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Tissue and subcellular localization of oligofurostanosides and their specific degrading β-glucosidase in *Dioscorea caucasica* Lipsky

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Abstract

The application of the histochemical technique using the Ehrlich reagent showed that the oligofurostanosides—the steroid glycosides of the furostan series—are localized in idioblasts (special cells—receptacles) of the epidermal layer of *Dioscorea caucasica* leaves. The activity of oligofurostanoside-specific β-glucosidase was localized mainly in the membrane fraction of the thylakoides and was also found in the *Dioscorea caucasica* leaves. We have suggested, that the differential tissue, the subcellular localization of the oligofurostanosides and their degrading enzymes provide the maintenance of the furostanol structure of steroid glycosides which is necessary for their transport within the leaf and from the leaf to the rhizome.

Keywords: *Dioscorea caucasica* Lipsky; *Dioscoreaceae*; Steroid glycoside; Oligofurostanoside-specific β-glucosidase; Aryl-β-glucosidase; Oligofurostanoside; Thylakoide; Localization

1. Introduction

The sterol glycosides—the spirostanol and furostanol derivatives—form the group of chemical compounds known as the saponins. These compounds are characterized by the haemolytic, oncostatic, fungicidal and antibacterial properties (Lacaille-Dubois and Wagner, 2000) as well as the cytotoxic (Yokosuka et al., 2002) immunoregulatory (Forse et al., 1995), antiviral (Arthan et al., 2002) and other types of biological activity. The oligofurostanosides contain a sugar chain at the C-3 position in addition to another glucose moiety at the C-26 position. Upon cell damage, they get immediately transformed into more aggressive antifungal active spirostanol glycosides, (Tschesche and Wulf, 1973). This process can be catalyzed by the β-glucosidases derived from various sources including the endogenic β-glucosidases (Kesselmeier and Urban, 1983; Grunweller and Kesselmeier, 1985). The existence of a significant amount of the oligofurostanosides in the *Dioscorea caucasica* leaves (approx. 1%) confirms that these glycosides and their decomposing enzymes get dissociated in the tissues or in the leaf cells. This assumption verifies the fact that when the integrity of the leaves is disturbed, a complete transformation of oligofurostanosides into oligospirostanosides occurs. The main purpose of our study was to investigate the localization of the oligofurostanosides and theirs degrading enzyme β-glucosidase in the *D. caucasica* leaves.

2. Results and discussion

Even though our knowledge of the biosynthetic pathways is rather extensive, the information regarding the conditions required to accumulate and store the steroid glycosides is comparably scarce. The research on the localization was delayed due to the lack of sensitive biochemical methods that would allow the detection of these compounds in the sections of different plant organs.

The colour reaction using the Ehrlich reagent was applied in order to detect the oligofurostanosides in the leave sections. The furostanolic glycosides gave the stable bright-crimson coloration for several hours while the spirostanolic glycosides have not developed any staining.

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The cross section of the leaves (Fig. 1) shows the accumulation of the oligofurostanosides in the special epidermal cells of *Dioscorea caucasica* leaves located at the bottom and top epidermis (Fig. 2). Apparently, these cells may be considered the idioblasts or the cell-receptacles of various compounds formed as a result of specific metabolism in plants. A histochemical study has not identified the oligofurostanosides in the mesophillous tissues of the leaf, which provides the evidence of their active transport (against the concentration gradient) from the mesophyll (where they are synthesized) to the epidermal layer where they are accumulated.

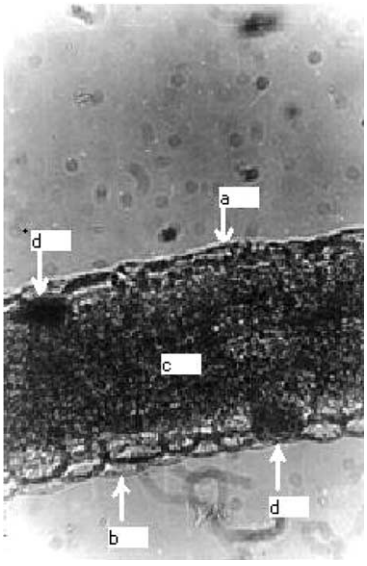

Fig. 1. Cross section of *Dioscorea caucasica* leaf. (a) the top epidermis; (b) the bottom epidermis; (c) mesophyll; (d) staining with the Ehrlich reagent, the blackened areas represent localization of oligfurostanosides. 7×40 magnification.

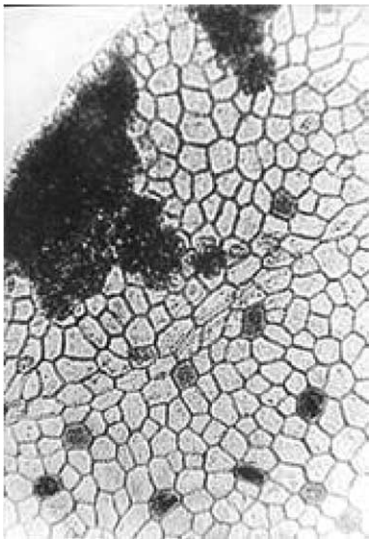

Fig. 2. The bottom epidermis of *Dioscorea caucasica*. Staining with the Ehrlich reagent, localization of oligofurostanosides is indicated by the black spotted areas. 7×40 magnification.

The distribution of the secondary plant products has been studied by means of the histochemical methods. An epidermal localization has been reported for many compounds, e.g. quinolizidine alkaloids, phenolics, steroid alkaloids and cyanogenic glycosides (Wink, 1984; Molisch, 1961). It is to be noted that the occurrence of many toxins in the epidermal tissues was interpreted in terms of a chemical defense barrier.

When the differential centrifugation of homogenates from young leaves of *Dioscorea caucasica* was applied, over a half of the whole activity of the deltoside—the oligofurostanoside glycoside degrading enzyme was detected in the fraction enriched with chloroplasts (the sediment at centrifugation with 5000 *g*). Respectively, 0.8 and 0.2% of the activities of the given enzyme were found in the fractions enriched with mitochondrias and microsomes. As for the water-soluble protein fraction, the oligofurostanoside specific β-glucosidase activity has not been observed. (Table 1).

A fraction of thylakoid membranes was isolated by means of centrifugation in the density gradient of sucrose of the chloroplast suspension destroyed by the osmotic shock. The crude thylakoid membranes obtained as a result of differential centrifugation of the cell homogenate, were loaded on a 0.5 and 1.5 M sucrose density gradient. The yellowish-brown bands were found accumulated at the interface between the 0.5 M sucrose and the sample loading zone (Fig. 3).

The main part of the activity was detected directly in this fraction. The upper layer containing the soluble proteins of chloroplast shows no activity of deltoside degrading enzyme (Table 2).

The treatment of the thylakoid membranes with acetone, 1% triton solution detergents and 1% of twin solution does not cause the solubilization of this enzyme. All the applied methods of solubilization have

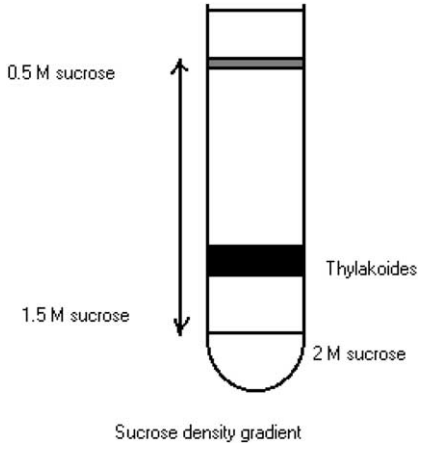

Fig. 3. Sucrose density gradient centrifugation profile of thylakoid membranes. Crude thylakoid membranes were loaded on a 0.5–1.5 M sucrose linear density gradient with 2 M sucrose cushion in a buffer containing 20 mM Tris–HCI (pH 7.5), 2 mM EDTA and centrifuged at 100 000 *g* for 2 h in a SW 41 rotor at 4 °C.

Table 1
Differential centrifugation of homogenates from young leaves of *Dioscorea caucasica*

Fraction	Protein (mg)	Aryl β-glucosidase		Furostanoside specific β-glucosidase	
		Cleavage of 4-nitrophenyl-β-D-glucopyranoside		Cleavage of deltoside	
		Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})	Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min)
Crude extract	489.0	2346.0	4.7	536.5	1.09
Fraction of chloroplasts (pellets at 1000 *g*)	35.0	13.6	0.38	3512.3	100.3
Fraction of mitochondrias (pellets at 40 000 *g*)	3.6	0	0	28.1	7.8
Fraction of microsomes (pellets at 100 000 *g*)	4.1	0	0	7.02	1.71
Water-soluble proteins (supernatante at 100 000 *g*)	428.2	2300.8	5.37	0	0

Table 2
Centrifugation of sucrose of the chloroplast suspension in the density gradient

Fraction	Protein (mg)	Aryl-β-glucosidase		Furostanoside specific β-glucosidase	
		Cleavage of 4-nitrophenyl-β-D-glucopyranoside		Cleavage of deltoside	
		Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})	Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})
Fraction of chloroplasts	35.0	13.6	0.38	3512.3	100.3
Crude thylakoid membranes	1.9	0	0	3506.2	1845.3
Water-soluble proteines of chloroplasts	32.4	12.9	0.39	0	0

visibly inactivated the enzyme. The above mentioned data indicate that the oligofurostanoside-specific β-glucosidase is located in the mesophyll of a leaf where the chloroplasts are concentrated. At the same time, the substrate of this enzyme is accumulated in the special cells—the so-called idioblasts which are located in the epidermal tissues. However, the oligofurostanoside-specific β-glucosidase was not found there. This fact points to a different compartmentation of oligofurostanosides and their degrading glucosidase in the leaves of *D. caucasica*.

The β-glucosidase, with its high substrate specificity, has been described in many plants (Hösel end Conn, 1982) and the existence of multiple forms of glucosidases has been documented (Dey and Del Campilo, 1984). It has been identified that the oat leaves contain β-glucosidase (avenacosidase) specific to the cleavage of the C-26 bound glucose moiety of the oat saponins avenacosides A and B. This transformation activates the fungitoxicities of the avenacosides. It is apparent that this enzyme is bound to the tonoplast membrane (Grunweller and Kesselmeier, 1985).

It should also be noted that the enzymes belonging to glycohydrolases can be located in different compartments of the plant cells. The dhurrin-specific β-glucosidase participating in the degradation of the lipopolysaccharides was found in chloroplasts of the sorghum leaves whereas the β-galactosidase in chloroplasts of the wheat leaves. The β-glucosidase in corn roots are the soluble enzyme located in the vacuoles or in the cytoplasm (Thayer and Conn, 1981; Bhallia and Dalling, 1984). The activity of β-glucosidase degrading ocinamic acids has been detected in the extracytoplasmatic space of the *Melilotus alba* leaves (Oba et al., 1981).

These data confirm that the subcellular localization of the glucosidases may change depending on the specificity (kinds of substrates) and also on the plants species. It may be closely related to their functions in a plant cell.

Summarizing of the present experimental data leads us to a conclusion that the intensive biosynthesis of the steroid glycosides in the *Dioscorea caucasica* leaves occurring from the products of photosynthetic transformation of CO_2 in the mesophyll tissues is accompanied by a fast outflow of these physiological active compounds in the special cells—idioblasts located in the epidermis of the sides of the leaves. A different intertissue localization of the oligofurostanosides and their degrading glucosidases provides the maintenance of the furostanolic structure necessary for the transport of the steroid glycosides not only inside the leaf, but also in

the phloem juice from the leaf to the rhizome where the oligofurostanosides partially get transformed to oligospirostanosides having a significant fungicidal and antibacterial activity. This protects rhizomes from various pathogenic microorganisms.

3. Experimental

3.1. Plant material

Dioscorea caucasica Lipsky was collected in the city of Tbilisi (Republic of Georgia) on a hill located to the west of the city center, Institute of Botany Botanical Garden. NY Specimen ID: 197411.

3.2. Histochemical studies

Histochemical detection of the oligospirostanosides in the leaves and other sections of *D. caucasica* has been carried out using the Ehrlich reagent modified by us in the following preparation: 100 ml mixture of CH_3OH-HCl conc. at a 66:34 ratio. The reagent was dropped on the leaf section placed on the slide, slightly heated for 1 min and analyzed by means of a light microscope (Zeiss photomicroscope).

3.3. Cell fractionation

Dioscorea caucasica leaves were homogenized in an isotonic medium according to Grunweller and Kesselmeier (1985). The filtered homogenate was fractioned by centrifugation at 1000 *g* (10 min), 40 000 *g* (20 min) and 10 000 *g* (3 h). The sediments 1–3 were carefully resuspended in a few ml of 0.05 M Tris–HCl (pH 6.8) and tested for β-glucosidase activity. A β-glucosidase supernatant was precipitated with NH_4SO_4 at 100 000 *g* by 80% of saturation; the precipitated proteins were redissolved in the same buffer and tested for β-glucosidase activity.

3.4. Thylakoid membrane isolation

Thylakoid membranes were isolated by the sucrose density gradient centrifugation according to Tanaka and Melis (1997). The cells were harvested by centrifugation at 1500 *g* for 10 min. Pellets were suspended in a buffer containing 20 mM Tris–HCl (pH 8.0), 1 mM aminocaproic acid, 1 mM aminobenzamidine, 0.2% polyvinylpyrrolidone and 15% (w/v) glycerol and centrifuged at 1500 *g* for 3 min. The pellet was resuspended in the same buffer, except that 1.5% of glycerol was used instead of 15%, which was centrifuged at 10 000 *g* for 10 min. The pellet was resuspended in the buffer containing 20 mM Tris–HCl (pH 7.5) 2 mM EDTA and 0.6 M sucrose using a tight fitting glass homogenizer. The homogenate was diluted with 2 volumes of 20 mM Tris–HCl (pH 7.5), 2 mM EDTA and loaded on a 0.5–1.5 M sucrose line density gradient with 2 M sucrose cushion in a buffer containing 20 mM Tris-HCL, 2 mM EDTA and centrifuged at 10 000 *g* for 2 h with an SW 41 motor at 4 °C. The green land was collected, diluted with the same buffer and centrifuged at 100 000 *g* for 1 h. The pellets (purified thylakoids) were used for the measurements given below.

The thylakoid membranes were solubilized in a buffer containing 0.05 M Tris–HCl (pH 6.8) 3.5% SDS and 10% glycerol. Then the membranes were separated by centrifugation during 30 min at 20 000 *g*. A deposit was washed out by the same buffer (but without a detergent) followed by a subsequent centrifugation; the activity of β-glucosidase in the supernatant and a deposit after its dissolution in the same buffer was determined.

3.5. Enzyme assay

The activity of the oligospirostanoside-specific β-glucosidase was determined by using the deltoside as the substrate that was isolated by the techniques described earlier in this paper (Gurielidze et al., 1986). The incubative mixture composed of 0.1 ml non-cellular extract added to 0.2 ml 1 mM solution of deltoside in the 0.05 M Tris–HCL buffer (pH 6.8) was incubated at 37 °C for 10 min. The deltoside amount was determined by the color reaction of 1% *p*-dimethylamino benzaldehyde solution in CH_3OH–HCl conc. mixture. To this end, the incubative mixture was thoroughly evaporated and 0.2 ml Ehrlich reagent was added to the sediment. The mixture was kept for 3 min at 60 °C, 3.8 ml ethanol were added and the crimson color was measured with the spectrophotometer SF-4A at 520 nm.

4-Nitrophenyl-β-D-glucopyranoside cleavage was measured according to Hosel and Barz (1975).

The activity of the enzyme catalyzing the substrate decomposition was accepted as 1 nmol min^{-1} activity unit. The specific activity of the enzyme was expressed in the 1 nmol min^{-1} mg^{-1}.

3.6. Protein determination

Protein was measured according to Schaffner and Weismann (1973).

References

Arthan, D., Svasti, J., Kittakoop, P., Pittayakhachonwut, D., Tanticharoen, M., Yodhathai Thebtaranonth, Y., 2002. Antiviral isoflavonoid sulfate and steroidal glycosides from the fruits of *Solanum torvum*. Phytochemistry 59, 459–463.

Bhallia, P., Dalling, M., 1984. Characteristics of β-glucosidase associated with the stroma of chloroplasts prepared from mesophyll protoplasts of the primary leaf of wheat. Plant Physiology 76, 92–98.

Dey, P.M., Del Campilo, E., 1984. Biochemistry of the multiple forms of glucosidases in plants. In: Meister, A. (Ed.), Advances in Enzymology and Related Areas of Molecular Biology, Vol. 56. Wiley-Interscience, New York, pp. 141–249.

Forse, R., Armour, L., Chavali, S., 1995. Enteral formulations for treatment of inflammation and infection. U.S. Patent 5,397,778. (March 14). (BIO# 11014).

Grunweller, S., Kesselmeier, J., 1985. Characterization of a membrane bound β-glucosidase responsible for the activation of oat leaf saponins. Phytochemistry 24, 1941–1943.

Gurielidze, K.G., Paseschnichenko, B.A., Vasilieva, I.S., 1986. Determination of furostanolic saponins in plants. Prikl. Biokhim. Mikrobiol. 22, 131–136 (in Russian).

Hosel, W., Barz, W., 1975. β-Glucosidases from Cicer arietinum L. purification and properties of isoflavone-7-*O*-glucoside-specific β-glucosidases. European Journal of Biochemistry 57, 607–616.

Hösel, W., Conn, E.E., 1982. The aglycone specificity of plant β-glycosidases. Trends Biochem. Sci. 7, 219–221.

Kesselmeier, J., Urban, B., 1983. Subcellular localization of saponins in green and etiolated leaves and in green protoplasts of oat (*Avena sativa* L.). Protoplasma 114, 133–140.

Lacaille-Dubois, M.A., Wagner, H., 2000. Bioactive saponins from plants. In: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry Series. Elsevier Science, pp. 633–687.

Molisch, H., 1961. Anatomie der Pflanze, 7. neubearb. Auflage. Karl Höfler, Jena.

Oba, K., Conn, E.E., Canut, H., Boudet, A.M., 1981. Subcellular localization of 2-(β-D-glucosyloxy)-cinnamic acids and related β-glucosidase in leaves of *Melilotus alba*. Plant Physiology 68, 1359–1366.

Schaffner, W., Weismann, C.A., 1973. Rapid, sensitive, and specific method for the determination of protein in dilute solution. Analytical Biochemistry 56, 502.

Tanaka, A., Melis, A.A., 1997. Irradiance-dependent changes in the size and composition of the chlorophyll a–b light-harvesting complex in the green alga *Dunaliella salina*. Plant Cell Physiology 38, 17–24.

Thayer, S., Conn, E., 1981. Subcellular localisation of dhurrin-β-glycosidase and hydroxynitrile lyase in the mesophyll cells of sorghum leaf blades. Plant Physiology 67, 617–622.

Tschesche, R., Wulf, G., 1973. Chemie und Biologie der Saponine. Fortschr. Chem. Org. Naturstoffe 30, 461–473.

Wink, M., 1984. Chemical defense of lupins. Mollusk-repellent properties of quinolizidine alkaloids. Z. Naturforsch. C, Biosci 39, 553–558.

Yokosuka, A., Mimaki, Y., Sashida, Y., 2002. Spirostanol saponins from the rhizomes of *Tacca chantrieri* and their cytotoxic activity. Phytochemistry 61, 73–78.