Decarboxylative metabolism of \([1'-^{14}C]\)indole-3-acetic acid by tomato pericarp discs during ripening

Effects of wounding and ethylene

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Abstract. The rate of decarboxylation of \([1'-^{14}C]\)indole-3-acetic acid (IAA) infiltrated into tomato (Lycopersicon esculentum Mill.) pericarp discs was much more rapid in green than in breaker and pink tissues. Studies were carried out in order to determine whether the decarboxylative catabolism occurring in the green pericarp discs was associated with ripening or was a consequence of wound-induced peroxidase activity and/or ethylene production. After a 2-h lag, the decarboxylative capacity of the green pericarp discs increased exponentially during a 24-h incubation period. This increase was accompanied by increases in IAA-oxidase activity in cell-free preparations from the intercellular space and cut surface of the discs. Although higher IAA-oxidase activity was detected in extracts from the tissue residue, which comprises mainly intracellular peroxidases, this activity did not increase during the 24-h incubation period. Analysis of the cell-free preparations by isoelectric focusing revealed the major component in all samples was a highly anionic peroxidase (pI = 3.5) the levels of which did not increase during incubation. However, the intercellular and cut-surface preparations contained additional anionic and cationic peroxidases which increased in parallel with the increases in both the IAA-oxidase activity of the preparations and the decarboxylative capacity of the green pericarp discs from which they were derived. Treatment of green discs with the ethylene-biosynthesis inhibitors aminoxyacetic acid and CoCl₂, inhibited the development of an enhanced capacity to decarboxylate \([1'-^{14}C]\)IAA but the inhibition was not counteracted by exogenous ethylene. Another ethylene-biosynthesis inhibitor, aminoethoxyvinyl glycine, also reduced ethylene levels but did not affect IAA decarboxylation, indicating that the decarboxylation was not a consequence of wound-induced ethylene production. The data obtained thus demonstrate that the enhanced capacity to decarboxylate \([1'-^{14}C]\)IAA that develops in green tomato pericarp discs following excision is not associated with ripening but instead is attributable to a wound-induced increase in anionic and cationic peroxidase activity in the intercellular fluid and at the cut surface of the excised tissues.

Key words: Ethylene and fruit ripening – Fruit ripening – Indole-3-acetic acid (decarboxylative catabolism) – Lycopersicon (auxin metabolism) – Peroxidase

Introduction

It has been proposed that auxins act as inhibitors of fruit ripening and that endogenous IAA levels fall either during or prior to the onset of ripening (Frenkel 1972; Frenkel and Dick 1973; McGlasson et al. 1978; Brady 1987). The size of endogenous IAA pools is controlled by the relative rates of IAA biosynthesis, catabolism and conjugation. Conjugation of IAA leads to the production of ester and amide conjugates (Cohen and Bandurski 1982) while catabolism can utilise nondecarboxylative oxidation pathways (Reinecke and Bandurski 1987) as well as decarboxylative routes (Sandberg et al. 1987). There are reports suggesting that peroxidase-catalysed decarboxylation of IAA reduces endogenous IAA levels during the ripening of apples (Gorin and Heidema 1976), grapes (Kochhar et al. 1979), papaya fruit (Da Silva et al. 1990) and tomato (Frenkel 1972; Thomas et al. 1981; Brooks 1986; Rothan and Nicholas 1989). These findings were based on in-vitro test systems in which peroxidase-IAA-oxidase activity was monitored in tissue homogenates and partially purified extracts. Caution should, however, be exercised when relating such data to intact fruit where cellular compartmentation is maintained. In-vivo metabolic studies with whole plants or organs are in turn not completely straightforward being compounded by tissue heterogeneity and the degree of penetration of the applied substrates to endogenous loci.

Abbreviations: AOA = aminoxyacetic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; AVG = aminoethoxyvinyl glycine; IAA = indole-3-acetic acid

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When investigating fruit ripening, the use of excised tissue discs offers an alternative to the traditional in-vitro and in-vivo test systems as it facilitates (i) the separation of various aspects of the ripening process by isolation of specific tissues, (ii) quantitative addition of substrates and inhibitors and (iii) the precise measurement of local processes through the analysis of replicate samples of uniform material (Campbell et al. 1990). However, since mechanical damage is incurred when discs of tissues are excised, it is important to assess the possible effects of wound-enhanced increases in ethylene production (Yang and Hoffman 1984) and peroxidase activity (Castillo 1986) on IAA metabolism.

Ethylene levels rise during the ripening of climacteric fruit, such as tomato, and there are reports that ethylene treatment increases decarboxylation of IAA in some plant tissues (Beyer and Morgan 1970; Aharoni 1985; Sagee et al. 1990). It is possible, therefore, that ethylene-induced ripening of climacteric fruit is a consequence of a lowering of endogenous IAA levels brought about by ethylene-mediated increases in IAA decarboxylation (Mousdale and Knee 1981). Ethylene is also produced as a wound response to mechanical damage incurred when discs of tissue are excised (Saltveit and Dilley 1978). In these circumstances, at least part of the decarboxylation of IAA occurring in excised discs may be attributable to wound-induced ethylene production. Similar complications arise when assessing the potential role of IAA-degrading peroxidases in regulating endogenous IAA levels during ripening as, like ethylene, peroxidase levels increase both during ripening and in response to damage incurred during the isolation of tissue-specific segments (Lagrimini and Rothstein 1987; Svalheim and Robertsen 1990).

In our previous studies with excised tomato pericarp discs, it was observed that [1-14C]IAA was metabolised primarily to indole-3-acetylserine, indole-3-acetyl-β-d-glucose and other polar, non-decarboxylated products (Catalfi et al. 1992). Decarboxylative oxidation pathways accounted for 10–16% of IAA catabolism (Catalá 1991). This report concerns an investigation of IAA decarboxylation in excised tomato pericarp discs in which experiments were designed to distinguish between metabolism associated with ripening and that occurring as a consequence of wound-induced peroxidase and/or ethylene production.

Materials and methods

Plant material. Fruits of tomato (Lycopersicon esculentum Mill. cv. Moneymaker) were grown in a greenhouse and fruit were selected at three stages, namely green at 80% of colour break, at colour break and red at 40% of colour break. In order to investigate the effects of wounding on the ability of the tomato pericarp to decarboxylate IAA, excision discs were placed in a moist environment in darkness at 25°C. After specified intervals, triplicate samples of discs were removed, infiltrated with [1-14C]IAA under vacuum and 14CO2 evolution during a 1-h incubation period determined as described above.

Incubation conditions. Fruits were surface-sterilised with 0.2% sodium hypochlorite before being rinsed with sterile distilled water. Pericarp discs were cut from the equatorial part of the fruit with a stainless-steel cork borer (8 mm i.d.). Disks were rinsed with distilled water, blotted with filter paper and triplicates weighing ca. 0.8 g were vacuum-infiltrated at −40 kPa for 5 min in 2 ml 20 mM citrate-phosphate buffer (pH 4.5), containing 50 μg chloramphenicol ml−1 and 3.4 μM [1-14C]IAA (specific activity 2.04 GBq mmol−1 (Amersham International plc, Amersham, Bucks., UK) or 222 MBq mmol−1 (Sigma, St. Louis, Mo., USA)). After releasing the vacuum, the discs were kept submerged in the incubating solution for 20 min before being rinsed in buffer for 1 min. The pericarp discs were then incubated in darkness at 25°C in 25-ml containers through which ethylene-free air was circulated at 22 ml min−1. To measure [1-14C]IAA uptake by the tomato pericarp tissues prior to incubation, triplicate excised discs were homogenised in methanol (4 ml g−1 FW) and extracted for 20 h at 4°C before being centrifuged at 3000 g for 10 min. Radioactivity in 100-μl aliquots of the methanolic supernatant was then determined by liquid scintillation counting using a Rackbeta 1217 liquid scintillation counter (LKB, Turku, Finland).

Decarboxylation measurements. In studies on the rate of decarboxylation of [1-14C]IAA, 14CO2 output, was determined by bubbling air leaving the 25-ml incubation container through a CO2 trap comprising 5 ml scintillant (0.7 g Fluoroloy dry mix [Beckman Instruments, Madrid] in toluene/phenylethylamine/water/methanol, 40:35:5:2 v/v) in a U-tube. At specified intervals, the CO2 trap was replaced and the 14C-content of the scintillant determined by liquid scintillation counting.

Effects of wounding on the decarboxylative capacity of pericarp discs. In order to investigate the effects of wounding on the ability of the tomato pericarp to decarboxylate IAA, after excision discs were placed in a moist environment in darkness at 25°C. After specified intervals, triplicate samples of discs were removed, infiltrated with [1-14C]IAA under vacuum and 14CO2 evolution during a 1-h incubation period determined as described above.

Assay of peroxidase-IAA-oxidase activity. Peroxidases released from the cut surface of excised pericarp discs were extracted by rinsing triplicate discs for 3 min in 2.5 ml 20 mM citrate-phosphate buffer (pH 4.5). A modification of the method of Rathmell and Sequeira (1974) and Castillo et al. (1984) was used to extract peroxidases from the intercellular spaces. This involved subjecting discs to vacuum-infiltration, in 2 ml 20 mM citrate-phosphate buffer (pH 4.5) containing 0.1 M KCl, for 1 min at −66.7 kPa. Ten minutes after releasing the vacuum the discs were blotted to remove excess liquid and triplicate samples placed in a 10-ml syringe with a stainless-steel mesh at the base of the barrel and the tip inserted in a 2-ml Eppendorf tube. After centrifugation at 1500 g for 15 min, the intercellular fluid of the Eppendorf tube was collected and the tissues ground in liquid N2 before being macerated in 20 mM citrate-phosphate buffer (pH 4.5) containing 0.1 M KCl and 1% (w/v) insoluble polyvinylpolypyrrolidone (5 ml g−1) with a Polytron homogeniser. The resulting homogenate was centrifuged at 22 000 g for 20 min and the supernatant collected and treated as the residual soluble fraction. Peroxidase-IAA-oxidase activity of the three fractions was assayed by measuring IAA decarboxylation in a 3-ml reaction mixture containing 73 mM acetate-phosphate buffer (pH 5.0) 1.0 mM [1-14C]IAA (1.3 kBq mmol−1), 1 mM 2,4-dichlorophenol and 1 mM MnCl2 incubated for 2 h at 25°C. Prior to isoelectric focusing the cell-free extracts were desalted by passing through a Sephacryl G-25 minicolumn (Pharmacia, Uppsala, Sweden) after which cut-surface washes were then incubated in darkness at 25°C in 25-ml containers and extracted for 20 h at 4°C before being centrifuged at 22 000 g for 10 min. Radioactivity in 100-μl aliquots of the methanolic supernatant was then determined by liquid scintillation counting using a Rackbeta 1217 liquid scintillation counter (LKB, Turku, Finland).

Isoelectric focusing. Flat-bed isoelectric focusing was carried out on Ampholine PAGplate 5% polyacrylamide according to the manufacturer’s instructions (Pharmacia). After 15 min pre-focusing at 2 W constant power, 10-μl samples and protein calibration standards (pH 3–10) were applied to the gel. Isoelectric focusing was then run at 3 W constant power for 90 min. Samples were stained for peroxidase with 1.3 mM benzidine and 3 mM H2O2 in 0.2 M sodium-acetate buffer (pH 5) for 30 min, while protein standards were stained with Coomassie brilliant blue.