PARTIAL STRUCTURE ELUCIDATION OF THE CARBOHYDRATE MOIETY OF 1,25–DIHYDROXYCHOLECALCIFEROL GLYCOSIDE ISOLATED FROM *Solanum glaucophyllum* LEAVES[1] /

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Resumen

La cromatografía de intercambio iónico de los complejos de boratos de los carbohidratos presentes en las hojas Solanum glaucophyllum *permitió el aislamiento del glicósido 1,25-dihydroxycholecalciferol. El esterol estaba unido a una serie de fructo-glucósidos. La investigación de su estructura fina mediante oxidación ácida peryódica demostró que la fructosa estaba enlazada a una unidad de disacárido* [Glc_p *α 1-2 Glc*] *repetida 1, 2 ó 4 veces.*

La aglicona estaba unida al extremo reductor del sacárido.

Introduction

Solanum glaucophyllum (conspecific with *S. malacoxylon* and *S. glaucum*) is a shrub growing in Argentina, Brazil and Uruguay, common in areas of low-lying poorly drained lands. The leaves of this plant contain the glycoside of 1,25-dihydroxycholecalciferol (8, 12, 23). Chronic ingestion of the leaves by cattle produces a vitamin-D intoxication state called "Enteque Seco" (24). The biological activity of the leaves appears to be influenced by geographical location or climatic conditions and by the development of the plant (it is higher in vegetative than in reproductive state, 18). Although no physiological function has been assigned to the glycoside or the aglycone, some experiments suggest that the latter may have rhizogenic activity (4). This paper reports a method for the isolation of the glycoside and details on the fine structure of its carbohydrate moiety.

Material and methods

S. glaucophyllum leaves were dessicated at 60°C for 12 hours and ground with a hand driven steel mill. The powder was sieved through a No. 140 net (U.S. Standard Sieve Series) to obtain a very fine powder of mesophile. The latter was extracted with chloroform in a Soxhlet apparatus for 16-24 hours, losing 9% of its weight and leaving a cream-colored powder, which was extracted with water at 37-40°C for 5 minutes (one gram of powder with five ml of water) and filtered with suction. The acqueous extract contained 85% of soluble carbohydrates; it was stored at −20°C.

The hexose content of the acqueous extract or chromatographic fractions was measured after acid hydrolysis: the aliquot was made 0.1 N in HCl and heated for 15 minutes in a boiling water bath.

Fructose and glucose were measured according to Roe (20) and Washko and Rice (22), respectively. Reducing sugars were measured with the Nelson (14) reagent. Paper chromatography of fructo-glucosans was carried out on Whatman paper No. 1

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with butanol-pyridine-water (4:3:4) (11). Location of spots was performed with the alkaline silver reagent (15).

Ion exchange chromatography of carbohydrates. Aliquots of the acqueous extract containing 15 mg of hexoses were mixed with 1 ml of 50 mM sodium tetraborate, adjusted to pH 8.6 (glass electrode) and diluted with 10 ml of distilled water. A flocculent precipitate was separated by centrifugation.

Chromatography of sugar-borate complexes was carried out according to Khym and Zill (10). Amberlite CG-400 (100-200 mesh) was slurried into the column (lx23 cm) and successively washed with 200 ml of N HCl, 300 ml of 100 mM sodium tetraborate (pH 8.6) and 50 ml of distilled water. After the column had been converted to the borate form, it was equilibrated with 50 ml of 5 mM tetraborate. The carbohydrate solution was passed through the column followed by 250, 150 and 150 ml of 5, 15 and 30 mM sodium tetraborate solutions, respectively. Fractions of 10 ml were collected while the UV absorption of the effluent was continuously recorded. The spectrum of selected fractions was determined with a Beckman DU-2 spectrophotometer.

The dihydroxycholecalciferol content of chromatographic fractions was determined extracting the sterol after pH 6 hydrolysis (see below) and measuring the absorbancy at 264 nm.

Stability of 1,25-dihydroxycholecalciferol glycoside and effect of enzymes. Aliquots of the pooled chromatographic fractions containing the glycoside were adjusted to pH 4, 5 or 6 and incubated at 30°C for 24 hours under nitrogen. A drop of 2% sodium azide was added to prevent bacterial growth. After incubation pH was adjusted to 8.6 and rechromatographed as indicated above, or extracted with organic solvents (8, 12, 23). In other experiments, forty units of α-amylase (Miles) or β-glucosidase (Sigma) were added to pooled chromatographic fractions containing ca. 20 μmoles of total hexoses, adjusted to pH 6 or 5, respectively. After 3 hours of incubation at 37°C, pH was readjusted to 8.6, chromatographed as indicated above.

Analysis of fructoglucosans structure. Oxidation of chromatographic fractions containing the nonsaccharide, with periodic acid, proceeded to completion at pH 4, 4°C in 16 hours. Periodate consumption (7), formate (1) and formaldehyde (13) production were measured. The product of periodate oxidation was reduced with sodium borohydride and hydrolyzed (2). The neutralized solution was deionized by passage through a mixed bed of ion exchange resins. The eluate was made 5 mM in sodium tetraborate, adjusted to pH 8.6 and chromatographed in the same column described above. Analysis of fractions was carried out measuring formaldehyde (13) produced by periodate oxidation, against suitable standards.

Biological activity of 1,25-dihydroxycholecalciferol glycoside. The ability to increase plasma phosphorus when administered to rats (5, 18) was employed to confirm the presence of the glycoside. Pooled chromatographic fractions (equivalent to 0.7 g of dried leaves) were freed of tetraborate (25) and injected intraperitoneally to each of five rats. Plasma phosphorus (6) was measured before and 6 hours after injection.

Results and discussion

Ion exchange chromatography of soluble carbohydrates present in the leaves of *S. glaucophyllum* revealed the presence of sucrose, a series of fructoglucosides, fructose and glucose (Figure 1). The first peak was identified as sucrose because: a) it is eluted in the position of authentic sucrose, b) equimolar amounts of glucose and fructose could be measured after acid hydrolysis, c) it gave a negative test for reducing sugars and d) the rate constant for hydrolysis in N HCl at 37°C (0.020 min^{-1}) was not significantly different from that obtained with the standard (0.019 min^{-1}). Some unknown substance with undefined UV spectrum was eluted simultaneously.

Inspection of analytical data of the second peak (Table 1) indicated it was composed by a series of fructoglucosides with glucose:fructose ratios 8:1, 4:1 and 2:1 (nona–, penta– and trisaccharide). Paper

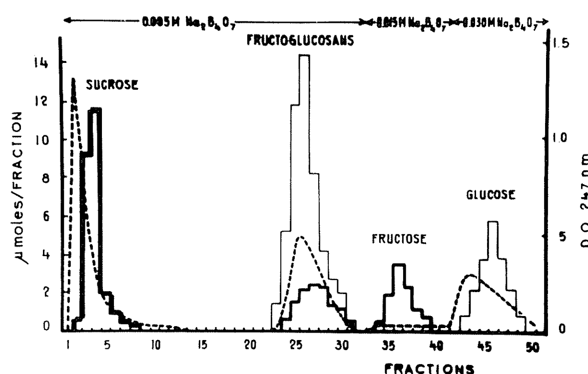

Fig. 1. Chromatographic pattern of soluble carbohydrates of *S. glaucophyllum* leaves, eluted as borate complexes from an Amberlite CG-400 column. Fructose or glucose content of fractions is represented by a thick or thin line, respectively. The dashed line indicates the UV absorbance.

Table 1. Analytical data of fructoglucosides isolated by ion exchange chromatography.

Fraction No.	Glucose μmoles	Fructose μmoles	Glucose Fructose	DHCC μmoles	Reducing sugars μmoles*
23	1.39	0.18	7.6	0.15	negative
24	5.20	0.60	8.6	0.52	negative
25	11.79	1.52	7.8	0.85	0.23
26	14.40	2.25	6.4	0.79	1.60
27	8.32	2.42	3.4	0.45	1.90
28	4.16	2.35	1.8	0.14	2.60
29	2.77	1.36	2.0	0.07	1.20
30	2.09	1.10	1.9	none	1.30

* Standard = glucose

chromatography of pooled fractions confirmed the presence of three oligosaccharides (Figure 2). The UV spectrum of these fractions showed a maximum absorbance at 282 nm, minimum at 225 nm (Figure 3). Biological activity of these fractions was confirmed by bioassay. Pooled chromatographic fractions equivalent to 0.7 g of dry leaves per rat increased plasma phosphorus 6 hours after intraperitoneal injection (Plasma P increase 1.40 ± 0.1 mg/dl, n = 5, $P < 0.05$).

Incubation of second peak fractions at pH 4, 5 or 6 for 24 hours produced some hydrolysis of the fructoglucosans (30, 29 and 27% , respectively) and complete hydrolysis of the linkage with the aglycone (no UV absorption could be detected in eluates, upon rechromatography of pH 4-6 incubates). Crystalline 1,25-dihydroxycholecalciferol in 5 mM tetraborate reacted with the resin and could be eluted with N HCl; the UV spectrum of the eluate revealed two maxima (225 and 276 nm) and two minima (210 and 255 nm) indicative of modification of the steroid by passage through the column when not bound to carbohydrates[1]. Extraction of the acid incubates with organic solvents gave, without further purification, the typical spectrum of the aglycone (chloroform, maximum 264 nm, minimum 228 nm).

After incubation with α-amylase, 49% of the fructoglucosans were hydrolyzed. The remaining oligosaccharides were eluted from the column

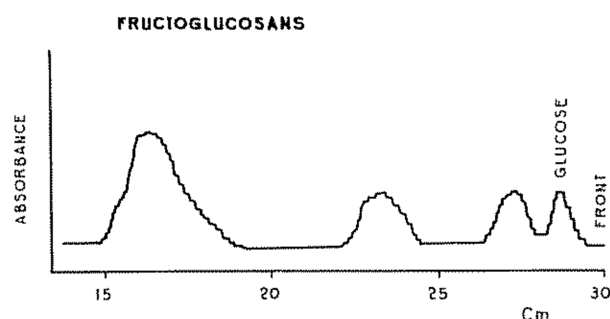

Fig. 2 Densitometric tracing of a paper chromatogram of fructoglucosans previously isolated by ion exchange chromatography. From left to right: nona–, penta– and trisaccharide. Standard: glucose

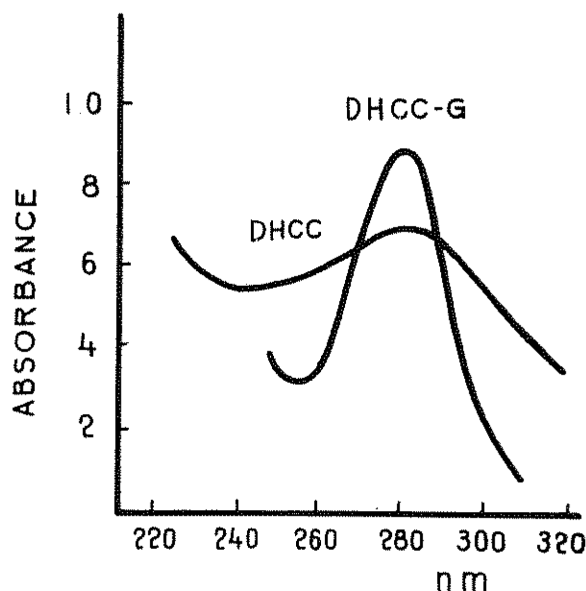

Fig. 3 Ultraviolet spectrum of 1,25-dihydroxycholecalciferol glycoside (DHCC-G) in 5 mM tetraborate solution compared with that of the free steroid (DHCC) in the same solvent

1 The UV spectrum strongly suggests the formation of an additional chromophore (unsaturated-α-β-ketone) by oxidation of the hydroxyl group at C-1. According to Woodward's rules (16) such a compound would have a calculated γ_{max} at 225 nm. The pre-existing triene system would suffer a small hypsochronic shift (γ_{max} 279 nm (calc.)) due to a smaller contribution of the exocyclic double bond (between C-10 and C-19) now engaged in the former chromophore.

associated with the steroid. When incubation was performed with β-glucosidase, hydrolysis of saccharides was 11% ; upon rechromatography no UV absorption could be detected in eluates. These experiments indicated that most probably the linkage between hexoses is α and that with the steroid is β. The latter conclusion agrees with other reports (8, 12, 23).

Periodic acid oxidation of the major component of fructoglucosides (the nonasaccharide) revealed that the reagent was consumed in almost equimolar amounts to the number of hexoses present (Table 2) without formic acid production. These findings discarded the 1→6 linkage between glucose units and suggested that fructose had a furanose configuration. Application of the Abdel Akker *et al.* (2) procedure for the determination of fine structure revealed glycerol and glyceraldehyde (in a 1.2:1.0 molar ratio) as the only products (Figure 4) indicating a 1→2 linkage between glucose units. The slight excess of glycerol over glyceraldehyde was interpreted to indicate that fructose was linked to the glucosan through its C-2. The 1→2 linkage explains the lability of the saccharide to mild acid medium (3).

The analytical data reported in Table 1 indicate that the aglycone is bound at the reducing end of the fructoglucosans. Note that in the first two fractions the number of moles of sterol and fructose coincide and no reducing power could be detected. In the next fractions, the sum of glucose equivalents of reducing power plus aglycone coincided, within experimental error, with the number of moles of fructose. The presence of reducing fructoglucosans can be explained by partial hydrolysis of the glycoside after collection of the leaves.

Present results confirm other reports indicating that the aglycone is bound to a sugar moiety of variable molecular weight (17, 21). The calculated molecular weights of the fructoglucosides (902, 1126, 1676) agree with the estimates of 1000-3000 (9) inferred from gel-filtration experiments.

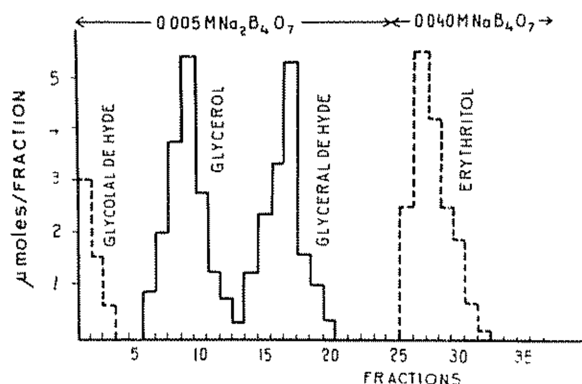

Fig. 4 Ion exchange chromatography of products obtained after periodate oxidation of 1,25-dihydroxycholecalciferol glycoside, followed by borohidride reductions and hydrolysis. The full line indicate the actual products obtained. The deshed line indicate the elution pattern of other possible products of the reaction with 1 → 4 or 1 → 6 polysaccharides.

The proposed formula (Figure 1) summarizes the data obtained. The disaccharide unit [Glc_p α 1-2 Glc], repeating 1,2 or 4 times is known as kojibiose (3). It is uncertain whether the linkage between fructose and glucose is α or β and whether the steroid is linked through its hydroxyl group at C-3 (as usual with steroidal aglycones), C-1 or C-25.

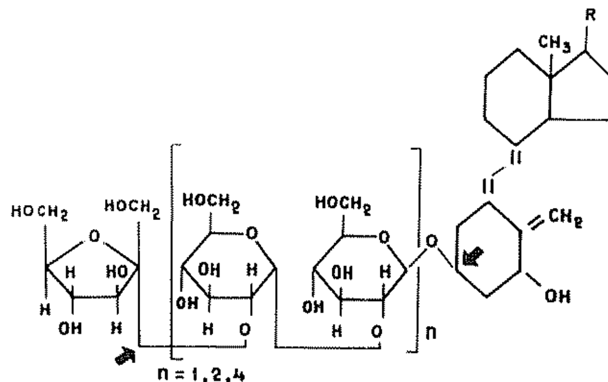

Fig. 5 Proposed structure of 1,25-dihydroxycholecalciferol glycoside present in *S. glaucophyllum* leaves. The arrows indicate uncertain details of structure (see text).

Table 2. Stoichiometry of periodic acid oxidation of fructoglucosans.

	Fructoglucosan (hexose content µmoles)	Periodate consumed µmoles	Formate produced µmoles	Formaldehyde produced µmoles
Expt. 1:	Fructose = 0.96 Glucose = 7.56	10.20	–	0.93
Expt. 2:	Fructose = 1.22 Glucose + 9.96	12.32	–	1.14

Ion exchange chromatography is the simplest method reported to date for the isolation of 1,25-dihydroxycholecalciferol glycoside.

Summary

Ion exchange chromatography of the borate complexes of soluble carbohydrates from *Solanum glaucophyllum* leaves allowed the isolation of 1,25-dihydroxycholecalciferol glycoside. The sterol was bound to a series of fructoglucosides. Investigation of their fine structure through periodic acid oxidation demonstrated that fructose was linked to a disaccharide unit [Glc_p α 1-2Glc] repeating 1, 2 or 4 times. The aglycone was bound to the reducing end of the saccharide

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