Progressive Release of Carboxylating Enzymes during Mechanical Grinding of Sugar Cane Leaves

C. W. BALDRY, C. BUCKE and J. COOMBS
Tate & Lyle Limited, Research Centre, Westerham Road, Keston, Kent, England

Received January 4, 1971

Summary. The progressive release of protein, chlorophyll, phenol oxidase activity and phenolic compounds during the mechanical disruption of sugar cane leaves has been correlated with the release of carboxylating enzymes. Enzymes of the photosynthetic carbon reduction cycle were released in parallel with chlorophyll, the bulk of which was recovered in grana-containing chloroplasts. PEP carboxylase activity followed the release of total protein. Increased activities of the carboxylating enzymes were obtained in the presence of thioglycollate. There is evidence that PEP carboxylase resides in the cytoplasm rather than in either type of chloroplast. These results are discussed in relation to the possible localisation of carboxylation reactions in the sugar cane leaf.

β-carboxylation reactions are assuming greater significance in studies of photosynthetic CO₂ fixation by tropical grasses such as sugar cane (Kortschak et al., 1965; Hatch and Slack, 1966). It has been suggested that CO₂ is first incorporated into four-carbon organic acids, malic acid in particular, within the grana-containing chloroplasts of the mesophyll cells. The malic acid is then translocated to the bundle sheath cells where the CO₂ may be liberated and further metabolised through the conventional photosynthetic carbon reduction (PCR) cycle to produce sugars. The evidence for such a scheme has been reviewed recently by Hatch and Slack (1970).

We now report results of experiments which suggest that the specific location of the carboxylation enzymes in sugar cane leaves is still open to question. In these experiments cane leaves were progressively disrupted using a mechanical blender, and the release of carboxylation activity correlated with the release of protein, chlorophyll and inhibitory phenolic compounds.

Materials and Methods

Sugar cane was grown in a heated greenhouse maintained above 10°. The mid-ribs were removed from mature leaves and the laminae cut into 1 cm lengths. Thirty grams of leaf laminae were submerged in 200 ml grinding medium (sorbitol, 0.33 M; Na₄P₂O₇, 10 H₂O, 0.01 M; MgCl₂, 2 mM; adjusted with HCl to pH 6.5 at
Grinding Sugar Cane Leaves

room temperature). The tissue was macerated for 10 seconds in a domestic blender (Waring) running at full speed. The resultant brei was filtered through 4 layers of muslin and squeezed dry. The supernatant was kept and the cellular debris added back to the blender containing 200 ml of fresh medium and ground for a further 20 seconds. The brei was again filtered through muslin and the supernatant kept. This process was repeated so that the cellular debris were ground for two more periods of 30 seconds. Similar preparations were carried out with \(10^{-3}\) M thioglycolate incorporated into the grinding medium. Mixed chloroplasts were harvested by centrifugation and the two types separated on discontinuous sucrose density gradients as previously described (Baldry et al., 1968). Methods used for assaying various constituents and enzyme activities were described previously (Baldry et al., 1970a). Briefly: Chlorophyll was determined spectrophotometrically at 652 nm. Protein was estimated using Folin reagent. Phenolic compounds were extracted using 80% methanol in place of the grinding medium, the extracts concentrated and the components separated by high-voltage paper electrophoresis in borate buffer. The phenolic compounds were identified and quantitated by recording their absorption spectra between 250 and 400 nm. Enzymic CO₂ assimilation in the dark was determined by measuring the incorporation of \(^{14}\)CO₂ into acid-stable products. \(\beta\)-carboxylation activity was estimated by measuring CO₂ fixation catalysed by PEP carboxylase (E.C.4.1.1.31) with phosphoenol-pyruvate supplied as substrate. Photosynthetic carbon reduction (PCR) cycle activity was estimated in a similar manner with ribose-5-phosphate and ATP as substrates.

**Results**

The sequence of events which occurred during the mechanical disruption of cane leaf tissue can be correlated with the structure of the leaf. As shown in Fig. 1, the chlorophyll-containing cells, consisting of the mesophyll layer (m) and bundle sheath (bs), surround the vascular bundles (vb). Each region comprising vascular tissue and ancillary photosynthetic tissue is separated from the next by large colourless (non chlorophyll-containing) bulliform cells (b) and parenchyma (p). During the first 10 seconds of grinding these colourless cells were ruptured as the leaves were shredded parallel to the vascular bundles (Fig. 1b). During the second period of grinding these shreds were torn at right angles to the long axis of the leaf (Fig. 1c). At the same time xylem elements and fibres were pulled from the vascular bundles (Fig. 1f). Further grinding led to a complete breakdown of the organised structure of the leaf (Figs. 1d, g). Blocks of mesophyll cells (m), strips of epidermis (e) and free vascular elements (j) were released. During the final 30 seconds' grinding the tissue was further disrupted so that finally almost all the cells had been ruptured.

Throughout the grinding process the progressive release of cellular components and enzyme activities was followed by recording the amounts recovered in a given supernatant. These were expressed as a percentage of the total amounts recovered in all four supernatants from each leaf sample. The total recoveries of various components investigated