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INTRACELLULAR DISTRIBUTION OF THE STEROIDAL SAPOGENINS IN *DIOSCOREA TOKORO**

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Abstract—Leaf blades and rhizomes of mature *Dioscorea tokoro* Makino were homogenized separately. The homogenates were separated into nuclear, chloroplastidic, mitochondrial, microsomal and supernatant fractions by means of differential centrifugation. Sapogenins found free and sugar-bound were almost completely in the organelles, only minute quantities being in the supernatant. Sapogenins of the chloroplastidic and mitochondrial fractions were not released into the medium by sonic disintegration. The sapogenins of this plant are presumed to be bound to the membrane systems of the organelles.

INTRODUCTION

UNDERGROUND parts of many species of genus *Dioscorea* are used as major source materials for the synthesis of steroidal hormones.[1] They contain large amounts of diosgenin (25D-spirost-5-en-3β-ol) and its C25 epimer, yamogenin. These sapogenins are usually extracted from the underground parts of these plants and there have been few reports of their isolation in quantity from aerial parts. This lack of research on the aerial parts is chiefly because of their economic disadvantage compared with the underground storage organs which often attain large weights. Other sapogenins such as gentrogenin (25D-spirost-5-en-3β-ol-12-one)[2] and chiapagenin (25L-spirost-5-en-3β,12β-diol)[3] have also been isolated from the underground parts. Japanese species of this genus, other than three edible species, *D. japonica* Thunb., *D. batatas* Decne. and *D. bulbifera* L., contain diosgenin only in their rhizomes[4] and, of these, only *D. tokoro* Makino[4-6] and the *D. tenuipes* complex[4,7] contain large amounts of steroidal sapogenins, which have α-hydroxyl groups at C3 in their aerial parts. The natural 3α-hydroxyl sapogenins are found only in these two plants, these 3α-sapogenins being contained both as free and sugar-bound sapogenins in the plant bodies.[8] This unusual situation with the sapogenins of *D. tokoro* provoked our interest in their localization in cells and in their role in the basic metabolism of the plant. The present report contains the results of such a study.

* Studies on the steroidal components of domestic plants, Part LXI. Part LX, G. SAUER, A. SHIMAOKA and K. TAKEDA, *J. Chem. Soc.* in press.

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RESULTS

The study was carried out on the sapogenins of mature plants. Of the sapogenins isolated from *Dioscorea tokoro*, only diosgenin, yonogenin (25D,5β-spirostane-2β,3α-diol), and tokorogenin (25D,5β-spirostane-1β,2β,3α-triol) were analysed. Other sapogenins were excluded from this study for the following reasons. Igagenin (25D-5β-spirostane-2β,3α,27-triol) is found only in female flowers.[9] Isodiotigenin (25D,5β-spirostane-2β,3α,4β-triol) appears in seedlings[10] and young plants propagated by rhizome cutting[11] but it gradually decreases in quantity as the plant ages and its detection in mature plants is almost impossible. Kogagenin (25D-spirostane-1β,2β,3α,5β-tetraol)[6] and isotenuipegenin* are present only in traces.

Because the separation of diosgenin and yamogenin is very difficult and they react in the same manner with the chromogenic reagent, these two sapogenins were analysed together and their combined total expressed as diosgenin in the tables.

The quantities of protein nitrogen and sapogenins in the subcellular fractions of leaf blades (80 g)† are summarized in Table 1(a). The protein content of the filtrate was not analysed. Protein was contained in the largest quantity in the 105,000 *g* supernatant, but the amounts of sapogenins were very low in this fraction. Diosgenin was not detected in any form, even by thin-layer chromatography. Yonogenin and tokorogenin were most concentrated in the 20,000 *g* sediment, but, when calculated on protein basis, their concentrations in this fraction were similar to those in the 105,000 *g* sediment (Table 3(a)).

The distribution of protein among the subcellular fraction in the rhizome was similar to, but the amounts in each fraction less than, those in the leaf blades. The major sapogenin of the rhizome was diosgenin, found only bound with sugars. The sapogenin content of the 105,000 *g* supernatant was low, as in the leaf blades, and diosgenin and tokorogenin were most concentrated in the 105,000 *g* sediment (Table 1(b)). This became more significant when calculated on protein basis (Table 3(b)).

The distributions of sapogenins in the subfractions of the 2000 *g* and 20,000 *g* sediments after sonication are summarized in Table 2. The sapogenins were not released into the supernatant, remaining almost completely in the membrane fractions. The amount of material available for sonication from the 2000 *g* fraction of the rhizome was too small to permit quantitative analysis of any sapogenin, except sugar-bound yonogenin, in the sub-fractions.

During fractionation, the amounts of free sapogenins increased slightly and those of sugar-bound sapogenins decreased.

DISCUSSION

The homogenates of leaf blades and rhizomes, after straining through gauze, were each separated into five fractions by differential centrifugation. The speeds and the time intervals for separating the chloroplasts, mitochondria and microsomes, were selected after a survey of the literature on the preparation of these organelles from higher plants. Because the original residues still contained large amounts of fibres and undisrupted cells, this fraction was not considered in the discussion on the distribution of the sapogenins, although they contained

* This is a 25D-tetrahydroxysapogenin,[10] whose structure has not yet been elucidated.

† Fresh weight. The moisture content of the leaf blades and rhizomes was 77·3 and 69·7% of fresh weight respectively.

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TABLE 1. SAPOGENIN YIELD FROM THE INTRACELLULAR ORGANELLES OF *Dioscorea tokoro*

(a) *Leaf blade*

Fraction	Protein (N)	Diosgenin		Yonogenin		Tokorogenin		Total		
		f*	s	f	s	f	s	f	s	f+s
Residue		—‖	—	377·89†	29·69	17·36	5·28	395·25	34·97	430·22
650 *g*	24·58	—	—	67·64	10·87	4·07	1·11	71·71	11·98	83·69
2000 *g*	7·12	—	—	23·66	1·08	1·79	+‡	25·45	1·08	26·53
20,000 *g*	26·09	—	—	131·62	44·87	10·73	4·12	142·35	49·00	191·35
105,000 *g*	3·91	—	—	18·38	6·28	1·26	0·61	19·64	6·89	26·53
Supernatant	99·22	—	—	9·12	2·65	0·82	0·52	9·94	3·17	13·11
Total		0	0	628·31	95·44	36·04	11·64	664·35	107·08	771·43
Whole leaf blade§		0	0	457·65	132·74	36·95	29·60	494·60	162·34	656·94

(b) *Rhizome*

Fraction	Protein (N)	Diosgenin		Yonogenin		Tokorogenin		Total		
		f	s	f	s	f	s	f	s	f+s
Residue		—	90·94	7·93	1·05	9·54	2·17	17·46	94·16	111·63
650 *g*	7·13	—	0·20	0·50	0·35	0·57	2·04	1·07	2·59	3·66
2000 *g*	0·83	—	1·66	+	0·38	+	5·82	+	7·86	7·86
20,000 *g*	2·25	—	17·60	2·38	1·45	2·17	9·82	4·56	28·88	33·43
105,000 *g*	0·43	—	69·22	0·78	1·06	0·51	16·15	1·29	86·43	87·72
Supernatant	27·71	—	5·44	0·69	0·35	3·03	3·39	3·72	9·18	12·90
Total		—	185·06	12·28	4·64	15·81	39·40	28·10	229·10	257·20
Whole rhizome§		—	175·51	+	8·30	0·75	55·75	0·75	239·57	240·32

* f, Free sapogenin; s, sugar-bound sapogenin.
† Figures denote mg of sapogenins and N contained in each fractions derived from 80 g of fresh material.
‡ The presence of the sapogenin was detected by TLC, but the estimation of its quantity was impossible.
§ Extracted from 80 g of materials by methanol.
‖ The presence of the sapogenin was not detected, even by TLC.

TABLE 2. SAPOGENIN DISTRIBUTION IN THE SUBFRACTIONS OF 2000 *g* AND 20,000 *g* FRACTIONS

(a) *Leaf blade*

Fraction	Protein (N)	Diosgenin		Yonogenin		Tokorogenin		Total		
		f	s	f	s	f	s	f	s	f+s
2000 *g* fract.										
2000 *g*	1·61*	—	—	5·92	0·56	0·20	0·05	6·12	0·62	6·73
105,000 *g*	3·34	—	—	14·74	1·10	1·29	0·06	16·03	1·15	17·18
Supernatant	0·08	—	—	0·16	+	0·06	+	0·22	+	0·22
20,000 *g* fract.										
20,000 *g*	24·44	—	—	99·70	20·94	5·89	1·79	105·60	22·73	128·33
105,000 *g*	4·80	—	—	28·24	1·16	1·71	0·12	29·95	1·29	31·24
Supernatant	0·21	—	—	1·51	+	0·13	+	1·64	+	1·64

(b) *Rhizome*

Fraction	Protein (N)	Diosgenin		Yonogenin		Tokorogenin		Total		
		f	s	f	s	f	s	f	s	f+s
2000 *g* fract.										
2000 *g*	0·46	—	+	—	+	—	+	—	—	—
105,000 *g*	0·40	—	+	—	0·16	—	+	—	0·16	0·16
Supernatant	0·17	—	+	—	+	+	+	—	—	—
20,000 *g* fract.										
20,000 *g*	0·64	—	+	+	0·10	+	+	—	0·10	0·10
105,000 *g*	0·95	—	21·51	2·40	1·73	1·92	3·07	4·32	26·31	30·63
Supernatant	0·07	—	+	+	—	+	—	—	—	—

* Mg of sapogenins and N contained in the subfractions of 2000 *g* and 20,000 *g* fractions derived from 80 g of fresh material.

TABLE 3. CONCENTRATION OF SAPOGENINS IN THE INTRACELLULAR ORGANELLES OF *D. tokoro*

(a) *Leaf blade*

Fraction	Diosgenin		Yonogenin		Tokorogenin		Total		
	f	s	f	s	f	s	f	s	f+s
650 *g*	—	—	2·75*	0·44	0·17	0·05	2·92	0·49	3·40
2000 *g*	—	—	3·32	0·15	0·25	—	3·57	0·15	3·73
20,000 *g*	—	—	5·04	1·72	0·41	0·16	5·46	1·88	7·33
105,000 *g*	—	—	4·70	1·60	0·32	0·16	5·02	1·73	6·78
Supernatant	—	—	0·09	0·03	0·01	0·01	0·10	0·03	0·13
2000 *g*†									
2000 *g*	—	—	3·68	0·35	0·12	0·03	3·80	0·38	4·18
105,000 *g*	—	—	4·41	0·33	0·39	0·02	4·80	0·34	5·14
Supernatant	—	—	2·02	—	0·78	—	0·28	—	0·28
20,000 *g*‡									
20,000 *g*	—	—	4·08	0·86	0·24	0·07	4·32	0·93	5·25
105,000 *g*	—	—	5·88	0·24	0·36	0·03	6·24	0·27	6·50
Supernatant	—	—	7·19	—	0·06	—	7·79	—	7·79

(b) *Rhizome*

Fraction	Diosgenin		Yonogenin		Tokorogenin		Total		
	f	s	f	s	f	s	f	s	f+s
650 *g*	—	0·03	0·07	0·05	0·08	0·29	0·15	0·36	0·51
2000 *g*	—	2·01	—	0·46	—	7·05	—	9·51	9·51
20,000 *g*	—	7·83	1·06	0·65	0·97	4·37	2·03	12·85	14·87
105,000 *g*	—	162·23	1·83	2·49	1·19	37·85	3·02	202·56	205·59
Supernatant	—	0·20	0·02	0·01	0·11	0·12	0·13	0·33	0·47
2000 *g*									
2000 *g*	—	—	—	—	—	—	—	—	—
105,000 *g*	—	—	—	0·40	—		—	0·40	—
Supernatant	—	—	—	—	—	—	—	—	—
20,000 *g*									
20,000 *g*	—	—	—	0·15	—	—	—	0·15	0·15
105,000 *g*	—	22·58	2·52	1·81	2·01	3·22	4·53	27·61	32·14
Supernatant	—	—	—	—	—	—	—	—	—

* Mg of sapogenins per mg N.
† Subfractions of the 2000 *g* fraction.
‡ Subfractions of the 20,000 *g* fraction.

considerable amounts of sapogenins. The 650 *g* sediment consisted of nuclei, starch grains and cell debris. The 2000 *g* sediment of the leaf blades was chiefly composed of chloroplasts. To precipitate mitochondria thoroughly, a speed slightly higher than normal was chosen, so the 20,000 *g* fractions are thought to contain a considerable amount of large-size microsomes besides mitochondria.

The steroidal sapogenins of *Dioscorea tokoro* were found mainly in the organelles and only traces in the 105,000 *g* supernatant. These sapogenins were not released into the medium by sonic disintegration. It seems likely that the sapogenins are bound to the membranes of the organelles and are not present as solutions in their matrix.

As mentioned above, the preparations obtained by the present procedure are not considered to be pure, since some contamination of one organelle with another is inevitable. The total sapogenin content of the mitochondrial fraction of the rhizome was apparently higher than that of the leaf blade, though this was considered to be due to the presence of diosgenin and to contamination with the large-size microsomes which contain very large amounts of the sapogenin.

Sterols have been found in the intracellular organelles of plants[12-14] as well as of animals,[15-17] the composition of the sterols differing from organelle to organelle[12] and from tissue to tissue.[15] In our present study, the proportions of the sapogenins are similar in all subcellular fractions of the leaf blade but these proportions are markedly different for the different fractions of the rhizome (Table 4). Knapp *et al.*[13] homogenized the seedlings of *Phaseolus vulgaris* fed mevalonic acid-2-^{14}C, separated the homogenates into chloroplastidic, mitochondrial, microsomal and supernatant fractions by means of differential centrifugation and detected labelled squalene in the supernatant fraction and labelled sterols principally in the microsomal and the supernatant fractions. From these results they estimated that sterols are synthesized in the microsomal and supernatant fractions in higher plants as in mammalian tissues.[18] Capstack *et al.*[19] referred the principal site of the β-amyrin synthesis in the seedlings of *Pisum sativum* to a 10,000 *g* supernatant fraction, and the microsomes were estimated to have been included in that fraction. The high concentration of diosgenin in the microsomal fraction of the rhizome of *D. tokoro* may indicate that it is synthesized in this organelle. The concentrations of yonogenin and tokorogenin in the microsomes of the leaf blade were not so high as that of diosgenin of the microsomes of the rhizome, possibly attributable to the arrest of active biosynthesis of these sapogenins in the fully developed leaves.

In view of the known role of cholesterol in the membrane permeability[20] and electron transport system[17] in animal cells, it is suggested that the existence of high concentrations of sapogenins in the membranous structures of the organelles of *D. tokoro* indicates that they may have a related metabolic function in plant cells.

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TABLE 4. COMPOSITION OF THE SAPOGENINS IN THE INTRACELLULAR ORGANELLES

(a) *Leaf blade*

Fraction	Free sapogenins			Sugar-bound sapogenins			Total sapogenins		
	Diosgenin	Yonogenin	Tokorogenin	Diosgenin	Yonogenin	Tokorogenin	Diosgenin	Yonogenin	Tokorogenin
Residue	0*	1	0·044	0	1	0·171	0	1	0·053
650 *g*	0	1	0·058	0	1	0·098	0	1	0·063
2000 *g*	0	1	0·072	0	1	0	0	1	0·069
20,000 *g*	0	1	0·078	0	1	0·088	0	1	0·081
105,000 *g*	0	1	0·066	0	1	0·093	0	1	0·073
Supernatant	0	1	0·086	0	1	0·190	0	1	0·110

(b) *Rhizome*

Fraction	Free sapogenins			Sugar-bound sapogenins			Total sapogenins		
	Diosgenin	Yonogenin	Tokorogenin	Diosgenin	Yonogenin	Tokorogenin	Diosgenin	Yonogenin	Tokorogenin
Residue	0	1	2·642	90·111	1	1·988	10·567	1	1·257
650 *g*	0	1	1·086	0·608	1	5·710	0·247	1	2·964
2000 *g*	0	1	0	4·596	1	14·881	4·596	1	14·881
20,000 *g*	0	1	0·877	12·629	1	6·514	4·783	1	3·012
105,000 *g*	0	1	0·626	68·118	1	14·687	39·201	1	8·718
Supernatant	0	1	4·263	16·064	1	9·238	5·464	1	5·955

* Figures are the molar ratios of the sapogenins.

EXPERIMENTAL

Materials

Mature plants of *Dioscorea tokoro* cultivated at Takarazuka, Hyogo Pref., were harvested on 29 July. Leaf blades were freed from petioles. Rhizomes were cut off the stems and washed with water to remove soil.

Isolation of Intracellular Organelles

The leaf blades and rhizomes (each 80 g wet wt.) were treated separately. Each part was cut into small pieces with scissors and homogenized in a blender with 100 ml of ice-cold medium (0·1 M potassium-phosphate buffer, pH 7·4, containing 0·5 M D-mannitol and 0·01 M mercaptoethanol) for four periods of 30 sec, interspaced by 30-sec intervals, at 18,000 rev/min. The homogenate was strained through two layers of gauze and centrifuged at 650 *g* for 5 min. All sediments were resuspended in the same medium by gentle homogenation in a Potter–Elvehjem teflon homogenizer and again centrifuged at the same speeds and for the same time intervals. The sediments were removed and the supernatants united with the original supernatants. The 650 *g* supernatants were centrifuged at 2000 *g* for 10 min. The 2000 *g* supernatants were then centrifuged at 20,000 *g* for 20 min. The 20,000 *g* supernatants were further centrifuged, at 105,000 *g*, for 60 min. All the procedures described above were carried out at 0–4°.

Sonic Disintegration of the 2000 g *and* 20,000 g *Fractions*

Aliquots of the 2000 *g* and 20,000 *g* sediments of the leaf blades and rhizomes were resuspended at 4° in 20 ml of the same medium as used for the fractionation and sonicated for 2 min in a sonic disintegrator (10 kc) at maximal output. The suspensions were then separated into 2000 *g*, 10 min (or 20,000 *g*, 20 min) and 105,000 *g* 60 min sediments and supernatants by the same means as above.

Extraction of Sapogenins

Each sediment and residue was stirred in 20 ml of ether for 30 min and then centrifuged at 2000 *g* for 10 min. The extraction with ether was repeated three times. The ether extracts were washed with water, dried over Na_2SO_4, evaporated to dryness and analysed for free sapogenins. Aliquots of the ether insoluble substances were analysed for protein. The remainder of the ether unsoluble substances were suspended in 50 ml 2 N HCl and hydrolysed for 5 hr on a boiling water bath. The sapogenins hydrolysed were extracted three times with 50 ml of ether. The ether extracts were washed, dried, evaporated and analysed for sugar-bound sapogenins.

The supernatants were continuously extracted with 100 ml of ether for 5 hr. The ether extracts were analysed for free sapogenins. Aliquots of the aqueous layers were added to equal vol. of 10% trichloroacetic acid. The precipitated proteins were collected by centrifugation and analysed. The remaining portions were made 2N by addition of 35% HCl and heated for 5 hr on a water bath. The hydrolysis products were extracted with ether and analysed for sugar-bound sapogenins.

The leaf blades (13 g fr. wt.) and the rhizomes (25 g) of the same origin were extracted three times with 50 ml of methanol for 4 hr under reflux. The methanol extracts were evaporated to dryness and extracted three times with 50 ml of ether for 15 min. The ether extracts were washed, evaporated and analysed for free sapogenins. The residues, after the ether extraction, were hydrolysed in 50 ml of 2 N HCl for 5 hr on a water bath. The hydrolysis products were extracted with ether and analysed for sugar-bound sapogenins.

Analysis of Protein and Sapogenins

Protein was analysed by the micro-Kjeldahl method.

Diosgenin and the other two sapogenins were estimated by the methods of Akahori *et al.*[21] and Okanishi and Togami[22] respectively. Aliquots of the sapogenin mixtures were dissolved in methanol, spotted on thin-layer plates (20 × 20 cm, Kiesel gel G, 0·25 mm) and developed with C_6H_6–EtOAc–HOAc (90:8:2, v/v, for the analysis of diosgenin) or with $CHCl_3$–Me_2CO–HOAc (80:20:5, v/v, for the analysis of yonogenin and tokorogenin). The thin-layer plates were freed from the solvents and sprayed with water. Sapogenins were detected as white spots on the grey backgrounds. The areas of Kiesel gel containing diosgenin were scraped off the plates into test tubes and extracted with $CHCl_3$. The extracts were evaporated to dryness, warmed with 0·5 ml of conc. H_2SO_4 and 5 ml of H_3PO_4 containing 8 mg of $FeCl_3$ at 70° for 9 min and, after cooling, the absorbances of the reaction mixtures were measured at 485 nm. The areas containing other sapogenins were scraped into centrifuge tubes and warmed with 0·1 ml of ethanol containing 0·5 mg of anisaldehyde for 15 min at 100°, then added to 5 ml of H_3PO_4 and again warmed at 100° for 70 min. After cooling, the reaction mixtures were centrifuged at 2000 *g* for 10 min, and the absorbances of the clear supernatants were measured at 540 nm.

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