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Provitamins and vitamins D_2 and D_3 in *Cladina* spp. over a latitudinal gradient: possible correlation with UV levels

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Abstract

Provitamin D_2, vitamin D_2, and vitamin D_3, were identified in the thallus of a lichen species, *Cladina arbuscula* (Wallr.) Hale and W.L. Culb. The identification of vitamin D_3 was supported by: (1) co-chromatography in both reverse and straight phase HPLC (high performance liquid chromatography), (2) ultraviolet absorption spectrum, and (3) molecular ion peaks demonstrated by ESI (electrospray ionisation) mass spectrometry. The contents of vitamin D_3 range from 0.67 to 2.04 mg/g dry matter in the thalli of *C. arbuscula* specimens grown under different natural conditions, while provitamin D could not be detected. The ranges for provitamin D and vitamin D_2 were 89–146 and 0.22–0.55 mg/g dry matter, respectively, while the contents of provitamin D were below the detection limit (0.01 mg/[g dry matter]). When *C. arbuscula* thalli collected at different latitudes from northern Finland to Greece were compared, a positive correlation of vitamin D and D_3 contents with modelled UV-B radiation at the collection sites was found. A single sample of *C. rangiferina* from northern Finland gave much higher values for the vitamins. A possible reason could be the lower content of UV-B absorbing pigment in the latter species.

Keywords: Cholecalciferol; *Cladina rangiferina*, *Cladina arbuscula*; ESI-MS; Provitamin D, Vitamin D; UV-B

1. Introduction

Since provitamin D_3 (7-dehydrocholesterol) was initially isolated from swine skin and was postulated to be the precursor of vitamin D_3 (cholecalciferol) [1], much research has been conducted to isolate, identify and understand the physiological functions of vitamin D_3 in mammals. Vitamin D_3 $(OH)_2$ plays an important role in the regulation of intestinal calcium and phosphorus absorption, calcium mobilization from bone and renal reabsorption of calcium and phosphorus. With the discovery of a vitamin D receptor, some new functions of vitamin D_3 have been found, such as modulation of osteoclast differentiation, suppression of parathyroid cell growth and parathyroid hormone gene expression, and effects on the growth and differentiation of keratinocytes in skin [2–4].

As vitamin D_3 is the form synthesized by vertebrates, it is often referred to as 'animal vitamin D', in contrast to vitamin D_2 , 'plant vitamin D'. Actually, this is a misconception; vitamin D_3 and vitamin D_3-like substances have been found in a variety of plants. Vitamin D_3 has been identified in the leaves of *Cestrum diurnum*, *Lycopersicon*

esculentum, *Solanum malacoxylon*, *Solanum tuberosum* (Solanaceae) and *Cucurbita pepo* (Cucurbitaceae) [5–7], as well as in tissues of the alfalfa plant (*Medicago sativa*) [8], and the grasses *Dactylis glomerata* and *Trisetum flavescens* [9]. Moreover, in the leaves of *S. malacoxylon*, *L.esculentum*, *C. diurnum* and *Trisetum flavescens*, pollen of *Pinus nigra* and *Pinus sylvestris* not only free vitamin D_3 but also its hydroxylated metabolites have been detected [5–7,10–12]. In *S. malacoxylon*, vitamin D_3 may be hydroxylated by a similar pathway as in animals [12]. It has been suggested that the synthesis of vitamin D in plants is similar to the photolytic reactions occuring in the skin of vertebrates [9,13], but in some plants or plant cells vitamin D_3 can be formed also without the action of ultraviolet-B radiation ([14] and own results to be published separately). The biological functions of vitamin D_3 and its metabolites in plants remain unclear.

Compared to the plant taxa mentioned above, limited information is available on the vitamin D_3 contents in lichens. *Cladina* is the second largest genus in the family Cladoniaceae [15]. It comprises 40 species, some of them (for example, *C. rangiferina*, *C. arbuscula* and *C. mitis*) being the most important winter food of reindeer and caribou, therefore commonly referred to as 'reindeer lichens'. To our knowledge no vitamin D_3 data have been reported for this genus. In the present study, vitamin D_3 in the thalli of *Cladina arbuscula* (Wallr.) Hale and W.L. Culb. grown under different natural conditions was identified and determined by utilizing high performance liquid chromatography (HPLC), electrospray ionisation mass spectrometry (ESI-MS) and UV spectrophotometry. The results indicate that the biosynthesis of vitamin D_3 in *C. arbuscula* is a light-dependent process. The established method can be applied to further investigation of vitamin D_3 content in other closely related lichens.

2. Materials and methods

2.1. *Materials*

The places and the dates of the collections of *Cladina arbuscula* (Wallr.) Hale and W.L. Culb. and *Cladina rangiferina* (L.) Nyl. samples analysed in this study are listed in Table 1.

2.2. *Chemicals*

Standard provitamin D_2 (ergosterol), vitamin D_2 (ergocalciferol), provitamin D_3 (7-dehydrocholesterol) and vitamin D_3 (cholecalciferol) were purchased from Sigma Chemical Co., St. Louis, USA. Chloroform (HPLC ap-plication-stabilized) used for extraction, acetonitrile, methanol, isopropanol and *n*-hexane (HPLC-grade) used for HPLC were from Labscan Ltd., Dublin, Ireland. Ammonium acetate (Guarantee analysis) was purchased from Riedel-de Haen, Seelze, Germany.

2.3. *Sample preparation for reverse phase HPLC*

The dry thallus of *Cladina arbuscula* was finely ground by using a mortar and pestle. For each gram powder, 10 ml chloroform was used for extraction. The extract was filtered, the filtrate collected and the residue re-extracted. The combined filtrates were evaporated. The dried residue was redissolved in acetonitrile–methanol (50:50) and stored at 22°C

until further analysis.

Provitamin D_2 and vitamin D_2 and vitamin D_3 contents of *Cladina arbuscula* (Wallr.) Hale and W.L. Culb. (first five lines) and *Cladina rangiferina* (L.) Nyl. (Rovaniemi, last line) samples[a]

Table 1.

Place			Date of collection	Contents mg (g dry weight)$^{-1}$ of		
				provit. D_2	vitamin D_2	vit. D_3
Sithonia, Greece	40.08N 23.88E	Open place	23-09-1999	149	0.57	1.73
						1.84b
Friseboda, Sweden	55.88N 14.28E	Deep shade	17-09-2000	106	0.54	1.04
Friseboda, Sweden	55.88N 14.28E	Open place	16-09-2000	112	0.46	1.24
Vombs Fure, Sweden	55.78N 13.68E	Sparse forest	16-09-2000	120	0.43	1.12
¨Sodankylä, Finland	67.48N 26.68E		26-09-2000	91	0.23	0.67
Rovaniemi, Finland	66.58N 25.78E	Sparse forest	05-10-2000	99	0.49	2.04

a Values are means of duplicate determinations differing by up to 20%.
b Two independent experiments, each with duplicate determinations.

2.4. *Assay of provitamins and vitamins D_2 and D_3*

Assay of provitamins and vitamins D_2 and D_3 by HPLC was carried out by a method modified from Ref. [16]. The extract was first subjected to reverse phase HPLC, after which the fractions having retention times corresponding to the substances to be assayed were rechromatographed by direct phase HPLC. The chromatography was monitored by UV absorption at 265 nm. Details will be described elsewhere.

2.5. *Ultraviolet spectroscopic measurements*

The ultraviolet absorption spectra of the vitamin D_3 standard and the putative vitamin D_3 fraction from the second straight phase HPLC were measured with a UV– VIS recording spectrophotometer (Shimadzu Corporation, UV-160A, Japan).

2.6. *Electrospray ionisation mass analyses*

Mass spectrometry was performed on a Hewlett Packard 5989B MS Engine (Hewlett Packard, Palo Alto, USA) quadrupole mass spectrometer, equipped with a high energy conversion dynode detector and operated in the positive ion mode. The quadrupole was scanned from *m*/*z* 30 to 750 at 1 scan s^{-1}. The samples were introduced into the electrospray ionization source with a syringe pump. Details will be given elsewhere.

2.7. *Estimation of ambient UV-B exposure*

Expected daily ambient UV-B exposure (time-integrated plant-weighted irradiance) at the top of the vegetation under cloud-free conditions was calculated for the day of sample collection using the programme of Björn and Murphy [17].

3. Results

3.1. *UV spectrum of the vitamin D_3 fraction*

The fraction corresponding to vitamin D_3 from the second straight phase analytical HPLC was collected for determining the UV absorption spectrum in the region 220 to 320 nm. As shown in Fig. 1, the spectrum had a maximum at 265 nm and minimum absorption at 228 nm, coinciding with the pattern of the spectrum of the standard. This demonstrated that a vitamin D-like *cis*-5,7,10(19)-triene chromophore was present in the fraction.

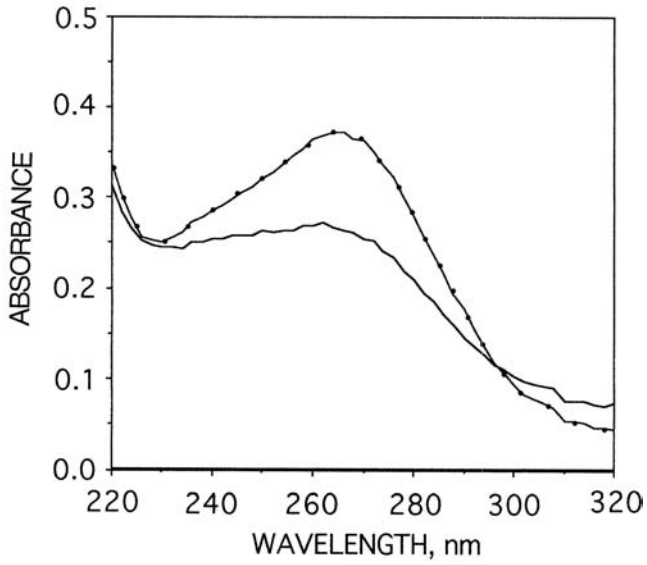

Fig. 1. Ultraviolet spectra of the putative vitamin D_3 fraction from *C. arbuscula* purified by two successive steps of HPLC (plain line) and standard vitamin D_3 (line with dots).

3.2. *ESI mass analysis*

Fig. 2 presents the mass spectrum of the vitamin D_3 isolate. Seven molecular ion signals were detected in the region between *m*/*z* 100 and *m*/*z* 700. The peak at *m*/*z* 385.4 attested that the sample contained vitamin D_3.

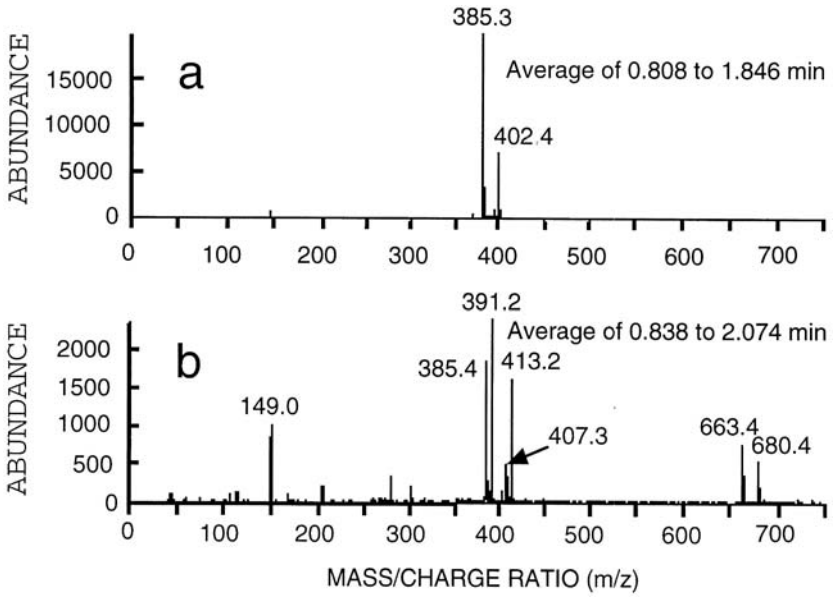

Fig. 2. ESI-MS analyses of authentic vitamin D (a) and *C. arbuscula* sample (b). Peaks at *m/z* 385.3 and *m/z* 402.4 represent $[M–H]^+$ and $[M–NH]_4^+$, respectively. The peak at *m/z* 385.4 shows that the sample contains vitamin D_3.

3.3. *Contents of provitamin and vitamins D_2 and D_3 in the Cladina arbuscula field samples*

Four grams of *C. arbuscula* or *C. rangiferina* thallus were needed for each assay of the vitamin D_3 content by the external standard method using vitamin D_3 solution of known concentration. The contents of vitamin D_3 quantified by HPLC range from 0.67 to 2.04 mg $(g_{dry\ matter})^{-1}$ in the thalli of *C. arbuscula* samples (Table 1). The recovery of vitamins D_2 and D_3 by the HPLC was 97 and 96%, respectively, and that of provitamins D_2 and D_3 98% and 99%, respectively. While the contents of provitamin D_3 were below the detection limit (0.01 mg $g\,[dry\ matter]^{-1}$), the contents of provitamin D_2 were much higher than those of the corresponding vitamin.

In Fig. 3 the vitamin content of *C. arbuscula* is correlated with the plant-weighted UV-B radiation dose from morning till noon computed for cloudless skies.

4. Discussion

The finding of vitamin D_3 in the thallus of *C.arbuscula* was conclusively supported by: (1) co-chromatography in both reverse phase and straight phase HPLC which was carried out under quite different experimental conditions,
(2) UV-spectrum showing the characteristic absorption unique to vitamin D and its derivatives and (3) intact parent molecular weight (384.4) information consistent with vitamin D_3 calculated from ESI mass spectrum. To our knowledge vitamin D_3 has not been found in lichens before.

In the skin of vertebrate animals and humans, vitamin D is formed by the processes: Provitamin D➔ Previtamin D➔ Vitamin D. The conversion of provitamin D to pre-vitamin D is a photochemical reaction requiring ultraviolet B photons. In the grass, *Trisetum flavescens* [9], as well as in tomato leaves [18,19], vitamin D_3 is formed only under UV-irradiation, which suggests a similar mechanism of vitamin D_3 biosynthesis in plants as that in the vertebrate and human skin [13]. There is a positive correlation between the vitamin D_3 content of *C. arbuscula* and the calculated UV-B exposure at the place and day of sample collection (Fig. 3), and also between vitamin D content and the UV-B exposure during several weeks before collection (not shown). However, the correlation between vitamin D content and UV-A exposure (not shown) is almost as strong. Thus the data are compatible with the view that vitamin D synthesis in the lichen is a UV-B dependent process, but no firm dependence on UV-B has been established. The content of provitamin D in the thallus of *C. arbuscula* is below the detection limit when the same experimental procedures were utilized for investigation. One possible explanation is that in vivo this provitamin is glycosylated or bound in some other way, such that it is not extracted with our procedure.

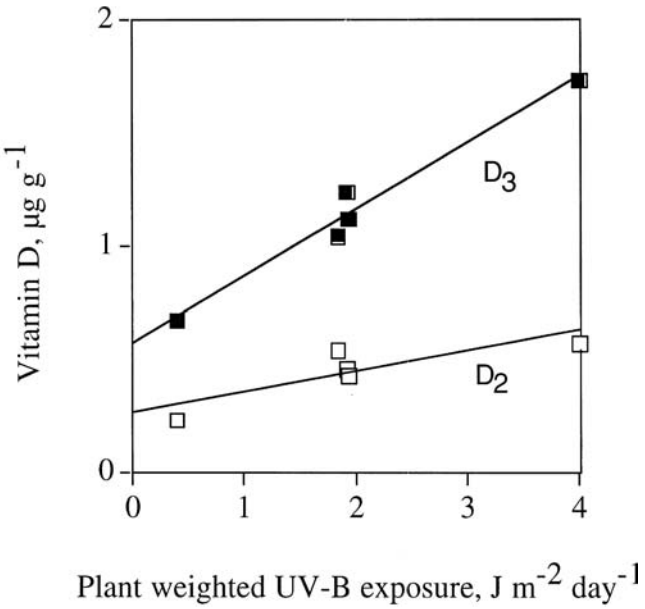

Fig. 3. Contents of vitamin D_2 and D_3 in dried *C.arbuscula* as a function of the expected ambient UV-B exposure at the day of sample collection calculated for cloud-free conditions at the top of the vegetation [17]. The values for *C. rangiferina* from Rovaniemi are not included.

The *C. rangiferina* sample from Rovaniemi contained considerably more vitamins D_2 and D_3 than *C. arbuscula* from nearby Sodankylä. One likely explanation is that *C. arbuscula*, in contrast to *C.rangiferina*, has a high content of usnic acid, which acts as a filter for UV-B radiation and allows less of it to reach the provitamins D. If lichens are to be used as 'biodosimeters' for UV-B, it is thus important to pay close attention to species.
The occurrence of vitamin D_3 in the lichen species, *C. arbuscula* and *C. rangiferina*, raises several questions. First, lichens are symbiotic organisms that are composed of fungi and algae (and/or cyanobacteria). Since provitamin D_2 (ergosterol) is the major

membrane sterol in many fungi, its presence (as well as the presence of vitamin D_2) is expected. However, the high content of vitamin D_3 in combination with the absence of provitamin D_3 is surprising. In future research it would be interesting to analyze the fungal and algal components separately. Secondly, *C. arbuscula* and *C. rangiferina* are important winter feeds for reindeer and caribou. Their content of vitamin D_3 may represent a vital source of vitamin D for the animals, especially during winter. Further investigations of the presence of vitamin D_3 in other chemotaxonomically related lichen species and in animal tissues based on the method established here may be helpful for elucidating their importance.

Previous investigations have shown that vitamin D_3 affects growth and development of plants and algae (see Refs. [18,19] for literature). Its possible physiological function in lichens remains to be found.

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