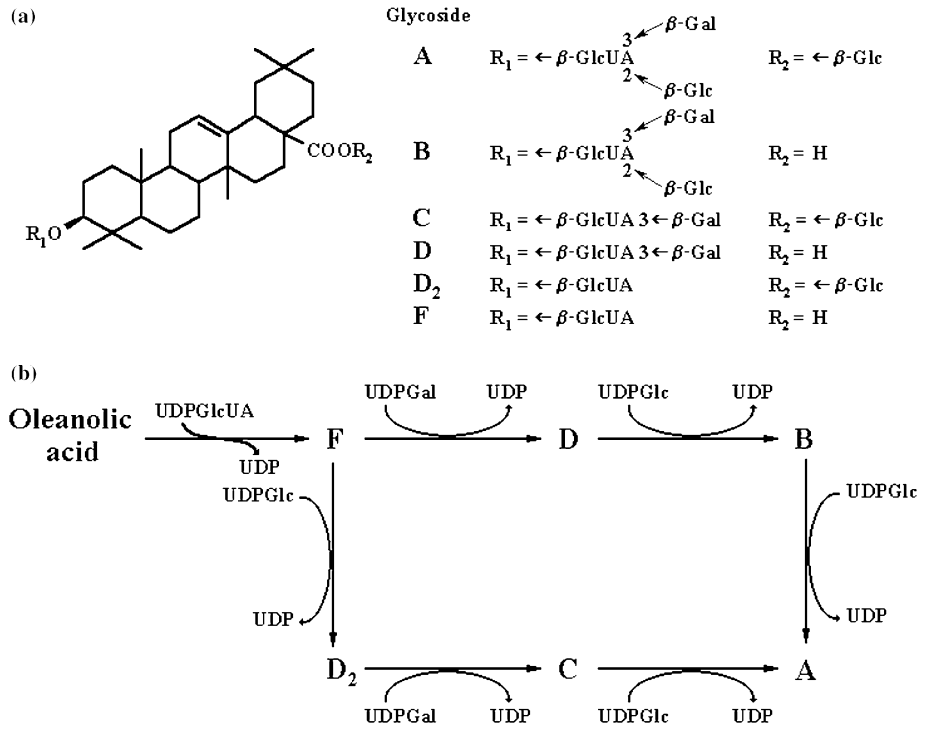

Figure 3. Saponins of pot marigold (*Calendula officinalis*) flowers and shoots (a) and their biosynthetic interrelations (b).

also seems to be a specific enzyme, since free oleanolic acid could not serve as the acceptor and monodesmosidic glycosides D and B were glucosylated at the carboxyl group at much lower rates than glycoside F. The consecutive steps of elongation of sugar chains at C-3 could be followed by incubations of membranous fractions from marigold seedlings with glycosides F and D_2 and labeled UDPGal or with glycosides D or C and labeled UDPGlc. In these cases, however, the most active were membranous fractions sedimenting between 300 and 15,000*g*, tentatively identified as Golgi and/or plasma membrane fragments.

Steroidal saponins

Steroidal saponins are particularly abundant in species belonging to the Liliaceae, Scrophulariaceae, Dioscoreaceae, Zygophyllaceae and Solanaceae families and can be divided into the furostane- and the spirostane-type compounds. The spirostane-type saponins are usually monodesmosides containing an oligosaccharide chain usually bound to the hydroxyl group at C-3 of the aglycone which consists of six fused rings A–F. The furostane-type saponins are bidesmosidic glycosides, containing, apart of a sugar chain bound to the hydroxyl group at C-3 of the aglycone, an additional sugar residue, usually a single β-D-glucosyl moiety, bound to the hydroxyl group present at C-26 (or C-27) of the aglycone in which the F-ring is opened. Examples of furostane saponins and their spirostane counterparts are protodioscin or protogracillin and dioscin or gracillin which occur e.g. in crape ginger (*Costus speciosus*) rhizomes (see Figure 4).

It is known for a long time that the removal of D-glucose moiety present in the furostane-type saponin at C-26 (or C-27) by enzymic hydrolysis with β-glucosidases or by mild acid hydrolysis leads to a spontaneous closure of the F-ring, i.e. to the conversion of the furostane-type saponin, e.g. protodioscin, into the corresponding spirostane-type saponin, e.g. dioscin. It is generally assumed that steroidal saponins are accumulated and stored in vacuoles of plant cells mainly in the form of bidesmosidic, furostane-type glycosides and they are converted to their spirostane-type counterparts only after a damage of plant tissue. For the above reason the furostane-type steroidal saponins are frequently termed 'protosaponins'. However, it should be pointed out that the biogenetic

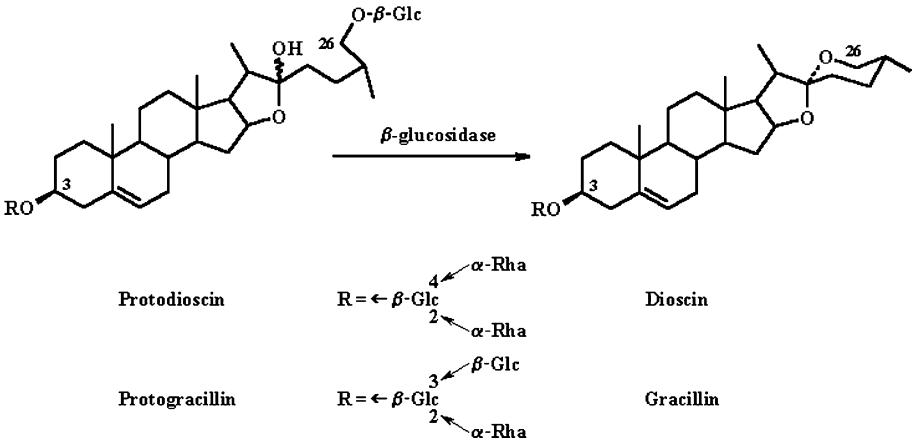

Figure 4. Conversion of bidesmosidic furostanol 'protosaponins', present in freshly harvested rhizomes of crape ginger (*Costus speciosus*), into monodesmosidic spirostanol glycosides, found in stored rhizomes.

relationship between the furostane- and spirostane-type analogues is controversial and will be discussed later.

Studies of the initiation of the sugar chains in steroidal saponins began about 15 years ago. Up to now, several GTases which seem to be highly specific for steroidal sapogenins have been isolated, partially purified and characterized (see Table 2). The chemical formulas of some sapogenins glycosylation of which will be discussed below are given in Figure 5.

The first enzyme effectively catalyzing glucosylation of a steroidal sapogenin was UDPGlc:nuatigenin GlcTase (EC 2.4.1.192) isolated from oat leaves (Kalinowska and Wojciechowski, 1986, 1987). This enzyme glucosylates, at C-3 hydroxyl group, nuatigenin ([20*S*,22*S*,25*S*]-22,25-epoxy-furost-5-ene-3β,26-diol) – a rare pseudospirostanol sapogenin which is the aglycone of oat saponins, avenacosides A and B. Avenacosides contain branched tri- or tetrasaccharide chains composed of glucose and rhamnose bound through a β-D-glucosyl moiety to the hydroxyl group at C-3 of the aglycone and a single β-D-glucosyl moiety bound to the hydroxyl group at C-26. The product of the reaction catalyzed by oat GlcTase was identified as nuatigenin 3-*O*-β-D-glucopyranoside – a putative intermediate in the biosynthesis of avenacosides A and B. It is of interest that this enzyme could not glucosylate nuatigenin at the C-26 hydroxyl group. UDPGlc:nuatigenin GlcTase, with an apparent molecular mass of ca. 60 kDa, was partially purified from the cytosol fraction of oat leaves (Kalinowska and Wojciechowski, 1988). Among various nucleoside-5′-α-diphosphate derivatives of glucose tested, UDPGlc was the most efficient sugar donor for nuatigenin glucosylation by this enzyme. Studies of its specificity with respect to the glucose moiety acceptor showed that, among a number of steroid compounds, nuatigenin was the best substrate but several other 3β-OH steroidal sapogenins, e.g. diosgenin, tigogenin, hecogenin or chlorogenin, as well as some steroidal alkaloids,

Table 2. Plant UDP-glucose-dependent glucosyltransferases acting on the C-3 hydroxyl group of steroidal sapogenins.

Plant species	Preferred UDPGlc acceptor(s)	Subcellular localization	References
Avena sativa (young leaves)	Nuatigenin, isonuatigenin	Soluble?	Kalinowska and Wojciechowski (1986, 1987, 1988)
Asparagus officinalis (shoots)	Sarsasapogenin, smilagenin	Soluble?	Paczkowski and Wojciechowski (1988)
Asparagus plumosus (shoots)	Yamogenin, diosgenin	Soluble?	Paczkowski et al. (1990)
Solanum melongena (leaves)	Diosgenin, yamogenin	Soluble?	Paczkowski and Wojciechowski (1993, 1994)

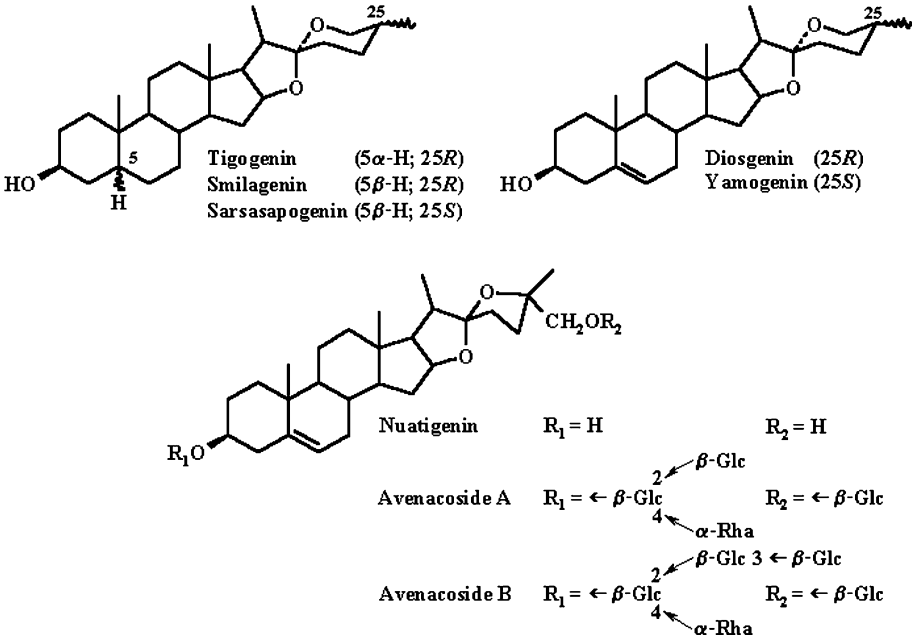

Figure 5. Chemical formulas of some steroidal sapogenins mentioned in the text.

e.g. tomatidine or solanidine, were more or less efficiently glucosylated. On the other hand, all tested 3α-OH steroids were poor substrates. These data strongly suggest that β-configuration of the hydroxyl group at C-3 is an indispensable condition for the enzyme action. It is noteworthy that typical phytosterols, such as sitosterol or stigmasterol, were also were poor substrates for the enzyme. It means that UDPGlc:nuatigenin GlcTase is not identical with UDPGlc:sterol GlcTase which is also present in oat leaves (Warnecke and Heinz, 1994; Warnecke et al., 1997). In contrast to UDPGlc:nuatigenin GlcTase, the latter enzyme is tightly bound to cell membranes and is strongly activated by some detergents, e.g. Triton X-100. Moreover, the nuatigenin-specific enzyme is almost completely inhibited by detergents at concentrations which considerably stimulate glucosylation of phytosterols by the membrane-bound, sterol-specific enzyme.

Subsequently, similar GlcTases showing high affinities towards steroidal sapogenins were isolated from leaves of two *Asparagus* species: garden asparagus, *A. officinalis* (Paczkowski and Wojciechowski, 1988) and asparagus fern, *A. plumosus* (Paczkowski et al., 1990) as well as from eggplant (*Solanum melongena*) (Paczkowski and Wojciechowski, 1993). Like the above described enzyme from oat, all these enzymes are soluble proteins, use UDP-glucose as sugar source and display a broad specificity pattern with respect to various steroidal sapogenins. Good substrates for the enzyme obtained from eggplant consisted of, apart from diosgenin and tigogenin which are natural sapogenins in this plant, some structurally related spirostanols regardless of the stereochemistry at C-25, presence or absence of a double bond in the ring system, or presence of an additional oxygen function in the ring system. However, the prerequisite for the eggplant enzyme activity consisted of a relatively planar ring system, i.e. *trans* coupling of the A and B rings (sapogenins of 5α-H series) or the presence of a double bond at C-5 (sapogenins of the Δ^5 series). Neither sarsasapogenin nor smilagenin which are 5β-H sapogenins with *cis* coupling of the A and B rings could be glucosylated by this enzyme (Paczkowski and Wojciechowski, 1994, 1996). The enzyme isolated from asparagus fern exhibited a similar specificity pattern. In the latter plant yamogenin (25*S*-epimer of diosgenin), belonging to the Δ^5 series, is the native steroidal sapogenin. It is noteworthy that, in contrast to the enzymes present in eggplant and

asparagus fern, the enzyme obtained from garden asparagus preferably glucosylated sapogenins of the 5β-H series containing a non-planar steroid nucleus, e.g. sarsasapogenin, which is a native sapogenin in this plant, as well as smilagenin which is 25*R*-epimer of sarsasapogenin (Paczkowski and Wojciechowski, 1988). These results indicate that there is an evident correlation between the type of sapogenin produced by a given plant species and the specificity of GlcTase isolated from the same plant.

It is well established (see e.g. Heftmann, 1983; Paseshnichenko, 1987) that steroidal sapogenins are formed via stepwise oxidation of cholesterol molecule at C-26 (or C-27), C-16 and C-22 and subsequent cyclization of the oxygenated sterol side chain to the spiroketal ring system (the heterocyclic rings E and F). Some authors postulated (Ronchetti et al., 1975; Tal et al., 1984; Gurielidze et al., 1987) that the glucosylation of the hydroxyl group at C-26(27) takes place at an early stage of the process described above, preventing the closure of the F-ring. The resulting furostanol 26-β-D-glucosides would be then glycosylated at the C-3 hydroxyl group to form bidesmosidic furostane-type saponins containing an oligosaccharide chain at C-3 and a single glucose moiety bound at C-26 of the aglycone. According to this hypothesis the spirostane-type saponins are formed secondarily by enzymatic removal of D-glucose bound at C-26 hydroxyl group of the furostane-type saponins with a concomitant spontaneous cyclization of the heterocyclic ring F (see Figure 6, counter-clockwise). There is, however, another possibility. Immediately after the introduction of oxygen functions at C-26(27), C-16 and C-22 into cholesterol molecule, the spiroketal system is formed, spirostanol aglycone is glycosylated at the C-3 and only afterwards the resulting monodesmosidic, spirostane-type saponin can be converted to its bidesmosidic, furostane-type analogue by an opening the ring F and subsequent glucosylation of the resulting C-26 hydroxyl group (see Figure 6, clockwise).

The studies discussed above on enzymatic glycosylation of steroidal sapogenins seem to speak in favor of the latter hypothesis. They prove that plants which synthesize steroidal saponins of the furostane-type contain fairly specific glucosyltransferases effectively glucosylating free spirostanol sapogenins. Moreover, experiments with glucosyltransferase from asparagus fern showed that this enzyme which efficiently glucosylated yamogenin was completely unable to glucosylate, at C-3 hydroxyl group, its furostane analogue, i.e. yamogenin 26-β-D-glucopyranoside (Paczkowski et al., 1990). These results clearly indicate that the presence of β-D-glucosyl moiety at C-26 is not essential for sugar chain formation at C-3. Therefore, the results discussed above suggest that the monodesmosidic, spirostane-type steroidal saponins are biogenetic precursors of the bidesmosidic, furostane-type saponins which are main storage form in plant cells. Some recent results obtained with cell suspension cultures of crape ginger confirm the above supposition. These cell cultures efficiently converted free diosgenin to its bidesmosidic, furostane-type derivative, i.e. 3-*O*-[β-D-glucosyl-(1$''$ $\rightarrow$ 2$'$)-β-D-glucoside]; 27-*O*-β-D-glucoside (Indrayanto et al., 2001). Thus, in the intact plant cells, an opening of the ring F in the spirostane-type aglycone with subsequent glucosylation of the resulting hydroxyl group at C-26(27) is evidently possible.

As mentioned earlier, steroidal saponins are usually accumulated in plant tissues in the form of bidesmosidic furostane-type compounds. Most likely they are biologically inactive, storage forms of steroidal saponins, i.e. they can be regarded as preformed chemical protectants against pathogen infections or herbivore attacks. When plant tissues are wounded, the bidesmosidic, furostane-type saponins are enzymatically converted into the biologically active, highly toxic, monodesmosidic analogues, i.e. into the corresponding spirostane-type compounds (Roddick, 1987; Osbourn, 1996). The occurrence of specific plant glycosylhydrolases (glycosidases) that carry out the above conversion, i.e. the removal of D-glucose bound to the hydroxyl group at C-26, were identified in several saponin-containing plants, e.g. in three yam species: *Dioscorea floribunda* (Joly et al., 1969), *Dioscorea deltoidea* (Gurielidze et al., 1987) and *Dioscorea caucasica* (Gurielidze et al., 2004), Spanish dagger – *Yucca gloriosa* (Gurielidze et al., 1992), *Allium erubescens* (Vardosanidze et al., 1991), oat (Kesselmeier and Urban, 1983; Gus-Mayer et al., 1994) and crape ginger (Inoue and Ebizuka, 1996a, 1996b; Inoue et al., 1996a, 1996b; Ichinose et al., 1999).

One of the best characterized is the oat enzyme – avenacosidase. This enzyme, which in intact cells

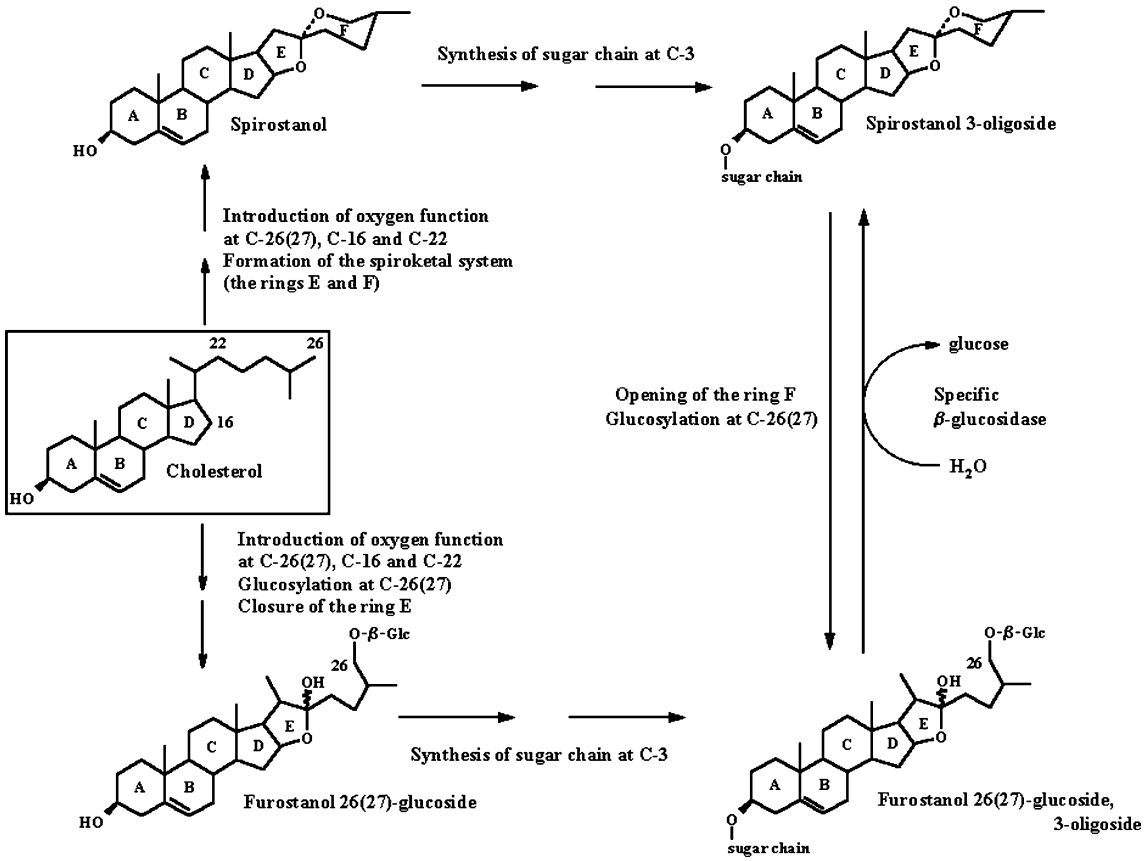

Figure 6. Possible biosynthetic interrelations between steroidal saponins of the furostane- and spirostane-type.

of oat leaves is located in the plastid stroma (Nisius, 1988), converts bidesmosidic oat saponins, avenacosides A and B, which are accumulated in the vacuoles (Kesselmeier and Urban, 1983), into the antifungally active, monodesmosidic saponins, 26-desgluco-avenacosides A and B. An injury of oat leaves arising from mechanical damage or pathogen attack causes a breakdown of compartmentalization of the enzyme and its substrates, allowing avenacosidase to come in contact with avenacosides A and B (Osbourn, 1996). Avenacosidase was isolated from etiolated oat seedlings and highly purified (Gus-Mayer et al., 1994). The enzyme consists of 60 kDa subunits, however, in the active form it is highly aggregated; it consists of 300–350 kDa aggregates or even multimers of these aggregates. Dissociation of these aggregates leads to a complete loss of enzyme activity. An anti-60 kDa subunit antiserum was prepared and used for isolation of a cDNA clone coding for the enzyme subunits. The presence of a N-terminal signal sequence containing 55 amino acid residues, directing the enzyme into the plastid stroma, was deduced from comparison of the cDNA sequence with N-terminal sequence of the purified enzyme protein.

Subsequently, a diosgenin-rich plant, crape ginger, was found to contain β-glucosidase converting the furostane-type glycosides, protodioscin and protogracillin, into their spirostane-type analogues, i.e. dioscin and gracillin (Inoue et al., 1996a, 1996b). The enzyme, furostanol glycoside 26-*O*-β-D-glucosidase, was purified to an apparent homogeneity (Inoue et al., 1996b) from crape ginger rhizomes as a heterodimer consisting of two subunits of 54 and 58 kDa. Substrate specificity of the enzyme, tested with protogracillin and protodioscin as well as with a number of natural or synthetic non-steroidal β-D-glucosides, e.g. gentiobiose, cellobiose, prunasin, amygdalin, *n*-octyl glucoside, phenyl glucoside, etc. as potential substrates, showed that the enzyme was highly specific

for the cleavage of β-D-glucosidic bond at C-26 hydroxyl group in steroidal protosaponins. Moreover, free diosgenin, the aglycone of gracillin and dioscin, strongly inhibited the enzyme indicating that this β-glucosidase can recognize the steroidal backbone of sapogenins (Inoue and Ebizuka, 1996b).

Degenerate oligonucleotide primers, based on amino acid sequences of several peptides obtained by digestion of the purified enzyme from crape ginger with endoproteinase, were used for amplification of cDNA encoding this enzyme. The recombinant enzyme expressed in *E. coli* cells consisted of 562 amino acids and showed β-glucosidase activity specific for cleavage of the C-26 β-D-glucosidic bond in steroidal protosaponins (Inoue et al., 1996b). The cloned cDNA encoding β-glucosidase from crape ginger was also introduced into a heterologous higher plant, tobacco (Ichinose et al., 1999). PCR analyses confirmed a successful integration of the cDNA into tobacco chromosomal DNA and β-glucosidase activity specific for the furostane-type saponins was detected in cell-free extracts of the transgenic tobacco seedlings. The amino acid sequences of both avenacosidase and β-glucosidase from crape ginger rhizomes, revealed high homologies to the sequences found in N-terminal regions of many other plant β-glucosidases, particularly those involved in plant defense mechanisms (Gus-Mayer et al., 1994; Inoue and Ebizuka, 1996b).

Steroidal glycoalkaloids

Oligoside derivatives of nitrogen-containing steroids, i.e. steroidal glycoalkaloids, are constituents of numerous plant species, most notably members of the Solanaceae family. Among other plant species, these compounds occur in such economically important cultivated plants as potatoes (*Solanum tuberosum*), tomatoes (*Lycopersicon esculentum*), sweet peppers (*Capsicum annuum*) or garden eggplants (*Solanum melongena*) (Friedman and McDonald, 1997, 1999).

Steroidal aglycones of glycoalkaloids synthesized in the Solanaceae can be divided into the solanidane-type (e.g. solanidine) and the spirosolane-type (e.g. tomatidine or solasodine). The sugar moiety of glycoalkaloids can range from a monosaccharide to a hexasaccharide, however, compounds containing three to four monosaccharide units forming a branched chain are particularly common. Monosaccharide directly bound to the C-3 hydroxyl group of aglycone is usually D-glucose or D-galactose (Maga, 1994; Friedman and McDonald, 1999). Structures of the most common glycoalkaloids occurring in Solanaceae and their aglycones are shown in Figure 7.

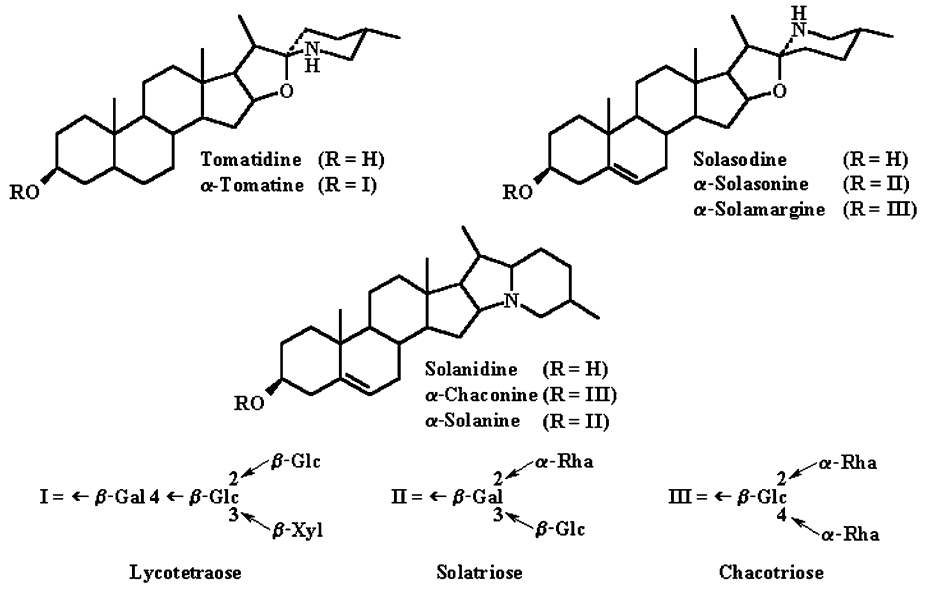

Figure 7. Chemical structures of steroidal glycoalkaloids accumulated in tomato, eggplant and potato.

For the first time, enzymatic glucosylation of a steroidal alkaloid, solasodine, was demonstrated by Liljegren (1971) in crude extracts from kangaroo apple (*Solanum laciniatum*) leaves incubated with UDP-[^{14}C]Glc. A few years later the glucosylation of solanidine by crude extracts from potato sprouts (Jadhav and Salunkhe, 1973) and tubers (Lavintman et al., 1977) was reported. In experiments with crude enzyme preparations from potato tubers incubations with labeled UDPGlc and solanidine led to the formation of several labeled products which were tentatively identified as solanidine trioosides, α-solanine and α-chaconine, which are the main glycoalkaloids of potatoes, as well as the corresponding biosides (β-solanine and β-chaconine) and monosides (γ-solanine and γ-chaconine). These results were explained by the presence, in the crude extracts, of enzymes converting UDPGlc into UDPGal and UDP-L-rhamnose as well as several GTases required for a further transfer of the monosaccharide moieties from these three nucleotide sugars. A stepwise addition of sugars to the aglycone was also confirmed by *in vivo* experiments in which radioactive solanidine, added to potato cell suspension cultures or tuber slices, was converted into its mono- and diglycoside derivatives (Osman and Zacharius, 1979; Osman et al., 1980). It should be pointed out, however, that in all these early studies, the identity of reaction products was not rigorously proved.

In the early nineties of the foregoing century, three research teams reported the isolation and partial purification of an UDPGlc-dependent GlcTase, showing high affinity for solanidine, from potato tubers (Stapleton et al., 1991), potato leaves (Zimowski, 1991), and potato sprouts (Bergenstrahle et al., 1992a) – see Table 3. The reaction product was unequivocally identified as solanidine 3-*O*-β-D-glucopyranoside (γ-chaconine) – a hypothetical intermediate in the biosynthesis of α-chaconine, one of the two major glycoalkaloids of potato. It is noteworthy that crude enzyme preparations from various organs of potato plants could also catalyze, in the presence of UDPGal, the synthesis of solanidine 3-*O*-β-D-galactopyranoside (γ-solanine) – a putative intermediate in the biosynthesis of α-solanine, another major glycoalkaloid of potatoes. The GalTase activity, however, was much lower than that of GlcTase and quite unstable during attempts to purify this activity. Moreover, it was reported that UDPGal was a competitive inhibitor in the solanidine glucosylation reaction, whereas UDPGlc showed non-competitive inhibition of the solanidine galactosylation (Bergenstrahle et al., 1992a). These results are most easily explained by assuming that UDPGlc:solanidine GlcTase and UDPGal:solanidine GalTase are indeed two separate enzyme proteins catalyzing the synthesis of γ-chaconine and γ-solanine, respectively. This assumption was then confirmed by a partial separation of the GlcTase and GalTase activities by ion-exchange chromatography (Zimowski, 1997).

It is noticeable that the solanidine glycosylating activities in various organs of potato plants were always present in the fraction of soluble proteins, i.e. in the cytosolic fraction, in contrast to the UDPGlc:sterol GlcTase activity which could be also detected in potato but was always found in membranous fractions. The UDPGlc:sterol and UDPGlc:solanidine GlcTases greatly differ in their specificities towards various steroid acceptors. The former enzyme has very low affinity for steroidal alkaloids, whereas the latter enzyme has very little

Table 3. Plant UDP-sugar-dependent glucosyltransferases acting on the C-3 hydroxyl group of steroidal alkaloids.

Plant species	Sugar donor	Preferred acceptor	Localization	References
Solanum tuberosum (tubers, leaves, sprouts)	UDPGlc, UDPGal	Solanidine, Tomatidine	Soluble?	Stapleton et al. (1991), Zimowski (1991), Bergenstrahle et al. (1992)
Solanum melongena (leaves)	UDPGlc	Solasodine, and other spirosolane-type alkaloids	Soluble?	Paczkowski and Wojciechowski (1994), Paczkowski et al. (1996, 1997, 1998)
Solanum lycopersicon (mature leaves)	UDPGal	Tomatidine and other spirosolane type alkaloids	Soluble?	Zimowski (1996, 1998)

activity towards typical plant sterols (Zimowski, 1992). It is noteworthy, however, that the solanidine glucosylating enzyme from potato plants is highly active not only with solanidane-type steroidal alkaloids but can also glucosylate spirosolane-type aglycones, e.g. tomatidine or solasodine, at comparable or even higher rates (Stapleton et al., 1991; Bergenstrahle et al., 1992a; Zimowski, 1992).

UDPGlc:solanidine GlcTase occurs in potato plants at a very low level, is relatively unstable and co-purifies with patatin, the major storage protein in potato tubers. Although an improved method enabling ca. 110-fold purification of the enzyme from greening potato peel was reported (Stapleton et al., 1992), attempts to purify the enzyme to homogeneity were unsuccessful. Therefore, an alternative approach was used in order to isolate cDNA encoding potato UDPGlc:solanidine GlcTase (Moehs et al., 1997). Based on the observation that solanidine, in contrast to its glycosidic derivatives, is highly toxic for baker's yeast, a cDNA encoding this enzyme was selected from a yeast expression library. The cDNA contained an open reading frame encoding a 483-residue polypeptide with a calculated molecular mass of 56 kDa. The coding sequence of the enzyme revealed significant similarity to a number of previously characterized nucleotide-sugar-dependent GTases involved in glycosylation of other plant secondary products, e.g. flavonoids, betanidins, limonoids, cyanohydrins or simple phenolics (Vogt and Jones, 2000). All these enzymes contain a highly conserved consensus sequence, so-called PSPG-box (Putative Secondary Product Glycosyltransferase-box) which was originally defined by Hughes and Hughes (1994) as a signature sequence for all plant GTases involved in glycosylation of various secondary plant metabolites. This consensus sequence consists of a short stretch of ca. 40 amino acids close to the C-terminal part of GTases, most likely representing the nucleotide-sugar binding site. Two highly conserved amino acids, histidine and glutamic acid, are proposed to be involved in a nucleophilic S_N2 reaction leading to the inversion of the anomeric sugar configuration during the synthesis of the glycosidic bond (Kapitonov and Yu, 1999). It is noteworthy, however, that with the exception of the PSPG-box, the overall homology of the amino acid sequences present in GTases participating in the synthesis of glycosides of various secondary plant metabolites is rather low, i.e. only ca. 10% (Vogt and Jones, 2000). Sequence alignments, phylogenetic analysis and the similar intron–exon organization suggest that plant GTases containing PSPG-box are, most probably, monophyletic, i.e. they emerged from a single ancestral GTase (Paquette et al., 2003).

The recombinant UDPGlc:solanidine GlcTase (SGT1) obtained from yeast exhibited a very similar specificity pattern towards steroid acceptors to that reported earlier for crude or partially purified enzyme preparations from various organs of potato plants (Stapleton et al., 1991; Bergenstrahle, 1992a; Zimowski, 1992), i.e. it glucosylated at high rates solanidine but the spirosolane-type alkaloids, i.e. tomatidine and solasodine, were even better sugar acceptors.

Enzyme preparations catalyzing glycosylation of steroidal alkaloids were also obtained from other solanaceous plants. Leaves of garden eggplant (*S. melongena*) contain a soluble enzyme catalyzing glucose transfer from UDPGlc to solasodine – a spirosolane-type steroidal alkaloid naturally occurring in this plant (Paczkowski and Wojciechowski, 1994, 1996; Paczkowski et al., 1997, 1998, 2001). This enzyme, UDPGlc:solasodine GlcTase, was purified ca. 50-fold and thoroughly characterized. The enzyme was almost completely inactive towards typical phytosterols and displayed only very limited activity with the solanidane-type steroidal alkaloids such as solanidine or demissidine (Paczkowski et al., 1997). In this respect the GlcTase present in eggplant leaves differs from the enzyme isolated from potato as the latter enzyme is highly active with both solanidane- and spirosolane-type aglycones. The purified enzyme was highly specific for UDPGlc and its apparent molecular mass was ca. 55 kDa.

Recently, it was demonstrated (Nawloka et al., 2003) that diethylpyrocarbonate (DEPC), a specific modifier of histidine residues, as well as Woodward's Reagent K (WRK) which is known to react with dicarboxylic amino acids, were potent inhibitors of the eggplant GlcTase. However, a substantial protection of the enzyme activity against the inactivation by DEPC could be observed in the presence of excessive amounts of the enzyme substrates, i.e. either solasodine or UDP-glucose. A similar protective effect of UDP-glucose was found when the enzyme was treated with WRK. These results strongly imply that

histidine and dicarboxylic amino acid residues are essential for the catalytic function of the eggplant GlcTase and suggest the presence of the earlier mentioned PSPG-box in this enzyme.

It is noteworthy, that in experiments with crude enzyme extracts from eggplant seedlings, incubations with solasodine and UDPGal yielded 3-*O*-*β*-D-galactopyranoside which could be easily separated by TLC from the reaction product formed in the presence of UDPGlc, i.e. from solasodine 3-*O*-*β*-D-glucopyranoside (Paczkowski et al., 1994). These results indicate the presence in eggplant, similarly as it was found earlier in potato, of separate GlcTase and GalTase participating in the initiation of sugar chain formation of the main glycoalkaloids occurring in eggplant, i.e. solamargine and solasonine.

The occurrence of UDPGal:tomatidine GalTase was reported in tomato leaves and the reaction product was identified as tomatidine 3-*O*-*β*-D-galactopyranoside, a putative intermediate in the biosynthesis of α-tomatine (tomatidine tetraoside) which is the primary steroidal glycoalkaloid present in leaves and green fruits of tomato plants (Zimowski, 1994, 1996, 1998). This GalTase, with an apparent molecular mass of ca. 50 kDa, was purified ca. 150-fold by gel filtration and subsequent ion-exchange chromatography.

The tomato GalTase shares several common properties with the above discussed GlcTases isolated from potato and eggplant. All these enzymes exhibit the highest activities within an alkaline pH range and do not require divalent metal cofactors. The molecular weights of all these enzymes are also similar, ranging from 50 to 56 kDa. The substrate specificity reported for UDPGal:tomatidine GalTase from tomato leaves differs, however, from substrate specificities of the GTases occurring in potato or eggplant. Enzymes from tomato and eggplant are specific for spirosolane-type alkaloids while potato enzyme is highly active with both spirosolane-type and solanidane-type aglycones. On the other hand, the enzyme from tomato leaves utilizes UDPGal at several-fold higher rate than UDPGlc (Zimowski, 1998), in contrast to the partially purified enzymes from eggplant leaves or potato tubers which are completely unable to galactosylate steroidal alkaloids.

The activities of all above discussed GTases involved in the synthesis of steroidal glycoalkaloids (as well as earlier described enzymes participating in glycosylation of steroidal sapogenins – see the foregoing paragraph) are present mainly in fractions of soluble proteins obtained from crude homogenates by high-speed centrifugation. Therefore, all these GTases, like many other GTases involved in the formation of glycosides of various secondary plant metabolites, are generally regarded as soluble, i.e. cytosolic proteins. However, it can not be precluded that in fact they are components of multi-enzyme complexes loosely associated with proteins anchored to some unidentified membrane structures, e.g. endoplasmic reticulum. There is evidence that the above suggestion is true for at least some plant GTases containing PSPG-box (Burbulis and Winkel-Shirley, 1999; Tattersall et al., 2001).

Quite recently, McCue et al. (2004) reported the isolation of a novel gene, designated SOLtu:Sgt 3, encoding a rhamnosyltransferase involved in the final step in the biosynthesis of chacotriose and solatriose – trisaccharide sugar moieties present in the two major glycoalkaloids of potato. This enzyme evidently catalyzes rhamnosylation of disaccharide sugar chains of *β*-chaconine (solanidine D-rhamnosyl(α 1 → 4)*β*-D-glucoside) and *β*-solanine (solanidine D-glucosyl(*β*1 → 3)*β*-D-galactoside) at C-2 of the hexose moiety bound to the aglycone, with the formation of branched trisaccharide sugar moieties i.e. chacotriose or solatriose, present in α-chaconine and α-solanine, respectively. Although the antisense constructs of SOLtu:Sgt 3 gene introduced to potato plants did not lower total glycoalkaloid content, a severe reduction in the level of α-chaconine and α-solanine was observed with a concomitant accumulation of *β*-chaconine and *β*-solanine. These results clearly indicate that, most probably, a single rhamnosyltransferase is responsible for the addition of the final L-rhamnosyl moiety in the biosynthesis of sugar chains of both α-chaconine and α-solanine.

Are glycosyltransferases involved in the regulation of triterpenoid glycoside biosynthesis and accumulation?

Our knowledge of mechanisms involved in the regulation of triterpenoid glycoside formation is still rather obscure. It is generally assumed that enzymes involved in the formation of specific

triterpenoid aglycones are key enzymes on the biosynthetic routes leading to the synthesis of saponins. Indeed, several recently published studies seem to confirm this point of view.

It was reported (Suzuki et al., 2002) that methyl jasmonate induced accumulation of the oleanane-type triterpene saponins, i.e. oligosides of soyasapogenols A and B, hederagenin, bayogenin and medicagenic acid, in barrel medic (*Medicago truncatula*) cell suspension cultures. The accumulation of saponins was associated with strong and co-ordinate induction of transcripts encoding β-amyrin synthase, squalene synthase and one form of squalene monooxygenase. Similarly, exogenously applied methyl jasmonate greatly stimulated accumulation of soyasaponin B, a derivative of soyasapogenol B, in cultured cells of liquorice (Hayashi et al., 2003). The increased biosynthesis of soyasaponin was accompanied by up-regulation of mRNA level, as well as the enzyme activity, of β-amyrin synthase – an oxidosqualene cyclase situated at the branching point for the biosynthesis of oleanane-type sapogenins, e.g. soyasapogenol B. The results obtained by Henry et al. (1992) not only supplied evidence that β-amyrin cyclase is the rate-limiting enzyme in the biosynthesis of saponins in tall gypsophyll (*Gypsophila paniculata*) cell cultures but also demonstrated a potent feedback inhibition of this enzyme activity by gypsogenin 3-*O*-glucuronopyranoside – the first glycosylated product on the way to the biosynthesis of saponins present in gypsophyll. These authors speculated that gypsogenin 3-*O*-glucuronopyranoside can affect the synthesis of β-amyrin synthase on the transcription or the translation level.

However, there are also some indications that GTases taking part in the initiation of sugar chain biosynthesis may be of importance for the regulation of triterpene saponin biosynthesis. Herold and Henry (2001) found that, in excised root cultures of tall gypsophyll, total saponin content and the activity of UDPGlcUA:β-glycyrrhetinic acid GlcUATase showed a very close correlation during the different phases of *in vitro* root growth. This gave rise to the hypothesis of a regulatory function of the enzyme in saponin biosynthesis. As mentioned above, experiments performed by Hayashi et al. (2003) showed that, in cultured cells of liquorice, exogenously applied methyl jasmonate greatly stimulated accumulation of soyasaponin. The increased biosynthesis of soyasaponin was accompanied not only by up-regulation of β-amyrin synthase but also by a high increase of the activity of glucuronosyltransferase catalyzing the formation of soyasapogenol B 3-*O*-β-D-glucuronopyranoside. These results confirm a possibility that early glycosylation events can be involved in the regulation of soyasaponin biosynthesis.

As mentioned earlier steroidal sapogenins and alkaloids are synthesized from cholesterol and it seems most likely that the level of cholesterol, which is usually present in plant cells in much lower amount than C-24 alkylated sterols, may be of particular importance for regulation of the formation of steroidal aglycones. Indeed, it was shown (Arnqvist et al., 2003) that transgenic potato plants overexpressing a soybean sterol C-24 methyltransferase cDNA had substantially decreased level of free cholesterol, presumably due to highly increased alkylation of cycloartenol which is a common precursor of cholesterol and 24-alkylated, C_{29} sterols, such as sitosterol and isofucosterol. Associated with this was a significant decrease of glycoalkaloid level in leaves and tubers, as compared to the wild-type potato plants. These results not only support the view of cholesterol as a precursor of steroidal glycoalkaloids but also clearly suggest that the rate of cholesterol production may control the rate of glycoalkaloid biosynthesis in potato plants.

Similarly as in the case of triterpene saponin biosynthesis, there are, however, reasons to believe, that early glycosylation steps may also be of importance in regulation of glycoalkaloid biosynthesis. Injury-induced synthesis and accumulation of α-solanine and α-chaconine in potato tubers is a well-known phenomenon (Friedman and McDonald, 1997, 1999). Bergenstrahle et al. (1992b) reported that the level of glycoalkaloids (α-chaconine and α-solanine) in sliced potato tubers started to increase after 24 h of incubation. This was due to *de novo* synthesis of glycoalkaloids since the accumulation of these glycosides was inhibited by a sterol synthesis inhibitor – tridemorph. Concomitant to the accumulation of glycoalkaloids, there was an increase in the activity of UDP-Glc:solanidine GlcTase, suggesting a possible regulatory function of the enzyme in glycoalkaloid biosynthesis. It was also found (Moehs et al., 1997; Rockhold et al., 2000) that mRNA encoding UDPGlc:solanidine GlcTase was

strongly induced in wounded potato tubers. These results seem to confirm earlier suggestions (Bergenstrahle et al., 1992b) that the reaction catalyzed by UDP-glucose:solanidine GlcTase, i.e. the synthesis of γ-chaconine, is a rate limiting step in the biosynthesis of α-chaconine – one of two main glycoalkaloids synthesized in potato. Therefore, it was postulated that down-regulation of UDPGlc:solanidine GlcTase by the technique of antisense RNA, can be used to reduce the level of toxic glycoalkaloids in potato plants (Moehs et al., 1999). In fact, recently published results (McCue et al., 2003) demonstrated that transgenic lines of potato expressing antisense UDPGlc:solanidine GlcTase constructs exhibited reproducible and statistically significant reductions (up to 40%) of glycoalkaloid content in potato tubers. Reduction in total glycoalkaloid content was accompanied by expression of UDPGlc:solanidine GlcTase antisense RNA transcripts and reduction in the enzyme protein level.

Conclusions and perspectives for future research

Substantial progress has been achieved over past two decades in the identification and biochemical characterization of GTases involved in the biosynthesis of saponins and glycoalkaloids. About 20 enzymes most probably taking part in the formation or rearrangements of sugar moieties in these compounds have been isolated from various plant species and more or less thoroughly characterized. On the other hand, very little is known about the genes involved in sugar chains synthesis and about the control of these genes.

Until quite lately almost all information on enzymes participating in the formation and modifications of sugar moieties in saponins and glycoalkaloids based on results obtained with the use of typical biochemical approaches. A typical biochemical approach involved: (a) demonstration of a particular chemical reaction in cell-free extract obtained from a plant tissue; (b) identification of the enzyme that catalyzed this reaction; (c) purification of the enzyme – if at all possible to homogeneity; (d) full characterization of the enzyme (e.g. its substrate specificity, cofactor requirement etc.); (e) sequencing of the enzyme protein, and, finally, (f) characterization of its three dimensional and subunit structure. However, due to low levels of enzymes involved in production of secondary metabolites in plant tissues and their unusually low stability, the isolation and purification of the target enzyme was an often very difficult, time-consuming and, in many cases, unsuccessful endeavor.

Functional genomics, i.e. the study of gene function on a large scale, is an alternative and very promising approach to the elucidation of chemical reactions taking part in the secondary plant product formation. Increasing availability of plant gene sequences or even complete plant genomes as well as dramatic improvements in DNA-sequencing technology facilitate more rapid identification and characterization of the enzymes involved in the secondary plant metabolism. Large scale and low-cost sequencing of cDNAs prepared from plant tissues with specific metabolic activities provides an alternative route toward the understanding of the metabolic pathways leading to various secondary plant products. Especially promising in this respect is expressed sequence tags (ESTs) analysis (Ohlrogge and Benning, 2000). Particularly successful examples of ESTs analysis in the area of secondary products biosynthesis include the isolation of genes encoding enzymes responsible for the biosynthesis of unusual fatty acids – such as for example ricinoleic acid (12-hydroxyoleic acid) synthesized in the endosperm of castor bean (*Ricinus communis*) seeds (Vande Loo et al., 1995). The ESTs analysis involves (a) isolation of mRNA from a particular tissue which is known to produce substantial amounts of a given secondary product; (b) preparation of a cDNA library with the use of reverse transcriptase; (c) sequencing of 200–500 nucleotides at both the 5′ and 3′ ends of each cDNA; (d) *in silico* analysis of available databases containing collections of gene and ESTs sequences looking for specific domains expected for a gene of interest; (e) based on the *in silico* data, identification of candidate genes among cDNAs cloned; (f) heterologous expression of full-length cDNAs; (g) testing the function of recombinant enzyme protein(s), i.e. its(their) ability to catalyze the chemical reaction under study; (h) examination of properties of these enzymes, e.g. their specificity.

Applying the above described ESTs analysis Jung et al. (2003) reported identification in ginseng (*Panax ginseng*) of four 2,3-oxidosqualene cyclase candidates, nine cytochrome P450-dependent

monooxygenase candidates and twelve GTase candidates, which may be involved in the synthesis of triterpene saponins of dammarane- and oleanane-type present in this plant. Among a total of 42 GTase candidates identified in ginseng, 12 ESTs contained the PSPG consensus sequence that is found in various plant enzymes involved in glycosylation of plant secondary metabolites. Cloning of full-length cDNA molecules (most cDNA candidates were isolated truncated at the 5′-ends) and their heterologous expression will enable functional characterization of these GTases (Choi et al., 2005).

Barrel medic (*Medicago truncatula*) is the model legume plant for genomics studies. The sequencing of its genome is far advanced. This plant is very rich in saponins, the aglycones of which are derivatives of β-amyrin, i.e. soyasapogenols B and E, medicagenic acid, hederagenin and bayogenin. There are preliminary reports (Achnine et al., 2003, 2004) that among 164 cDNA clones isolated from this plant, encoding putative GTases, 33 candidates were up-regulated in response to methyl jasmonate and 14 of them exhibited co-ordinated expression with β-amyrin synthase suggesting their possible involvement in saponin biosynthesis.

Oat is another interesting model plant for studies on biosynthesis of sugar moieties in saponins (Jenner et al., 2005). Besides steroidal saponins, i.e. avenacosides, which are produced in oat leaves, root epidermal cells synthesize triterpene saponins with β-amyrin skeleton – so-called avenacins, which seem to play a key role in resistance to fungal attacks (Osbourn, 2003). A number of saponin-deficient (*sad*) mutants of the diploid oat species *Avena strigosa* were generated by treatment with the chemical mutagen – sodium azide (Trojanowska et al., 2001). Some of these mutants are defective in glycosylation of avenacin aglycone indicating possible defects in GTase activities. For example, the *sad* 3 mutant produces mono-desglucoavenacin A-1 suggesting that there is a mutation affecting GlcTase needed to convert mono-desglucoavenacin A-1 into the biologically active avenacin A-1 (Qi et al., 2004). Analysis of these *sad* mutants using functional genomics and targeting metabolomics approaches will enable characterization of GTases that are required for the synthesis of avenacins. The identification of candidate avenacin GTase genes from oat root-tip ESTs resource has been already reported (Townsend and Jenner, 2003).

Note added in proof: During the final editing of this article, several papers have been published of great importance for the reviewed field. Using a genomics approach which involved metabolite profiling, ESTs profiling and DNA array analyses, Achnine et al. (2005) identified, in methyl jasmonate-induced cell cultures of barrel medic, two UDPGlc-dependent GTases acting on triterpene aglycones, denoted UGT73K1 and UGT71G1. Functional characterization of the recombinant proteins revealed that the former enzyme was specific for hederagenin and soyasapogenols B and E while the latter one was specific for medicagenic acid. UGT73K1 and UGT71G1 are the first cloned GTases involved in the biosynthesis of triterpene saponins. Kohara et al. (2005) reported the cloning, from *Solanum aculeatissimum*, of cDNA encoding a GlcTase (SaGT4A), most probably involved in the initiation of sugar chains biosynthesis in aculeatisides A and B, nuatigenin-derived steroidal saponins synthesized in this plant. Recombinant enzyme expressed in *E. coli* was highly specific for UDPGlc but showed rather broad specificity with respect to the steroid acceptor. It glucosylated, apart from nuatigenin, some other steroidal sapogenins, such as diosgenin and tigogenin as well as some steroidal alkaloids, such as solanidine, solasodine and tomatidine. McCue et al. (2005) reinvestigated substrate specificity of the recombinant solanidine glycosylating enzyme from potato (SGT1) with respect to the sugar donor. They found that this enzyme was many-fold more active with UDPGal than with UDPGlc. It means that SGT1 is not, as previously believed (Moehs et al., 1997), solanidine GlcTase involved in the synthesis of α-chaconine, but rather it is solanidine GalTase involved in the formation of α-solanine. The *in vivo* role of SGT1 as UDP-Gal:solanidine GalTase was confirmed by experiments with transgenic potato lines containing antisense *SGT1* constructs. In these potato lines an almost complete inhibition of α-solanine synthesis was observed and this inhibition was compensated by an elevated accumulation of α-chaconine. It is noteworthy that the activity of recombinant SGT1 was strongly inhibited *in vitro* by the addition of either α-solanine or α-chaconine suggesting that the accumulation of final products of the glycoalkaloid biosynthetic pathway may be regulated by

feedback inhibition at the primary glycosylation step (McCue et al., 2005).

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