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Composition and (in)homogeneity of carotenoid crystals in carrot cells revealed by high resolution Raman imaging

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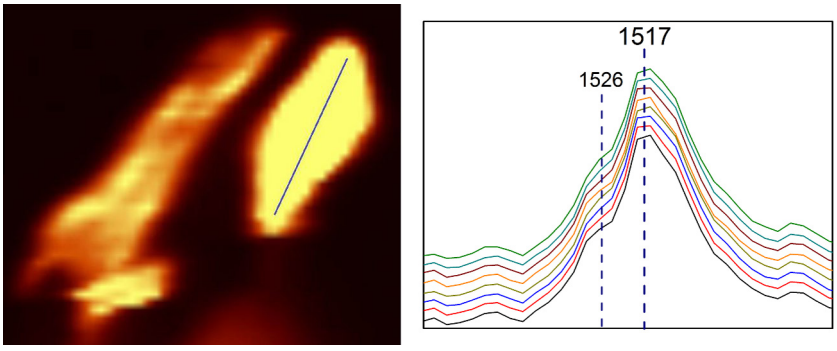
HIGHLIGHTS

- Carotenoid crystals in carrot cells are composed of a mixture of various carotenoids.
- β-Carotene predominates in carotenoid crystals, but it is accompanied by α-carotene.
- Crystal constituents are distributed homogeneously throughout a crystal.
- Crystalline and amorphous carotenoids co-exist in carrot cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Three categories of roots differing in both β/α-carotene ratio and in total carotenoid content were selected based on HPLC measurements: high α- and β-carotene (HαHβ), low α- and high β-carotene (LαHβ), and low α- and low β-carotene (LαLβ). Single carotenoid crystals present in the root cells were directly measured using high resolution Raman imaging technique with 532 nm and 488 nm lasers without compound extraction. Crystals of the HαHβ root had complex composition and consisted of β-carotene accompanied by α-carotene. In the LαHβ and LαLβ roots, measurements using 532 nm laser indicated the presence of β-carotene only, but measurements using 488 nm laser confirmed co-occurrence of xanthophylls, presumably lutein. Thus the results show that independently on carotenoid composition in the root, carotenoid crystals are composed of more than one compound. Individual spectra extracted from Raman maps every 0.2–1.0 μm had similar shapes in the 1500–1550 cm^{-1} region indicating that different carotenoid molecules were homogeneously distributed in the whole crystal volume. Additionally, amorphous carotenoids were identified and determined as composed of β-carotene molecules but they had a shifted the ν_1 band probably due to the effect of bonding of other plant constituents like proteins or lipids.

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Introduction

Chemical extraction of compounds from biological materials usually relies on the use of solvents that may interfere with the investigated compound causing its structural modifications. Fourier-transform (FT) Raman spectroscopy provides an opportunity for *in situ* investigation of compounds without any sample preparation [1,2]. Both Raman and IR spectroscopic methods were successfully used for organelle differentiation in human [3] and plant [4,5] cells. Most of available reports showing application of Raman microspectroscopy, including the use of mapping and imaging techniques, for plant cell analysis were focused on cell walls composition providing information on essential polymers such as cellulose and lignin [6,7] as well as on lipids [8]. The first attempt to study other constituents like carotenoids at the subcellular level *in situ* was done by Baranska et al. [9].

Carotenoids belong to important plant secondary metabolites and play many crucial functions in photosynthesis and photooxidative protection. Some of them are also pigments that determine tissue color when synthesized in high amounts [10]. The most important carotenoid for human health is β-carotene, which is converted to retinol (vitamin A). Other carotenoids also have antioxidative properties (lycopene) or are essential components of human eye macular yellow spot (lutein) [11,12]. Carotenoids are delivered to human body mainly with vegetables and carrot is one of the richest sources of β-carotene. It is well recognized that common carrot developing orange colored storage roots contains mainly β-carotene constituting up to 80% of all carotenoids and that it is accompanied by significant amounts of α-carotene. These two compounds may constitute up to 95% of total carotenoids in a root [13]. The third abundant carotenoid in orange carrot is lutein that belongs to xanthophylls [14]. In carrot, carotenoid biosynthetic pathway splits to two branches. Lycopene, a precursor of the above mentioned compounds, is converted to either β-carotene or α-carotene, the later is then converted to lutein. In non-photosynthetic tissues, carotenoids may occur in chromoplasts forming crystal structures, which is well observed in carrot storage root cells [15].

Carotenoids are built from a polyene chain and cyclic end groups. The former is responsible for three characteristic and strong (due to resonance effect) Raman bands called ν_1, ν_2, and ν_3 [16]. The most intense band (ν_1) in Raman spectrum comes from the C = C stretching mode and is positioned in the 1500–1550 cm^{-1} region. The number of the conjugated double bonds in the polyene chain influences Raman spectra of carotenoids. Furthermore, the position of the ν_1 band depends strongly on the length and terminal substituents of the polyene chain as well as on their interaction with environment [17–20]. For example, for carrots containing β-carotene as a predominant component and traces of lutein and α-carotene, the ν_1 band has been reported to be symmetric and located at *ca.* 1520 cm^{-1} [19,21]. On the other hand, the ν_1 band in Raman spectra of carrots with higher level of lutein and α-carotene is asymmetric and exhibits an additional shoulder at *ca.* 1527 cm^{-1} [21]. Thus, this band can be applied for identification of various carotenoids as well as determination of interaction between carotenoid molecules and environment. Although some carotenoids like α-carotene and lutein cannot be distinguished in raw plant material. The most updated information on carotenoid spectroscopic properties supplemented by their biosynthesis and function in plants is reviewed in Ref. [22].

Spectroscopic measurements of carotenoids in macro scale showed tissue specific distribution of these compounds [21,23]. In micro scale, i.e., at a subcellular level, carotenoid crystals in carrot cells were measured and mapped using Raman spectroscopy without their extraction from plant tissue [9]. That was the first attempt for analytical investigation of carotenoid crystal composition and revealed that such crystals contain β-carotene. The presence of other constituents was also postulated but due to technical limits of Raman spectrometer it was not possible to show conclusive evidence.

In this work, for the first time, single carotenoid crystals were measured directly in carrot root cells without compound extraction using high resolution Raman imaging technique. The use of high spectral resolution and new 488 nm excitation laser technology have allowed verification of the hypothesis that carotenoid crystals have complex composition and consist of β-carotene accompanied by other carotenoids. Moreover, spatial distribution of these compounds in a crystal is shown.

Material and methods

Plant material

Plant materials used in this study were storage roots of an F_4 carrot (*Daucus carota* L.) population derived from a cross between an orange-rooted carrot inbred, B493, and a white-rooted wild carrot [24] segregating for color and carotenoid content. Plants were grown in the greenhouse under a 14 h photoperiod and harvested approx. 105 days after sowing. Samples for carotenoid analysis were taken from mid-root, frozen, and lyophilized.

HPLC analysis

Carotenoid analysis was performed as described by Santos and Simon [24]. Briefly, carotenoids were extracted from lyophilized root tissue of individual roots with hexane and extracts were analyzed with HPLC using a Rainin Microsorb-MV column with a mobile phase of acetonitrile: methylene chloride: methanol, 55: 23: 22 at a flow rate of 1 ml/min. Data were collected from 192 to 600 nm (1.2 nm resolution), at 0.5 s intervals, and peaks were quantified using Millennium version 2.1software.

Spectroscopic measurements

Microscopic slides with monolayers of carrot cells were used as specimens. Carrot root secondary phloem (flesh) tissue was hand squashed on a microscopic glass slide using a cover slip, which was removed after deep freezing at −80 °C. Observations were made using the Nikon Eclipse E600 light microscope and images were captured using the Canon 450D digital camera.

Measurements of carrot specimens were performed using a Confocal Raman Imaging system Witec alpha 300 with the application of a 100× air objective (Olympus, MPlan FL N, NA = 0.9). The spectrometer was equipped with a back-illuminated CCD (a Newton EMCCD DU970-BV) camera as well as 600 and 1800 grooves per mm gratings (for 532 and 488 nm lasers). Measurements were performed with two excitation wavelengths, i.e. 532 and 488 nm, and as a result spatial resolution of images was 0.36 and 0.33 μm, respectively. The maximum power of the used 532 nm laser at the source was *ca.* 40 mW, whereas for the 488 nm laser the power was *ca.* 30 mW. Spectra were collected with 0.3 s exposure in the range of 0–3600 cm^{-1} and with spectral resolution of 3 and 1 cm^{-1} for 532 and 488 nm, respectively. Raman images were obtained by integrating intensity of the ν_1 band.