Food Chemistry **48** (1993) 391–394

Purification and kinetic characterization of a basic peroxidase isoenzyme responsible for lignification in Gamay rouge grape (*Vitis vinifera*) berries

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(Received 13 January 1993; revised version received and accepted 2 February 1993)

Gamay rouge grape (*Vitis vinifera*) berries contain a peroxidase isoenzyme of basic pI, the peroxidase isoenzyme B_5, which is the major component of peroxidase polymorphism in the whole fruit, and is localized in xylem vessels of grape berries at pigmentation. This isoenzyme has been purified by preparative isoelectric focusing in glycerol-stabilized 3·0–10·0 pH gradients, and characterized as regards its catalytic properties against coniferyl alcohol. The results showed that this isoenzyme is capable of oxidizing coniferyl alcohol with an optimal pH in the range 3·0–6·0. K_m values were 0·149 mM for coniferyl alcohol and 0·206 mM for H_2O_2. These results suggest that, although the affinity of Gamay rouge peroxidase B_5 towards the lignification substrates is low compared with that shown by other peroxidases involved in lignin biosynthesis, participation of this isoenzyme in the lignification of xylem vessels of Gamay rouge grape berries should be taken into account.

INTRODUCTION

Peroxidase (EC 1.11.1.7) is a ubiquitous enzyme in plant cells and is related to food quality in processing, particularly as regards the flavour of both raw foods and processed products (Vámos-Vigyázó, 1981). 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 nutritional qualities of processed foods and their products (Robinson, 1991).

In Gamay rouge grape (*Vitis vinifera*) berries at pigmentation, peroxidase activity is mainly due to a basic peroxidase isoenzyme (isoenzyme B_5) located in both mesocarp and hypodermal tissues (Calderón *et al.*, 1993). At mesocarp level, this peroxidase isoenzyme is located in the vascular bundles, probably associated with xylem vessels (Calderón *et al.*, 1993). Since this isoenzyme is located in cell walls (García-Florenciano *et al.*, 1991; Calderón *et al.*, 1992*a*), its participation in the lignification of xylem vessels embedded in the mesocarp tissues cannot be discounted. In fact, Abeles & 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).

With this in mind, the aim of the present work was to purify and to characterize this peroxidase isoenzyme with respect to its catalytic properties during the oxidation of coniferyl alcohol, a substrate of lignin biosynthesis.

MATERIALS AND METHODS

Plant material

The *Vitis vinifera* cultivar Gamay rouge was grown in fields at the 'Hacienda Nueva' Viticultural Experimental Station of the CRIA (Murcia, Spain) and sampled in August 1992 at pigmentation. Clusters were transported to the laboratory and frozen immediately at −30°C for later analysis.

Tissue homogenization and peroxidase fractions

After removal of the seeds, the grapes were homogenized in a mortar and pestle at 4°C in the presence of

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50 mM ammonium tartrate, 6 mM ascorbic acid, 1·0 M LiCl, 250 mM Tris [tris(hydroxymethyl)aminomethane]–HCl buffer (pH 7·5), containing polyvinylpolypyrrolidone in a ratio of 0·1 g/1·0 g fresh tissue (Calderón *et al.*, 1993). The homogenate was centrifuged at 20 000 *g* for 15 min and the supernatant dialysed overnight against 50 mM Tris–HCl buffer (pH 7·5). The dialysed extracts constituted the soluble protein fraction used in further studies.

Purification of the basic peroxidase isoenzyme B_5 by preparative isoelectric focusing

Preparative isoelectric focusing (IEF) was carried out using a Rotofor™ preparative IEF cell (Bio-Rad Laboratories, Richmond, CA, USA). For this, protein (115 mg) from grape berry homogenates was dialysed overnight against 4 litres of twice-distilled deionized water. This protein fraction was supplemented with 10% (v/v) glycerol and 1·5% (v/v) Bio-Lyte Ampholytes (Bio-Rad), pH range 3–10, and loaded into the Rotofor cell. Isoelectric focusing followed the *Rotofor Cell Instruction Manual* (Bio-Rad) with minor modifications. Pre-running was carried out: the focusing chamber was filled with 55 ml of distilled water and run at 5 W constant power for 5 min, using 0·1 M H_3PO_4 as electrolyte for the anode and 0·1 M NaOH as electrolyte for the cathode. For the purification of basic peroxidase isoenzyme B_5 in the Rotofor cell, optimal conditions were a constant voltage of 800 V for 16 h at 4°C for a starting current of 15–20 mA.

Once focusing was completed, the contents of the electrofocusing cell were fractionated in 20 aliquots ranging from acidic to basic pI proteins. Ampholytes and glycerol were removed from protein fractions by incubation in 1·0 M NaCl for 45 min at 4°C, and further dialysis overnight against 5 mM Tris–HCl buffer (pH 7·5).

Determination of peroxidase activity and protein

The determination of peroxidase activity with 4-methoxy-α-naphthol, *o*-phenylenediamine and guaiacol was carried out as previously described (Zapata *et al.*, 1992*b*). The assay of peroxidase activity with syringaldazine was performed as reported by Goldberg *et al.* (1985).

Unless otherwise noted, the measurement of peroxidase activity with coniferyl alcohol was performed as described by Pedreño *et al.* (1989) and Calderón *et al.* (1992*b*), in 0·1 M Tris–acetate buffer (pH 5·0), in the presence of 5·0 mM H_2O_2 and 0·1 mM coniferyl alcohol. An ε_{260} value of $2{\cdot}2 \times 10^3$ M^{-1} cm^{-1} was used for the calculation of the oxidation rate.

The protein content in each fraction was estimated according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Analytical isoelectric focusing and peroxidase staining

Separation of peroxidase isoenzymes by analytical isoelectric focusing on polyacrylamide gels was carried out as described by Calderón *et al.* (1990). Staining of peroxidase isoenzymes with 4-methoxy-α-naphthol was performed using 1·0 mM 4-methoxy-α-naphthol, 1·0 mM H_2O_2 and 0·1 M Tris—acetate buffer (pH 5·0) (Ferrer *et al.*, 1990).

RESULTS AND DISCUSSION

The measurement of peroxidase activity in Gamay rouge grapes at pigmentation (harvested 16 August 1992) with four different hydrogen donors (4-methoxy-α-naphthol, *o*-phenylenediamine, coniferyl alcohol and guaiacol) reveals that coniferyl alcohol is the best substrate (Zapata *et al.*, 1992*b*). This is especially apparent when comparing the peroxidase activities with different hydrogen donors normalized by dividing them by the activity measured when using guaiacol as substrate. Although the use of this ratio for comparing enzymic activities is debatable, several authors have considered the enzymic activity ratio against guaiacol as suitable for comparative purposes (Gove & Hoyle, 1975; Mäder *et al.*, 1977; Ferrer *et al.*, 1992). Thus, this ratio was 0·944 for 4-methoxy-α-naphthol, 3·48 for *o*-phenylenediamine and 11·0 for coniferyl alcohol. This suggests that coniferyl alcohol is easily oxidized by Gamay rouge peroxidase, in support of a role of Gamay peroxidase isoenzymes in the lignification process.

Gamay peroxidases also show a great specificity for

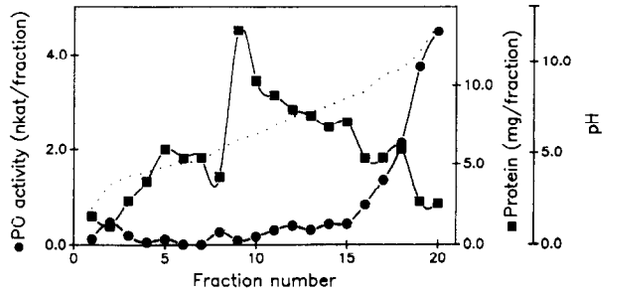

Fig. 1. Preparative isoelectric focusing of peroxidase from Gamay rouge grapes (*Vitis vinifera*) in glycerol-stabilized 3·0–10·0 pH gradients. At the end of the run, peroxidase (PO), protein and pH values were determined in 20 fractions recovered from the focusing cell.

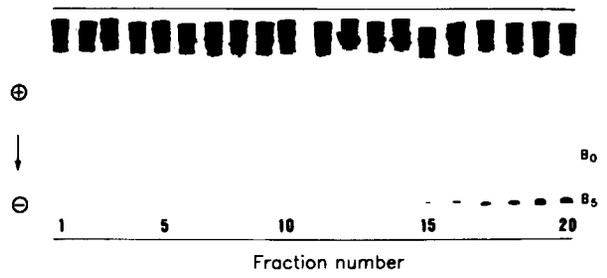

Fig. 2. Analytical peroxidase isoenzyme patterns of fractions recovered from the preparative isoelectric focusing described in Fig. 1. The arrow indicates the direction of protein migration during focusing.

Table 1. Purification of peroxidase isoenzyme B_5 from Gamay rouge grape berries

Fraction	Peroxidase activity		Purification factor
	Total[a]	Specific[b]	
Crude extract	23·1	0·135	1·0
Preparative IEF	15·5	1·79	13·2

[a] Total peroxidase activity in nkat.
[b] Specific activity of peroxidase isoenzyme B_5 in nkat mg^{-1} protein (fraction 20 of the isoelectric focusing cell).

coniferyl alcohol as lignification substrate since syringaldazine, a substrate commonly used for peroxidases related to the final stages of lignin biosynthesis (Goldberg *et al.*, 1985; Imberty *et al.*, 1985), is not oxidizable by this enzyme fraction even in the presence of H_2O_2. This inability to oxidize syringyl moieties is also shown by other grapevine peroxidases (Zapata *et al.*, 1992*a*).

Gamay rouge grapes, at pigmentation, show a very simple peroxidase isoenzyme pattern when subjected to analytical isoelectric focusing. This shows the major presence of peroxidase isoenzyme B_5 (numbered in accordance with previous nomenclature in Gamay peroxidases; see García-Florenciano *et al.*, 1991), and the weak presence of the isoenzyme B_0 (Calderón *et al.*, 1993).

In order to characterize kinetically the oxidation of coniferyl alcohol by Gamay rouge peroxidase isoenzyme B_5, it was purified by preparative isoelectric focusing in glycerol-stabilized 3·0–10·0 pH gradients (Fig. 1). Preparative isoelectric focusing of the protein fractions from homogenates of Gamay rouge grapes shows the most basic peroxidase isoenzyme B_5 in fractions 15–20 of the isoelectric focusing cell (Fig. 2). With this one-step purification procedure, a purification factor of 13·2 was attained (Table 1). Examination by analytical isoelectric focusing of the purified isoenzyme B_5 reveals

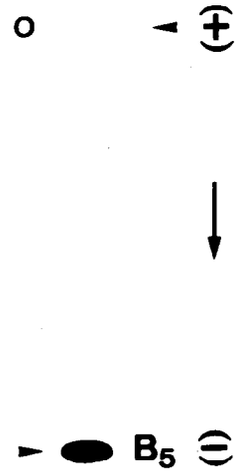

Fig. 3. Analytical isoelectric focusing of grapevine peroxidase isoenzyme B5 purified by preparative isoelectric focusing from tissue homogenates of *Vitis vinifera* cv. Gamay rouge stained with 4-methoxy-α-naphthol as substrate. The arrow indicates the direction of protein migration during focusing. O, origin.

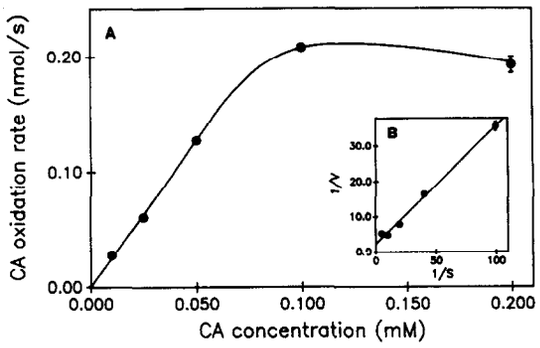

Fig. 4. (A) Dependence of the coniferyl alcohol (CA) oxidation, catalysed by basic peroxidase isoenzyme B_5, on coniferyl alcohol concentration in a reaction medium containing 0·1 M Tris–acetate buffer (pH 5·0), 5·0 mM H_2O_2 and 48 pkat of enzyme. (B) Lineweaver–Burk plot of data shown in (A).

that it migrates as the only isoperoxidase band during electrophoresis (Fig. 3).

The oxidation of coniferyl alcohol by the basic peroxidase isoenzyme B_5 is strictly dependent on the presence of H_2O_2. This was easily monitored by studying the spectral changes in a reaction medium with time. Consecutive spectra of a reaction medium containing coniferyl alcohol and H_2O_2 showed maximal spectral changes at 262 nm which were similar to those found for other grapevine peroxidases (Calderón *et al.*, 1992*b*) and which did not take place in the absence of H_2O_2.

The dependence of the coniferyl alcohol oxidation, by this basic peroxidase isoenzyme, on coniferyl alcohol concentration shows kinetic behaviour of the Michaelis–Menten type at low substrate concentrations, with inhibition at high substrate concentrations (Fig. 4(A)). Although true K_m values cannot be defined for oxidations catalysed by peroxidases, since these reactions show no sign of reversibility or of enzyme–substrate complex formation (Dunford & Stillman, 1976), apparent K_m values were calculated from the Lineweaver–Burk plot (Fig. 4(B)). From this plot, values of apparent K_m = 0·149 mM and k_{cat} = 9·27 nmol s^{-1} $nkat^{-1}$ were calculated.

In the case of H_2O_2, the dependence of the coniferyl

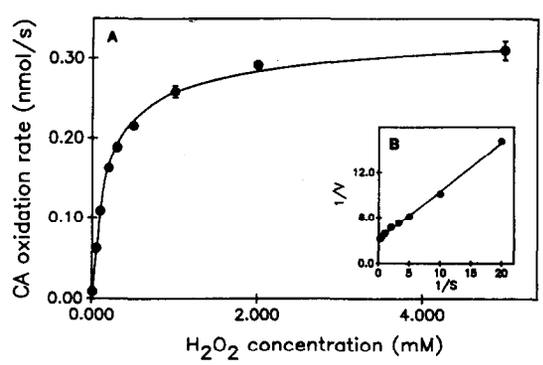

Fig. 5. (A) Dependence of the coniferyl alcohol (CA) oxidation, catalysed by basic peroxidase isoenzyme B_5, on H_2O_2 concentration in a reaction medium containing 0·1 M Tris–acetate buffer (pH 5·0), 0·1 mM coniferyl alcohol, and 49 pkat of enzyme. (B) Lineweaver–Burk plot of data shown in (A).

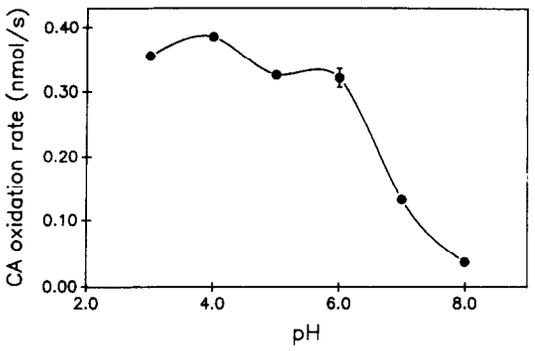

Fig. 6. Dependence of the coniferyl alcohol (CA) oxidation, catalysed by basic peroxidase isoenzyme B_5, on the pH of the reaction medium containing 0·1 M Tris–acetate buffer of variable pH, 5·0 mM H_2O_2, 0·1 mM coniferyl alcohol, and 66 pkat of enzyme.

alcohol oxidation, by the basic isoperoxidase B_5, on H_2O_2 concentration reveals a Michaelis–Menten type of kinetics with no inhibition at high substrate concentrations (Fig. 5(A)). From the Lineweaver–Burk plot (Fig. 5(B)) values of apparent K_m = 0·206 mM and k_{cat} = 6·57 nmol s^{-1} $nkat^{-1}$ were calculated. These analytical constants are in contrast with those shown by peroxidases of acidic pI, for which the values K_m = 0·010 mM for coniferyl alcohol, and K_m = 0·160 for H_2O_2, were found (Pedreño *et al.*, 1989).

Finally, the dependence of the coniferyl alcohol oxidation rate on pH was studied. The results (Fig. 6) illustrate that oxidation of coniferyl alcohol by the basic peroxidase isoenzyme B_5 shows an optimal pH for maximal activity in the pH range 3·0–6·0.

These results suggest that the Gamay rouge basic peroxidase isoenzyme B_5 is capable of oxidizing coniferyl alcohol and, although its affinity towards the lignification substrates (i.e. H_2O_2 and coniferyl alcohol) is low compared with that shown by peroxidases of acidic pI (Pedreño *et al.*, 1989), participation of this isoenzyme in the lignification of xylem vessels of grape berries should be taken into account.

ACKNOWLEDGEMENTS

This work was partially supported by a grant from the CICYT (project no. 296/89). A. A. Calderón holds a fellowship (BPP) from the MEC (Spain). Thanks are given to A. Martínez Cutillas and J. Carreño Espín (CRIA, Murcia, Spain) for providing the grape berries used in this study.

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