ULTRASTRUCTURE, ORGANIZATION AND CYTOCHEMISTRY OF CYTOPLASMIC CRYSTALLINE INCLUSIONS IN ORIGANUM DICTAMNUS L. LEAF CHLORENCYMA CELLS

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SUMMARY

In the cytoplasm of chlorenchyma cells in primary Origanum dictamnus leaves, rectangular prism-like crystals occur, which, as shown by pepsin digestion, are proteinaceous. In sections they usually show either a square lattice or striations running parallel to the short or long axis. In both cases the interspacings are estimated to be about 100 Å, suggesting that the arrangement of the crystallographic planes possesses tetragonal symmetry. High magnifications of the crystalline inclusions together with analysis of their diffraction patterns showed that the striations are composed of double helices of protein macromolecules. In leaves 3–4 mm long, eroded crystals are often observed. When leaves are larger than 5 mm, no crystalline bodies can be identified in chlorenchyma cells. It is possible that they represent a storage form of protein, which is used later by the developing cells.

INTRODUCTION

Crystalline inclusions of proteinaceous nature have often been observed in plant cells under the light and electron microscopes (Nageli, 1862; Heinricher, 1891; Frey-Wyssling, 1955; Sitte, 1958). These structures are either located within cell organelles, such as plastids (Behnke, 1973; Sprey, 1977), mitochondria (Tsekos, 1972; Gailhofer & Thaler, 1975), endoplasmic reticulum (Brunt & Stace-Smith, 1978), microbodies (Frederick, Gruber & Newcomb, 1975; Silverberg, Fischer & Sawa, 1976) and nucleus (Schnepf, 1971; Heinrich, 1972), or free in the cytoplasm (Williams & Kermsie, 1974; May & Künkell, 1975). Cytoplasmic crystalline inclusions vary in size and shape. Their outline is usually square, rectangular, rhomboid or polyhedral (Jensen & Bowen, 1970; Burgess, 1971; Metitiri & Zachariah, 1972), while their internal structure usually appears as a square lattice or a honeycomb, or in the form of parallel striations (Schnepf & Deichgräber, 1969; Thaler & Amelunxen, 1975; Renaudin & Cheguillaume, 1977). In sieve elements of Papilionaceae, P-protein crystals seem to consist of a spindle-like central body, which may or may not have a long thin tail at each end (Palevitz & Newcomb, 1971; Lawton, 1978; Arsanto, 1982).

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Cytoplasmic crystals present in chlorenchyma cells of *Origanum dictamnus* mesophyll have been studied in the present investigation by means of electron microscopy and cytochemistry in order to determine their form, organization and chemical constitution.

**MATERIALS AND METHODS**

Primary leaves of 12-day-old seedlings of *Origanum dictamnus* L. (Lamiaceae) were used. Small pieces of the tissue were fixed in a cacodylate-buffered glutaraldehyde/paraformaldehyde mixture (Karnovsky, 1965) for 3 h at room temperature. After washing in buffer the material was post-fixed in 1% osmium tetroxide, similarly buffered for 3 h in a refrigerator. Dehydration was carried out in a graded ethanol series and was followed by infiltration and embedding in Spurr's Epoxy resin. For light microscopy, semithin sections of plastic-embedded tissue were stained with 1% Toluidine Blue O in 1% borax solution. Ultrathin sections for electron microscopy were collected on copper or gold grids covered with a Formvar supporting film. They were stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope equipped with a goniometric stage.

For enzyme digestion, ultrathin sections of double-fixed material were treated for 20 min with 10% H2O2 before incubation in 0.5% pepsin (Serva, research grade) in 0.1 M HCl for 3–24 h at 37°C. After washing in distilled water the sections were stained as described above (Behnke, 1975).

**OBSERVATIONS**

Mesophyll chlorenchyma cells of primary leaves are characterized by the presence of large and numerous chloroplasts usually located in the parietal cytoplasm. The chloroplast matrix is electron-dense, containing a well-developed grana-free system with either a few small starch grains or none at all. The numbers of mitochondria and microbodies are normal and close associations between them and the chloroplasts have often been observed. The cytoplasm is densely filled with ribosomes. There is a large nucleus. Rough endoplasmic reticular cisternae are short and few in number, while the dictyosomes are scarce, producing smooth vesicles with electron-translucent contents.

During this stage of leaf development, crystalline inclusions located in the peripheral cytoplasm, or in plasma strands traversing the central vacuole, are frequently observed in the chlorenchyma cells (Fig. 1). They occur isolated or in groups and display an organized internal structure. In Fig. 2 an almost square crystal is in contact with two connected elongated ones, which appear broken in their middle
Cytoplasmic inclusions in O. dictamnus leaf cells

Figs 1-3
region. It is possible that the square crystal was pushed by the central vacuole (extending movement) into the rectangular crystal causing it to break. A similar ramming movement that was probably caused by a chloroplast is shown in Fig. 3.

The crystal contours most frequently encountered are rectangular (Figs 4, 5), trapezoidal (Fig. 6) and triangular (Fig. 7). These different outlines correspond to

Figs 4–7. Crystal contours usually encountered in the cytoplasm of leaf chlorenchyma cells. They may be rectangular (Fig. 4, ×37 000; Fig. 5, ×33 000), trapezoidal (Fig. 6, ×34 000) or triangular (Fig. 7, ×40 000).
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sections cut parallel or obliquely to the crystal surfaces (Fig. 8A–D). When observing the internal structure of a crystal, it usually appears either amorphous (Figs 2, 6, 7, 12) or in the form of striations running parallel to the long or short axis (Figs 2, 9, 10). Less frequently, crystals appear in sections as a square lattice of electron-dense dots (Figs 4, 5, 11).

Fig. 8. A diagram to illustrate the possible three-dimensional form of the crystal (rectangular prism). When crystals are sectioned parallel to the AA'–BB' or BB'–CC' planes, their contours appear to be rectangular (A, a). Obliquely sectioned crystals give trapezoidal (c) and triangular (d) profiles.

Figs 9–12. A crystal tilted at various angles to the electron beam. Its internal structure appears to consist of striations running parallel to the long axis (Fig. 9, ×70 000) or short axis (Fig. 10, ×70 000). It also appears as a square lattice (Fig. 11, ×70 000) or amorphous (Fig. 12, ×70 000). The diffraction patterns are shown in the corresponding insets.

Fig. 13. A crystal in which the boxed portion is highly enlarged to demonstrate the substructure of the striations (cf. Figs 14, 15). ×56 000.

Figs 14, 15. The same highly magnified image in which striations appear as double helices. These helices are locally cross-bridged via short fibrils (arrowheads in Fig. 14). ×180 000.

Fig. 16. Accumulation of small blocks of aligned dots (striations), variably oriented. ×77 000.

Fig. 17. A group of well-developed crystals demonstrating a radial arrangement. Note the presence and orientation of the striations. ×46 000.

Fig. 18. Enlarged view of the edge of a striated crystal. The boundary striations appear stair-like (arrows). ×74 000.

Fig. 19. A cytoplasmic crystalline inclusion undergoing erosion. This can be identified in both the outer and inner parts of the crystal (single and double arrowheads). ×108 000.

Fig. 20. An ultrathin section treated with pepsin. Electron-transparent regions (asterisk) can be seen in areas of the cytoplasm where crystals have been digested. ×43 000.
Figs 9–12. For legend see p. 235.
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Figs 13–15. For legend see p. 235.
Figs 16–20. For legend see p. 235.
To interpret the structure and then determine the crystal interspacings, the specimens were rotated/tilted with respect to the electron beam and diffraction patterns were recorded at various angles. Thus, when a crystal is oriented in the specimen so that the electron beam becomes parallel to the crystallographic plane along its long axis, the image shows a sequence of parallel striations that correspond to the parallel planes (Fig. 9). The spacings between the striations are estimated to be about 100 Å. Tilting and rotating the specimen until it is observed parallel to the crystallographic planes along the short axis of the crystal, causes the orientation of the striations to change, but the spacings remain the same (Fig. 10). When the electron beam becomes simultaneously parallel to both of these planes, the internal structure of the crystal appears as a square lattice of electron-dense dots 70–80 Å in diameter, spaced at about 100 Å centre-to-centre (Fig. 11). Crystals oriented randomly with respect to the electron beam appear amorphous (Fig. 12). This is due to the disappearance of the crystallographic planes that are not in a Bragg reflection position. The diffraction patterns of the above images are shown in the corresponding insets to the figures.

Under high magnification the striations seem to consist of double helices bridged together via short fibrils (Figs 13, 14, 15). Our selection of right and left-handed helices, illustrated in Fig. 15, was arbitrary.

The formation of the crystalline inclusions seems to start in cytoplasmic regions where aligned dots accumulate (Fig. 16). The latter increase further in length and become organized into typical crystals, which are often grouped (Fig. 17). When observing the edges of striated crystal profiles they either appear sharp or demonstrate a stair-like construction (Fig. 18).

In the next stage of leaf differentiation, crystalline bodies in mesophyll chlorenchyma cells appear to be undergoing erosion (Fig. 19). This process begins from the outer part of the crystal and extends progressively into the inner lumen until finally the crystal completely disappears. This has been verified in leaves larger than 3 mm.

Identification of the proteinaceous nature of the crystals was done by treating the specimens with pepsin. As a result electron-transparent regions were formed where crystals initially existed (Fig. 20).

**DISCUSSION**

Profiles of cytoplasmic crystalline inclusions in leaf chlorenchyma cells of *Origanum* usually appear rectangular, trapezoidal and triangular. Combining these shapes leads us to conclude that, most probably, the three-dimensional form of these inclusions corresponds to a rectangular prism or a derivative (Fig. 8). Thus, when a crystal is sectioned parallel to the AA′-BB′ or BB′-CC′ planes, rectangles similar to those illustrated in Figs 4 and 5 are seen. Trapezoidal or triangular contours are obtained when crystals are obliquely sectioned (cf. Figs 6, 7).

Large or small aggregations of variously oriented aligned dots are thought to represent initial stages of crystal formation (cf. Wrischer, 1973, for plastid inclusions). These aligned dots later increase in length and become organized into parallel striations. Crystals seem to grow by deposition of new striations at their edges.
This process probably does not take place simultaneously throughout the crystal, as shown in Fig. 18, where boundary striations appear unequal (one striation is overlaid by another shorter one).

High magnification of the striations showed that they consist of double helices. This fact is further supported by the form of the diffraction patterns recorded when the crystal was tilted at various angles (Figs 9–11). Thus, the existence of three spots, i.e. the central one and the ± 1 order, indicates that the striations comprise a sinusoidal grating. This implies that the density of the two groups of striations follows the sinusoidal distribution, which occurs if each striation can be projected as an ellipsoid of revolution (Moore, 1972). Since the crystals have been proved by pepsin digestion to be proteinaceous, the above-mentioned helices most probably represent protein macromolecules. Lawton (1978), studying the P-protein crystals in sieve elements of pea hypocotyl, proposed a model according to which striations consist of helically wound (perhaps double) filaments. A similar twisted form of striation was also described by Reanudin & Cheguillaume (1977), in the cytoplasmic crystalline inclusions of Thesium humifusum haustoria. Observations made by Arsanto (1982) on the P-protein crystals of some dicotyledons, revealed that striations are composed of double helices 10–15 nm in diameter termed double helices 1 (DH 1). In cytoplasmic crystals of O. dictamnus the double chains constituting the striations wind around each other rather loosely, i.e. they appear in some sections as aligned loops (cf. Fig. 15). Hence, it is suggested that the dots identified in the striations or the square lattices correspond to the described loops, which become obvious when images are greatly enlarged.

Striations are 70–80 Å wide and are spaced about 100 Å apart in both dimensions. This indicates that the arrangement of the crystallographic planes possesses tetragonal symmetry. Several authors have measured the width and periodicity of the striations of cytoplasmic crystalline inclusions present in both plant and animal cells. As shown in Table 1, these parameters vary over a wide range among different species. Since such inclusions are generally believed to be proteinaceous (Thaler & Amelunxen, 1975; Perry & Snow, 1975), the disparity in their spacing could be attributed to internal factors determining their growth and organization (Cronshaw, Gilder & Stone, 1973).

With respect to the factors affecting crystal formation, May & Künkel (1975) have reported that under certain growth conditions the protein concentration of yeast cells increases, so that after osmotic shock crystalline bodies are formed. The latter disappear within 4 h, provided that osmotic shock is followed by growth of the cells. Physiological stress (removal of the root system) was also considered by De Greef & Verbelen (1973) to be a cause of crystal formation. Cran & Possingham (1972) found that in spinach leaves when photosynthetic activity ceased crystalline inclusions were formed in the chloroplast matrix, and disappeared after the leaves were illuminated.

Crystals may be formed in the cytoplasm as a result of a surplus protein (Yoo, 1970; Baker, Hall & Thorpe, 1978) or due to the presence of numerous ribosomes (Krishan, 1970). Bennet & Smith (1979) consider that cytoplasmic crystalline bodies are composed of tubulin derived from disintegrated microtubules (see also De Brabander,
Table 1. *Width and periodicity of the striations of cytoplasmic crystalline inclusions in plant and animal cells*

<table>
<thead>
<tr>
<th>Species</th>
<th>Width of striation (Å)</th>
<th>Spacings between striations (Å)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cicer arietinum</em> (sieve elements of hypocotyl)</td>
<td>100–150</td>
<td>100–150</td>
<td>Arsanto (1982)</td>
</tr>
<tr>
<td><em>Dryopteris filix-mas</em> (root apical meristem)</td>
<td>35</td>
<td>70</td>
<td>Elfvin (1971)</td>
</tr>
<tr>
<td><em>Gonatobotryum apiculatum</em> (conidiogenous cell apices)</td>
<td>70–90</td>
<td>Edmonds &amp; Nagy (1973)</td>
<td></td>
</tr>
<tr>
<td><em>Armadillo</em> (principal and holocristal cells of epididymis middle segment)</td>
<td>100</td>
<td>Burgess (1971)</td>
<td></td>
</tr>
<tr>
<td><em>Mouse</em> (follicular cells of thyroid)</td>
<td>120 (central body) 450 (tail)</td>
<td>Lawton (1978)</td>
<td></td>
</tr>
<tr>
<td><em>Human</em> (leukemic lymphoblasts)</td>
<td>250–280</td>
<td>Krishan (1970)</td>
<td></td>
</tr>
<tr>
<td><em>Phaseolus multiflorus</em> and <em>Pisum sativum</em> (sieve elements of hypocotyl)</td>
<td>50 105</td>
<td>Nagano &amp; Ohtsuki (1971)</td>
<td></td>
</tr>
<tr>
<td><em>Human</em> (adrenal cortex)</td>
<td>66</td>
<td>76</td>
<td>Magalhaes (1972)</td>
</tr>
<tr>
<td><em>Penicillium</em> (all cells in penicillus except phialides and conidia)</td>
<td>140</td>
<td>Metitiri &amp; Zachariah (1972)</td>
<td></td>
</tr>
<tr>
<td><em>Chick</em> (posterior necrotic zone of developing limb)</td>
<td>530–550</td>
<td>Mottet &amp; Hammar (1972)</td>
<td></td>
</tr>
<tr>
<td><em>Human</em> (testicular interstitial cells)</td>
<td>50 150</td>
<td>Renaudin &amp; Cheguillaume (1977)</td>
<td></td>
</tr>
<tr>
<td><em>Thesium humifusum</em> (haustoria)</td>
<td>100</td>
<td>80</td>
<td>Thaler &amp; Amelunxen (1975)</td>
</tr>
<tr>
<td><em>Salvia glutinosa</em> (glandular hairs of leaf and flower buds)</td>
<td>105</td>
<td>105</td>
<td>Thaler &amp; Amelunxen (1975)</td>
</tr>
<tr>
<td><em>Lilium tigrinum</em> (epidermal cells of pedicle, ovary and leaf)</td>
<td>70</td>
<td>250</td>
<td>Wergin &amp; Newcomb (1970)</td>
</tr>
<tr>
<td><em>Glycine max</em> (sieve elements of hypocotyl)</td>
<td>115</td>
<td></td>
<td></td>
</tr>
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Aerts, Van de Veire & Borgers, 1975). According to Newcomb (1967) and Deshpande
(1974), coated vesicles, presumably originating from the Golgi apparatus or the
endoplasmic reticulum, may also contribute to the formation of P-protein crystals in
sieve elements. Crystalline inclusions have also been identified in tissues infected by

The erosion often observed in the cytoplasmic crystals of young Origanum
mesophyll cells indicates that protein exists in a storage form and is used later by the
developing cells (see also Lyshede, 1980). Ross, Thorpe & Costerton (1973), studying
tobacco callus cultures, speculated that disintegration of the cytoplasmic crystals
results in the release of amino acids that were used for further synthetic processes in
the meristem. It is possible that kinetin plays an important role during this process
(Jakubek & Mlodzianowski, 1978).

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