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Cytochemical Localization of Cellulase in Glandular Trichomes of *Cannabis* (Cannabaceae)

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Cellulase reaction product was localized cytochemically at the ultrastructural level in the cell wall of disc cells, the secretory cavity and in the subcuticular wall of glands in *Cannabis*. Cellulase reaction product was evident in the less dense region of the disc cell wall prior to secretory cavity formation. Reactivity in this region was associated with separation of an outer zone, forming the subcuticular wall, from the inner wall zone adjacent to the plasma membrane of the disc cells. Reaction product was associated with the disc cell wall and fibrillar matrix extending from it into the secretory cavity. Reactivity remained evident over the subcuticular wall throughout enlargement of the secretory cavity. Reaction product also was present over fibrillar matrix in the secretory cavity associated with both the inner wall and the subcuticular wall. The distribution of cellulase reaction product supports an interpretation that cellulase is involved in formation of the secretory cavity and subsequent redistribution of wall products to form the subcuticular wall during development of the secretory cavity.

Key words: *Cannabis*, glands, celluase, cytochemistry, secretory cavity

The secretory cavity of glandular trichomes of *Cannabis* is formed by the periclinal splitting of the outer disc cell wall to initiate formation of an intrawall cavity(Hammond and Mahlberg, 1977; Kim and Mahlberg, 1991). The outer zone of this wall contributes to the subcuticular wall which, along with the cuticle, forms the sheath of the gland. The inner wall zone forms the wall of the disc cells facing the secretory cavity. Enlargement of the secretory cavity is associated with the accumulation of cannabinoids and other compounds secreted into this cavity from the disc cells (Lanyon, Turner and Mahlberg, 1981). Histochemical studies indicate that the subcuticular wall contains cellulosic components (Hammond and Mahlberg, 1978). The subcuticular wall and cuticle increase in thickness as the secretory cavity enlarges (Kim and Mahlberg, 1991; Mahlberg and Kim, 1991). This increase in thickness of the subcuticular wall during cavity enlargement requires a pool of precursors within the cavity. The fibrillar matrix derived from the disc cell wall appears to bethe source of new wall precursors transported through the cavity to fuse with the subcuticular wall (Kim and Mahlberg, 1995: Mahlberg and Kim, 1992). The splitting of the disc cell wall during formation of the secretory cavity suggested to the authors that wall-forming enzymes, such as cellulase, may contribute to the wall separation process. This report describes the cytochemical localization of cellulase in the glandular trichomes of *Cannabis*, and thus provides the first evidence for the possible involvement of this enzyme in secretory cavity formation in developing glands.

MATERIALS AND METHODS

Glandular trichomes were obtained from floral bracts on pistillate plants of a Mexican strain of *Cannabis* grown under greenhouse conditions (Hammond and Mahlberg, 1973). Tissues for morphological studies were prepared as previously described (Kim and Mahlberg, 1991). Cytochemical localization of cellulase reaction product as crystals of cuprous oxide at the ultrastructural level was performed as described by Bal (1974). Bractscontaining glands at different developmental stages were cut into small pieces and fixed in Karnovsky fixative (1965) at 0 C for 1 hr, rinsed in 0.1 M buffer, pH. 7.2, and stored in this buffer at 0 C for 16 hr. Tissues were incubated in 0.01 M phosphate containing 0.02% carboxymethyl

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cellulose (CMC) for 20 min at room temperature and then transferred to hot (85-95 C) Benedict's reagent for 5-10 min. Control tissues were incubated in phosphate buffer without CMC and also placed in Benedict's reagent. Other control tissues were fixed as above but not incubated in CMC or Benedict's reagent. Tissues were dehydrated in an ethanol-acetone series and embedded in Spurr's resin (1969). Thin sections were cut on an LKB-IV ultramicrotome, and collected on 200-mesh copper grids. Sections were stained with uranyl acetate followed by lead citrate, and examined with a Philips EM 300 transmission electron microscope at 60 kV accelerating voltage. Illustrations were selected from numerous micrographs of glands at different stages in development.

RESULTS

Gland formation

Gland initiation begins upon vertical enlargement of an epidermal cell that is subsequently bisected by an anticlinal division (Fig. 1; Kim and Mahlberg, 1991). A periclinal division separates an upper pair of cells, which will form the secretory tier, from the lower auxiliary tier. The lower tier typically undergoes a periclinal division giving rise to a tier of stipe cells and a lower tier of basal cells embedded in the epidermis. The secretory tier undergoes additional anticlinal divisions to form a tier of 8-13 disc cells. An intrawall secretory cavity is initiated by periclinal splitting of the outer wall of the disc cells. The outer portion of this wall forms the subcuticular wall under the cuticle. This wall and the cuticle compose the sheath of the secretory cavity. The accumulation of secretions in this secretory cavity results in an enlarged gland head delimited by the sheath.

Cellulase reaction product in the gland was detected by the formation of electron dense deposits associated with the disc cells and the developing secretory cavity. Although the heat required for the Benedict's reaction disrupted the fine structure of the cell, the position of crystalline deposits of reaction product and the secretory cavity associated with the disc cells were readily recognized.

Cellulase reaction product was detected in the cell wall at the periphery of the secretory cells prior to formation of the secretory cavity. It was evident in the outer zone of the cell wall around the entire periphery of the disc cells prior to secretory cavity formation (Fig. 2). The zone of reaction product in the

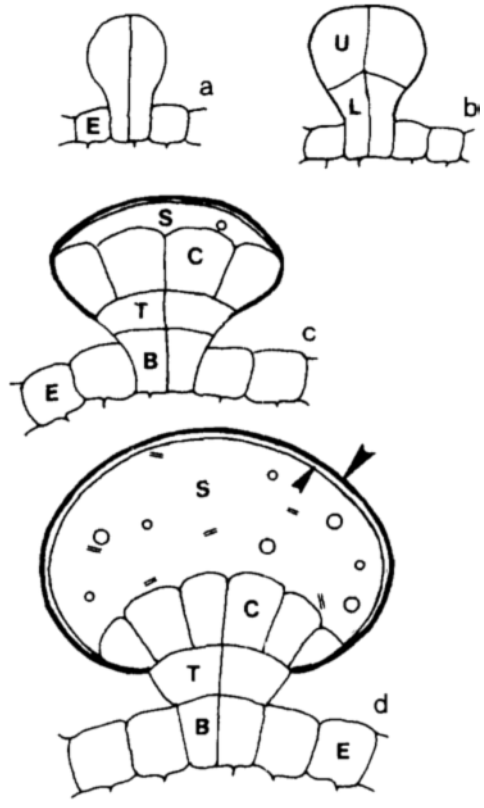
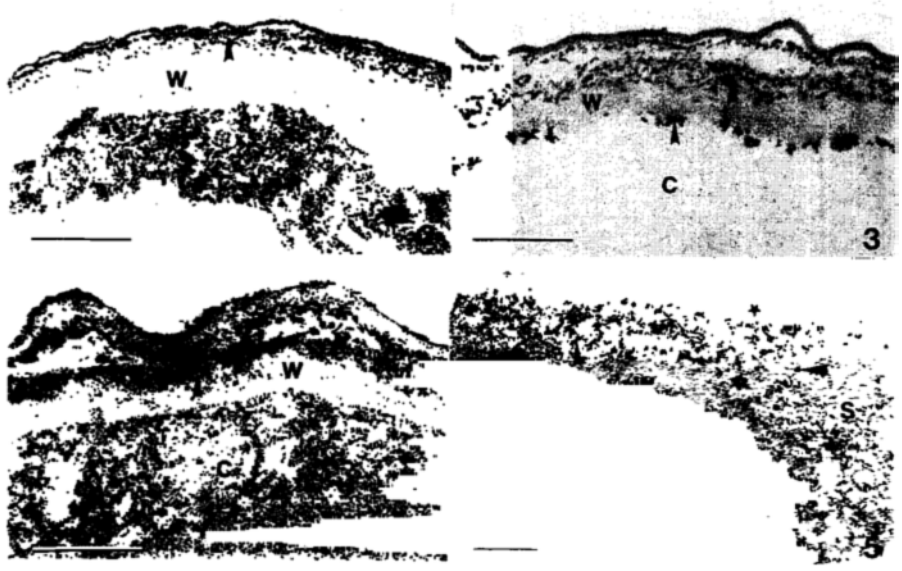


Fig. 1. Diagrammatic representation of glandular trichome development. a. Enlarged glandular trichome initial in epidermis (E) following anticlinal cell division. b. Periclinal division separates an upper (U) secretory cell tier from a lower (L) tier. c. Anticlinal divisions in upper tier form a tier of secretory disc cells (C) the outer wall of which splits tangentially to form the secretory cavity (S). A periclinal division in the lower tier forms the stipe (T) and basal (B) cells of gland embedded in epidermis (E). d. Mature gland consists of an enlarged secretory cavity (S) covered with a sheath consisting of cuticle (large arrowhead) and subcuticular wall (small arrowhead). Secretions and other components are present in the secretory cavity. O, secretory vesicle; =, fibrillar matrix.

wall appeared to increase in width and some reaction product became evident as a narrow nearly continuous layer immediately under the cuticle upon initiation of the secretory cavity (Fig. 3).

In glands with a more developed discoid head, but still prior to secretory cavity formation, the reaction product in disc cells was associated with the outer zone of the cell wall under the cuticle (Fig. 3). Reaction product also was evident along the plasma membrane surface of the cell wall at this developmental stage of the gland, but in subsequent stages it was not detectable at this position.



Figs. 2-5. Cellulase reaction product in glands. 2. Disc cell (C) in transection showing uniformity of reaction product (arrow) in outer zone of wall (W) under cuticle. Bar=0.5 μm. 3. Disc cell (C), in longisection showing reaction product along wall (W) surface adjacent to plasma membrane (small arrow) and in outer zone of wall (large arrow) under cuticle . Bar=0.5 μm. 4. Reaction product (arrow) was present in the less dense zone of the wall (W) and extended to the cuticle (star) as this zone separated from the more dense wall zone during initiation of secretory cavity above disc cells (C). Undulation of the thick cuticle indicated initial separation from wall layer. Bar=0.5 μm. 5. Reaction product (arrows) was abundant at and above surface of less dense wall (W) of disc cell (C) and in developing secretory cavity (S). It was also evident under cuticle (star). Fibrillar matrix in secretory cavity resembled wall material of the less dense zone of the disc cell wall. Bar=1.0 μm.

Secretory cavity stage

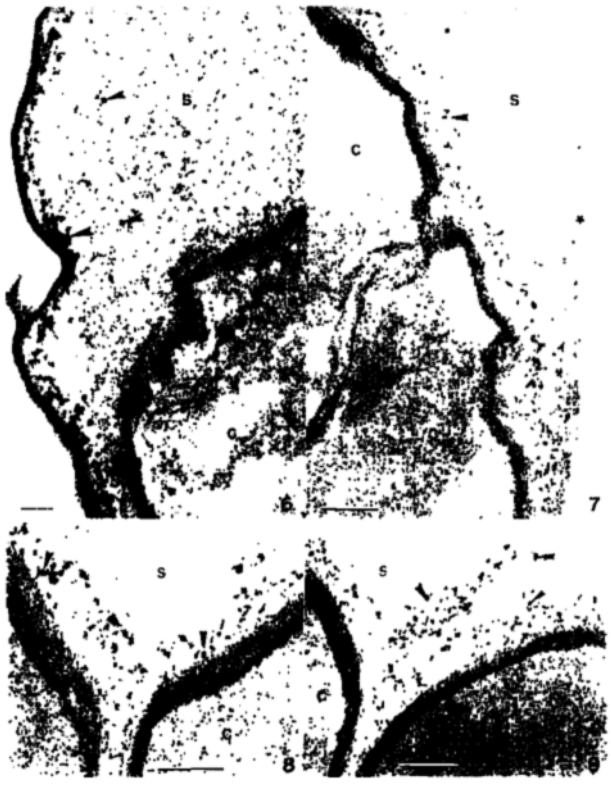
Upon initiation of the secretory cavity the wall region under the cuticle became differentially dense with the more dense region facing the plasma membrane surface (Fig. 4). Deposits of reaction product were most abundant in the less dense region facing the cuticular surface. The undulate character of the cuticle and subjacent wall was indicative of the initial phase in secretory cavity formation.

Reaction product remained associated with both the disc cell wall and subcuticular wall as they separated from each other during early enlargement of the secretory cavity (Fig. 5). The differential density of the wall became more evident in the early stage of secretory cavity development. The electron dense zone of the wall faced the disc cells whereas a less dense zone, approximately twice the thickness of the dense zone (Fig. 5), faced the secretory cavity.

In glands with an enlarged secretory cavity the reaction product was present along the subcuticular wall (Fig. 6). It also was evident in the secretory cavity as large deposits among a very small granule-like content of unknown character. Reaction product was less evident in the nonmedian (Fig. 6) than median sections of the disc cell wall where it occurred along the surface facing the secretory cavity (Fig. 7). Deposits of reaction product also were evident at localized sites in the cytoplasm of the disc cells (Figs. 6, 7). The particle size of these deposits was smaller than that associated with the wall and secretory cavity.

The relationships of the reaction product with the differentially dense wall and secretory cavity interface was evident in enlargements of this surface (Figs. 8, 9). The fibrous character of the wall was evident throughout the wall, including both the dense and less dense zones (Fig. 8). Reaction product was localized at the surface of the less dense wall as well as interspersed among fibrous-like wall materials. Little or no reaction product was associated with the more dense zone of the wall. The fibrillar matrix containing reaction product also was evident in the secretory cavity. Some of this matrix also was continuous with fibrous material in the wall. Quantities of fibrillar matrix containing reaction product, and separated from the wall in sectional view, were evident in the secretory cavity (Fig. 9).

Reaction product was associated with fibrillar ma-



Figs. 6-9. Cellulase reaction product in glands. 6. Reaction product lined the subcuticular surface (large arrow) of enlarged secretory cavity (S) and also was present (medium arrow) in secretory cavity. Localized deposits of reaction product(small arrow) occurred in disc cell (C). Cuticle at star. W, wall of disc cell. Bar=0.5μm. 7. Reaction product (large arrow) occurred along outer surface of less dense zone of disc cell wall (W) and extended to cell-cuticle juncture (lower right). It also occurred along the subcuticular surface above region shown here. Accumulations of reaction product of small-sized crystals (small arrows) occurred in cytoplasm of disc cell (C). Cuticle at star. Bar= 0.5 μm. 8. Fibrillar nature (small arrow and area above it) of wall (W) for adjacent cells was evident as fibrillar continuity with wall fibrils extending into secretory cavity (S). Reaction product was associated with surface of the less dense wall zone (large arrows) and with fibrillar matrix in secretory cavity(area between large arrows). Bar=0.5 μm. 9. Fibrillar matrix (large arrow) from less dense zone of wall (W) and associated reaction product appeared to be separated from the wall and present in secretory cavity (S). Individual particles of reaction product can be surrounded by fibrillar wall matrix (small arrows). The less dense wall also extended a short distance along the lateral wall region between the adjacent disc cells (C). Bar=0.5 μm.

Fig. 10-13. Cellulase reaction product in glands. 10. Reaction product at wall (W) surface typically consisted of individual particles (small arrow). Upon dissociation from wall surface the reaction product appeared to aggregate into clusters (large arrow) with wall matrix material in the secretory cavity (S). C, disc cell. Bar=0.5 μm. 11. Sheath and secretory cavity (S) showing reaction crystals of large size in the secretory cavity (large arrows) and adjacent to the subcuticular wall under cuticle (star). Crystal size of reaction product in subcuticular wall (small arrows) is smaller than that of crystals in secretory cavity. material Bar=0.5 μm. 12. Control showing absence of reaction product along wall (arrow) of the disc cell (C) and absence of reaction product in secretory cavity (S) and cytoplasm of disc cell (C). Bar=1.0 μm. 13. Control showing absence of reaction product along subcuticular cell wall (arrow) under the cuticle and in secretory cavity (S). Bar= 1.0 μm.

trix throughout the secretory cavity (Figs. 10, 11). Whereas the crystals of reaction product appeared small in size at the disc cell wall surface facing the secretory cavity, they frequently appeared to occur as aggregates over the fibrillar matrix in the secretory cavity.

Abundant reaction product was present in the subcuticular wall under the cuticle (Fig. 11). Crystalline deposits in this wall were small in size in contrast to larger aggregates associated with fibrillar matrix in the secretory cavity and with fibrillar matrix in contact with the subcuticular wall.

Epidermal cells of the bract also contained some cellulase reaction product in their outer wall, but it

was less dense than in the wall of disc cells. Epidermal cells did not develop the differential density of their wall or a secretory cavity as observed for disc cells (not shown).

Controls showed no cellulase reaction product in the cytoplasm or along the wall surface of the secretory cells (Fig. 12). Similarly, no reaction product was evident in the secretory cavity or in the subcuticular wall of controls (Fig. 13).

DISCUSSION

Cellulase activity enhanced by CMC substrate results in the formation of glucose which at high temperature reduces the cupric salts in Benedict's solution to form observable crystalline deposits of cuprous oxide (Bal, 1974). Bal reported that crystal size may be relatively small (about 20 nm) or they may aggregate into large clusters, often linear in form, within the cellulose fibril matrix. Cellulase localization was demonstrated with this method in cells of *Allium* (Bal, 1974) and the shoot apex of *Pisum sativum* (Maclachlan and Perrault,1964). This reaction product also demonstrated the association of cellulase activity with the development of wall articulations between component cells of articulated laticifer in *Papaver somniferum* (Nessler and Mahlberg, 1981).

The sheath of the secretory cavity of the gland in *Cannabis* has been described to consist only of cuticle (Amelunxen, 1965, Fridvalszky *et al.*, 1970). More recently it was shown that a subcuticular wall, including a possible cellulosic component, is present under the cuticle (Hammond and Mahlberg, 1978; Mahlberg *et al.*, 1984). Our morphological studies show the presence of a subcuticular wall originating from a periclinal splitting of the outer disc cell wall to form an intrawall secretory cavity (Kim and Mahlberg, 1991). The occurrence of cellulase activity in the subcuticular wall supports the interpretation that it functions to partially hydrolyze or loosen polysaccharide components in this wall, or to join fibrils of wall matrix into the developing subcuticular wall, or both activities, during expansion of the secretory cavity.

The presence of cellulase reaction product in the disc cell wall during progressive stages in secretory cavity development indicates its continuing role in wall development during enlargement of the secretory cavity. Cellulase may perform a role in the release of wall fibrils, as the fibrillar matrix, into the secretory cavity. This matrix appears to function as precursors for thickening of the subcuticular wall during enlargement of the secretory cavity (Kim and Mahlberg, 1991). Cellulase is a wall-bound enzyme affecting cellulose wall development (Fry, 1988), and its association with the disc cell wall and subcuticular wall, therefore, is consistent with data from other cells. Its association with cavity formation suggests that it performs the roles of loosening or partially degrading wall fibers in the disc cell wall during initiation of the cavity as well as dispersing fibrillar matrix into the secretory cavity where upon it contributes to the incorporation of this matrix into the subcuticular wall.

The detection of occasional deposits of apparent reaction product in the cytoplasm requires further study. Because crystals were very small in size they may represent a spurious deposits.

The distribution of reaction products of the secretory cavity during its development is interpreted to reflect its probable distribution although we recognize that the fixation process potentially can alter images within the cell. The differences in size of crystalline particles, those of small size associated with the disc cell wall and subcuticular wall in contrast to aggregates of particles within the secretory cavity, suggest that the reaction product reflects an actual distribution and was not mixed during tissue processing. Similarly, deposits in the cytoplasm are localized, not scattered, again supporting an interpretation that these images reflect the localization pattern at the time of fixation and processing.

Other wall-related enzymes may be associated with the formation of the intrawall cavity and the synthesis of the complex sheath of the secretory cavity. This cytochemical study, however, demonstrates a role for cellulase in development of the secretory cavity in the outer wall of the disc cells and in the sheath of the secretory cavity of these glandular trichomes.

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