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Peroxidase in unripe and processing-ripe strawberries

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The activity, enzymatic polymorphism and tissue localization of peroxidase in unripe and processing-ripe strawberries (*Fragaria ananassa* var. Oso Grande) was studied. The results showed that both unripe and processing-ripe strawberries contain a peroxidase isoenzyme of basic pI, which is the only component of peroxidase polymorphism in the fruits and which is mainly localized in the concentric array of the vascular bundles and in the vascular connections with the seeds. This isoenzyme is also located in the epidermal cells. The activity of this basic peroxidase isoenzyme, which is apparently bound to acidic polysaccharides during extraction and from which it may be solubilized by treatment with Caylase 345L, did not vary with ripening. From these results, it may be concluded that this basic peroxidase isoenzyme, similar to its homologous isoenzyme in grape berries, is involved in the lignification of the vascular tissues during fruit development.

INTRODUCTION

The regulation of fruit ripening at the molecular level is clearly understood in climacteric fruit but not so well understood in non-climacteric fruit such as the strawberry (Given *et al.*, 1988; Cheng & Breen, 1991). The principal distinctive developmental features of strawberries, such as the loss of astringency and the appearance of the characteristic colour at ripening, are related to changes in the synthesis and accumulation of phenolic compounds (Cheng & Breen, 1991). Information concerning phenol metabolism is important because phenols influence the quality of ripe strawberries and their related processed products.

To understand the phenolic metabolism of strawberries, it is necessary to study their biosynthetic and degradative reactions. These are mainly catalysed by enzymes, among which is found peroxidase (EC 1.11.1.7). Robinson (1991) reviewed the physiological role of peroxidases in postharvest fruits and vegetables and attributed many of the physiological functions to phenol oxidation, including lignin biosynthesis. In fact, phenolic oxidation mediated by peroxidase is believed to be associated with a deterioration in flavour, colour, texture and the nutritional qualities of processed foods (Haard, 1977; Vámos-Vigyázó, 1981; Robinson, 1991).

With this in mind, the aim of the present work was to study the histochemical localization of peroxidase and its activity and enzymatic polymorphism in unripe and processing-ripe strawberries.

MATERIALS AND METHODS

Plant material

Strawberries (*Fragaria ananassa* var. Oso Grande) were grown in field at Lepe (Huelva, Spain) and sampled at the unripe (pale green to whitish coloured berries) and at the processing-ripe (dark red colour, slightly soft) stage. Fruits were frozen at –20°C for 8 months until analysis.

Histochemical localization of peroxidase

Dry 0·45 μm nitrocellulose membrane filters (Bio-Rad Laboratories, Richmond, CA) were placed on microscope slides and blotted with a tissue section (Calderón *et al.*, 1993). A thin section from the opposite extremity to the pedicle of frozen unripe strawberries was cut freehand with a scalpel, and the sections thawed at 25°C. Thawing sections were placed on the nitrocellulose membrane, and then lightly pressed on it for 30 s. The sections were carefully removed and the dry membrane incubated at 25°C in a solution containing 1·0 mM 4-methoxy-α-naphthol, 0·33 mM H_2O_2, and 0·1 M Tris (tris–[hydroxymethyl]-aminomethane)–acetate buffer, pH 5·0 (Calderón *et al.*, 1993). Controls were carried out in the absence of H_2O_2. After staining, the membranes were rinsed with distilled water and air-dried.

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Tissue homogenization and peroxidase fractions

Thawed strawberries were homogenized in a mortar with pestle at 4°C in the presence of 5 mM $CaCl_2$, 6 mM ascorbic acid, 1·0 M LiCl, 250 mM Tris–HCl buffer, pH 7·5, containing polyvinylpolypyrrolidone in the ratio of 0·1 g/1·0 g fresh tissue. The homogenate was centrifuged at 27000 *g* for 20 min, and the supernatant dialysed overnight against 50 mM Tris–HCl buffer, pH 7·5. Unless otherwise stated, the dialysed extracts were treated with 1·0 mg/ml Caylase 345L (Cayla, Toulouse, France) for 1 h at 30°C and centrifuged at 15000 *g* for 15 min. The supernatant constituted the soluble protein fraction used in further studies.

Determination of peroxidase activity

Peroxidase activity was determined using 1·0 mM 4-methoxy-α-naphthol, and 0·33 mM H_2O_2 in 100 mM Tris–acetate buffer, pH 5·0 (Ferrer *et al.*, 1990). Enzyme amounts were expressed in nkat using a $\epsilon_{593} = 2{\cdot}1 \times 10^4$ M^{-1} cm^{-1} for the dye product.

Analytical isoelectric focusing and peroxidase staining

Peroxidase isoenzymes were separated by analytical isoelectric focusing on polyacrylamide gels, as described by Calderón *et al.* (1990). The staining of peroxidase isoenzymes with 4-methoxy-α-naphthol was performed using 1·0 mM 4-methoxy-α-naphthol, 0·33 mM H_2O_2 and 0·1 M Tris–acetate buffer, pH 5·0 (Ferrer *et al.*, 1990).

RESULTS AND DISCUSSION

Peroxidase localization in unripe and processing-ripe strawberries

Tissue printing has become an important tool in visualizing peroxidase localization in plant tissues, especially in fleshy fruits (Calderón *et al.*, 1993; Bernal *et al.*, 1994; Ros Barceló *et al.*, 1994). This technique consists of blotting fresh tissue sections on to nitrocellulose membranes, using the properties of nitrocellulose to absorb and retain proteins (Nibbering *et al.*, 1986), and subsequent detection of the enzyme activities on the blots by means of simple histochemical procedures.

Unripe and processing-ripe strawberries were used in this study. In unripe strawberries, blotting of thawed sections on nitrocellulose membranes and subsequent staining with 4-methoxy-α-naphthol and H_2O_2 revealed that most of the peroxidase activity is present in the concentric array of the vascular bundles (Fig. 1) and in the vascular connections with the seeds (Fig. 1, arrowheads). Peroxidase-mediated reaction products are also located in the epidermal cells (Fig. 1, arrow). These reactions were sensitive to the omission of H_2O_2 and were therefore due to peroxidase activities (data not shown). Processing-ripe strawberries showed a similar pattern for peroxidase localization in the vascular tissues.

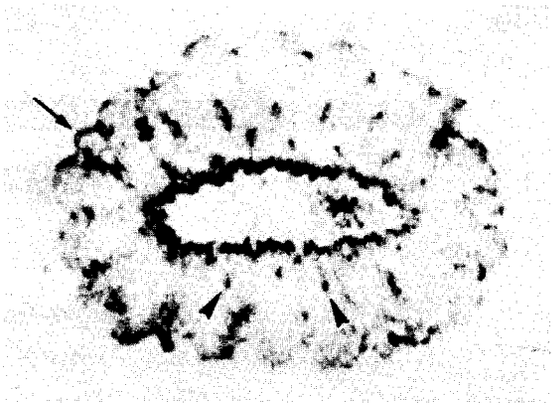

Fig. 1. Histochemical blot of thawing sections of an unripe strawberry after staining with 4-methoxy-α-naphthol and H_2O_2, showing the presence of peroxidase-mediated reaction products at the level of the concentric vascular bundles, vascular connections with the seeds (arrowheads), and epidermis (arrow).

A common characteristic of all the blotted thawed strawberry sections was the preservation of the vascular structure, as can be observed from the concentric distribution of the vascular bundles in the cross-sections (Fig. 1). These results suggest that tissue blotting of thawed tissues followed by histochemical staining may be a valuable tool in determining the localization of peroxidase in strawberries.

Peroxidase activity in unripe and processing-ripe strawberries

The measurement of peroxidase activity in homogenates of strawberries, as in other plant materials (Draetta & Ben-Shalom, 1984), is severely affected by the formation of protein–pectin complexes (Spayd & Morris, 1981). These last authors used $CaCl_2$ to selectively precipitate pectins. However, this treatment in itself did not solve the problem since it did not provoke

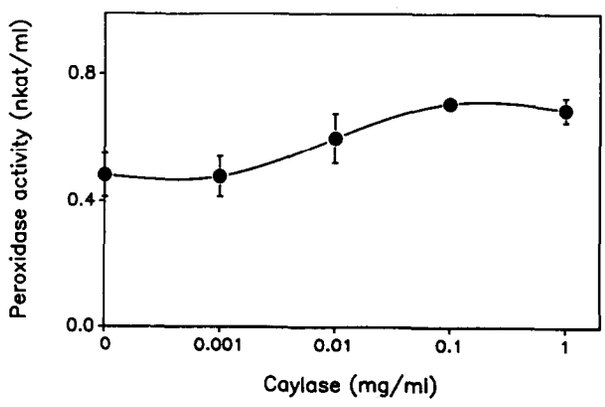

Fig. 2. Effect of the Caylase 345L concentration on the level of peroxidase activity recovered in the supernatants of processing-ripe strawberry homogenates after centrifugation at 15000 *g* for 15 min. Bars show SE ($n = 4$).

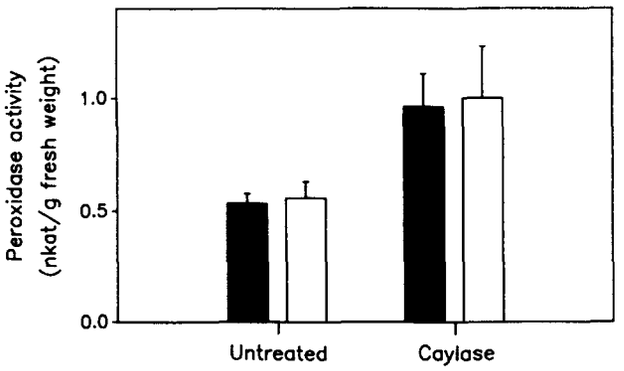

Fig. 3. Levels of peroxidase activity in unripe (■) and processing-ripe (□) strawberries when homogenates were untreated or treated with 1·0 mg/ml Caylase 345L for 1 h. Bars show SE (n = 4).

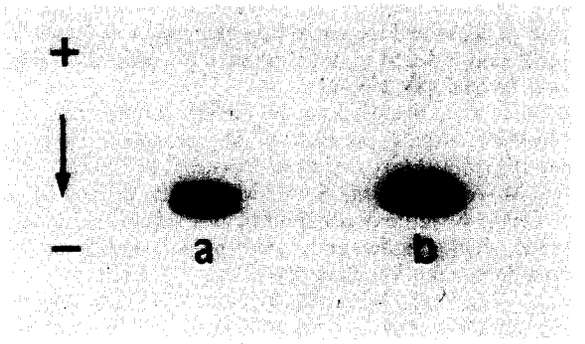

Fig. 4. Isoenzyme patterns of the peroxidase activity present in unripe (lane a) and processing-ripe (lane b) strawberries after staining with 4-methoxy-α-naphthol and H_2O_2, showing the presence of only one strongly basic peroxidase isoenzyme (pI > 9·0). Arrow indicates the direction of protein migration during isoelectric focusing.

the clarification of the protein extracts. For this reason, enzyme extracts of strawberries were treated with several concentrations of Caylase 345L, a highly active cellulolytic and pectolytic enzyme. These treatments were followed by centrifugation of the Caylase-treated protein extracts. The results showed (Fig. 2) that Caylase, used at 0·1–1·0 mg/ml, was an effective clarifier of protein extracts, solubilizing some peroxidase activity, which was estimated in the order of 45–50%.

The estimation of peroxidase activity in crude protein extracts treated with Caylase 345L showed that peroxidase activity in strawberries did not vary with ripening (Fig. 3). In fact, the peroxidase activity of unripe and processing-ripe strawberries was almost identical in both untreated and Caylase-345L-treated protein extracts (Fig. 3). These results are not surprising if it is remembered that peroxidase activity in strawberries is mainly located in the vascular vessels (Fig. 1), and that the vascular development in strawberries has presumably reached its last stages in unripe strawberries. These results differ from those obtained in other non-climacteric fleshy fruits, in which peroxidase activity is mainly located in the epidermis (Calderón *et al.*, 1993; Bernal *et al.*, 1994; Ros Barceló *et al.*, 1994), and in which peroxidase activity increases with ripening (Calderón *et al.*, 1993).

Peroxidase isoenzyme patterns in unripe and processing-ripe strawberries

Peroxidase isoenzyme patterns are frequently age-specific (Ros Barceló & Muñoz, 1992). For this reason, isoenzyme analysis of the peroxidase activity found in unripe and processing-ripe strawberries was carried out by protein isoelectric focusing in 3·5–10·0 pH gradients. The results shown in Fig. 4 illustrate the similarity between the peroxidase isoenzyme patterns of unripe and processing-ripe strawberries. This is characterized by the presence of one sole peroxidase isoenzyme of a basic nature and homologous, as regards isoelectric point, to the grapevine basic peroxidase isoenzyme, B_5, previously identified in cell walls and vacuoles (Calderón *et al.*, 1992; Pedreño *et al.*, 1993), and for which a role in the lignification of xylem vessels embedded in the mesocarp tissues has been proposed (Morales *et al.*, 1993). A similar role may be played by this basic peroxidase isoenzyme in strawberries, since this isoenzyme is mainly expressed in the vascular bundles (Fig. 1). In this context, Abeles and Biles (1991) have recently reported that a basic peroxidase isoenzyme is responsible for lignification in peach fruit endocarp, similar to that which occurs during tracheid differentiation in plant cell cultures (Church & Galston, 1988).

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