ELSEVIER

0308-8146(95)00077-1

Food Chemistry, Vol. 55, No. 2, pp. 133–137, 1996

0308-8146/96 $15.00 + 0.00

Purification and characterization of a basic peroxidase isoenzyme from strawberries

M. López-Serrano & A. Ros Barceló*

Department of Plant Biology (Plant Physiology), University of Murcia, E-30100 Murcia, Spain

(Received 21 December 1994; revised version received 10 March 1995; accepted 10 March 1995)

Canned syrup strawberries (*Fragaria ananassa* var. Oso Grande, Tudla and Chandler) after appertization showed significant signs of red pigment decay and the appearance of visible browning reactions. Even after this industrial processing, residual peroxidase activity may be measured in drained (syrup-free) canned strawberries, suggesting that a partially thermostable peroxidase activity may be involved in these browning reactions. Strawberries contain a peroxidase isoenzyme of basic pI, which is the only component of peroxidase polymorphism in the whole fruit. For this reason, this isoenzyme was purified by preparative isoelectric focusing in glycerol-stabilized 3.5–10.0 pH gradients and by liquid chromatography on CM-cellulose, and characterized as regards its catalytic properties against several phenols. The results showed that this isoenzyme is capable of oxidizing phenols only in the presence of H_2O_2, lacking oxidase (catecholase, cresolase, and laccase) activities. These results, and the previous observation that its homologous isoenzyme in other plant species may play a role in anthocyanin turnover and degradation, suggest that a participation of this peroxidase isoenzyme in browning reactions in canned syrup strawberries should be taken into account.

INTRODUCTION

It has been known for a long time that pigment instability in canned strawberries (*Fragaria ananassa*) is one of the main undesirable phenomena which appears during the processing of this food (Adams & Ongley, 1973). The principal red pigment of strawberries is pelargonidin-3-glucoside, and this has been shown to degrade in canned syrup strawberries and other strawberry-containing processed foods (Adams & Ongley, 1973).

Strawberries contain a peroxidase (EC 1.11.1.7) isoenzyme, belonging to the group of BPrx HpI (basic peroxidases of high isoelectric point) according to the nomenclature of Pedreño *et al.* (1993), which is the only component of peroxidase polymorphism in the whole fruit (López-Serrano & Ros Barceló, 1995). This BPrx HpI isoenzyme in strawberries mainly occurs in the concentric array of the vascular bundles and in the vascular connections with the seeds (López-Serrano & Ros Barceló, 1995). Since a property recently assigned to the BPrx HpI isoenzyme group is its participation in anthocyanin turnover and degradation in some plant species (Ros Barceló *et al.*, 1994*b*), we now describe attempts to purify and to characterize this peroxidase isoenzyme with respect to its catalytic properties during the oxidation of several phenols. Results shown later confirm the role of this peroxidase isoenzyme in pigment decay in canned syrup strawberries since it remains active after the common process of appertization.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Plant material

Strawberries (*Fragaria ananassa* var. Chandler, Oso Grande and Tudla) were grown in field at Lepe (Huelva, Spain) and sampled at the processing-ripe (dark red colour, slightly soft) stage. Fruits were stored at −20°C for 1 month until use or immediately processed by appertization as canned syrup strawberries by Anukka Foods S.A. (Murcia, Spain). In this last case, appertized cans were rapidly cooled up to 35–45° C, and then to room temperature. After appertization and, within 5 h, drained (syrup-free) strawberries were used to measure peroxidase activity to calculate peroxidase inactivation. Unprocessed and canned strawberries were characterized by measuring the percentage of soluble solids (Brix degree, expressed in % sucrose) and total acidity (% citric acid),

as previously reported by Spayd & Morris (1981) with slight modifications.

Tissue homogenization and peroxidase fractions

Strawberries were homogenized with a mechanical blade (Sorvall Omnimixer™ 230) at 4°C in the presence of 1.0-M $CaCl_2$, 6-mM ascorbic acid, 250-mM Tris–HCl buffer, pH 7.5. The homogenate was centrifuged at 27 000 *g* for 20 min, and the supernatant dialyzed overnight against 50-mM Tris–HCl buffer, pH 7.5. In order to remove pectins from this protein fraction, the dialysate was further incubated in the presence of 1-M KCl for 2 h at 4°C and clarified by centrifugation at 27 000 *g* for 20 min. The supernatant was dialyzed overnight against 50-mM Tris–HCl buffer, pH 7.5, and this constituted the soluble protein fraction used in further studies.

Ammonium sulfate precipitation

The above-mentioned dialyzed protein extracts were incubated with ammonium sulfate up to 95% (w/v) saturation for 3 h at 4°C. After centrifugation at 27 000 *g* for 20 min, the protein precipitate was washed twice with 95% ammonium sulfate in water, and redissolved in double-distilled deionized water (milli Q). Soluble protein was dialyzed overnight at 4°C against water (milli Q).

Purification of the basic peroxidase isoenzyme by preparative isoelectric focusing

Preparative isoelectric focusing was carried out using a Rotofor™ preparative IEF cell (Bio-Rad Laboratories, Richmond, CA). For this, the strawberry protein (32 mg), dialyzed overnight against 4 litres of double-distilled deionized water (milli Q), was supplemented with 12% (v/v) glycerol and 1.5% (v/v) Ampholine ampholytes (Pharmacia) of 3.5–10 pH range and loaded into the Rotofor cell. Isoelectric focusing, without prerunning, followed the Rotofor Cell Instruction Manual (Bio-Rad) with minor modifications (Morales *et al.*, 1993). For the purification of the basic peroxidase isoenzyme in the Rotofor cell, optimal conditions were 800 V of constant voltage for 16 h at 4°C for a starting current of 15–20 mA.

Once focusing was completed, the electrofocusing cell was fractioned 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 water (milli Q).

Purification of the basic peroxidase isoenzyme by ion-exchange chromatography on CM-cellulose

The basic peroxidase isoenzyme purified by preparative IEF was further purified by ion-exchange chromatography on CM-cellulose using the Econo™ FPLC system (Bio-Rad Laboratories, Richmond, CA). For this a flow rate of 0.5 ml/min and a volume fraction of 0.5 ml were used. Buffer A consisted of 20-mM Tris–Mes buffer, pH 6.0, while buffer B consisted of 20-mM Tris–Mes buffer, pH 6.0, containing 1-M KCl, using the sequential steps: (i) 0–5 min (100% A, 0% B), (ii) 5–25 min (100% A, 0% B to 0% A, 100% B; lineal gradient) and (iii) 25–35 min (0% A, 100% B). Peroxidase activity, assayed using 4-methoxy-α-naphthol (see later), was eluted from the ion-exchange column as a single peak at the end of the lineal gradient, and was recovered and dialyzed overnight against 20-mM Tris–Mes buffer, pH 6.0.

Determination of enzymatic activities and protein

The determination of peroxidase activity with 4-methoxy-α-naphthol was carried out as described (Ferrer *et al.*, 1990), and expressed in nkat (nmoles of substrate oxidized s^{-1}) The determination of catecholase and laccase activities of the basic peroxidase isoenzyme fraction with 4-methylcatechol and syringaldazine were performed as reported by Calderón *et al.* (1994). The determination of the cresolase activity of this basic peroxidase isoenzyme with tyramine and 3-methyl-2-benzothiazolinone hydrazone (MBTH) was carried out as described by Rodriguez-López *et al.* (1994).

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

Peroxidase isoenzymes were separated by analytical isoelectric focusing on polyacrylamide gels in 3.5–10.0 pH gradients, 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

The measurement of peroxidase activity in homogenates of three cultivars of processing-ripe strawberries reveals that levels of peroxidase activity are below 1 nkat g^{-1} FW (Table 1). These levels are low compared with that found in other plant materials (Ros Barceló *et al.*, 1987), but of the same order as the peroxidase activity found in other non-climacteric fruits (Calderón *et al.*, 1993; Ros Barceló *et al.*, 1994*a*). This peroxidase activity was partially thermostable, since it remained active in canned syrup strawberries after appertization (Table 2). Thus, the percentage of residual peroxidase activity after appertization for the three cultivars tested varied from 6.4% in Chandler variety to 26.2% in Tudla variety (Table 2). Residual peroxidase activity could be responsible for undesirable browning reactions which appear in canned syrup strawberries after 3 months. In fact, such undesirable browning reactions

Table 1. Brix degree (% sucrose), acidity (% citric acid) and peroxidase activity (nkat g^{-1} FW) in three varieties of processing-ripe strawberries ($n = 1 \times 3$)

Variety	°Brix	Acidity[a]	Peroxidase activity
Tudla	7.2	0.93 (3.26)	0.206 ± 0.014
Oso Grande	7.9	0.79 (3.51)	0.419 ± 0.020
Chandler	9.4	1.00 (3.00)	0.326 ± 0.009

[a]pH values are given in brackets.

Table 2. Brix degree (% sucrose), acidity (% citric acid) and peroxidase activity (nkat g^{-1} FW) in three varieties of drained (syrup-free) canned strawberries 5 h after appertization ($n = 2 \times 3$)

Variety	°Brix	Acidity[a]	Peroxidase activity[b]
Tudla	12.1	0.73 (3.43)	0.054 ± 0.001 (26.2%)
Oso Grande	12.5	0.50 (3.82)	0.038 ± 0.005 (9.1%)
Chandler	13.8	0.86 (3.48)	0.021 ± 0.002 (6.4%)

[a]pH values are given in brackets.
[b]Peroxidase activities given between brackets correspond to the % of residual peroxidase activity after appertization, and calculated from values given in Table 1.

have been associated with degradation of anthocyanin pigments (Adams & Ongley, 1973), and a metabolic role has recently been proposed for peroxidase in anthocyanin turnover and degradation (Ros Barceló *et al.*, 1994*b*).

Processing-ripe strawberries have a very simple peroxidase isoenzyme pattern when analyzed by analytical isoelectric focusing which shows the exclusive presence of a basic peroxidase isoenzyme, belonging to the group of basic peroxidases of high isoelectric point, and named as BPrx HpI by Pedreño *et al.* (1993). This peroxidase isoenzyme is located in vacuoles (Pedreño *et al.*, 1993), and is the only component of peroxidase polymorphism in the whole fruit (López-Serrano & Ros Barceló, 1995). At tissue-specific level, this BPrx HpI isoenzyme is mainly localized in strawberries in the concentric array of the vascular bundles and in the vascular connections with the seeds (López-Serrano & Ros Barceló, 1995). Since a property recently assigned to the BPrx HpI isoenzyme group is its participation in anthocyanin turnover and degradation (Ros Barceló

Table 3. Purification of the basic peroxidase isoenzyme from processing-ripe strawberries

Fraction	Peroxidase activity		Purification factor
	Total[a]	Specific[b]	
$(NH_4)_2 SO_4$ fractionation	12.90	0.40	1.00
Preparative IEF	6.02	3.30	8.25
CM-cellulose	1.95	19.33	48.32

[a]Total peroxidase activity in nkat.
[b]Specific activity of basic peroxidase isoenzyme in nkat mg^{-1} protein (fraction 20 of the isoelectric focusing cell or fraction 27 of the CM-cellulose chromatography).

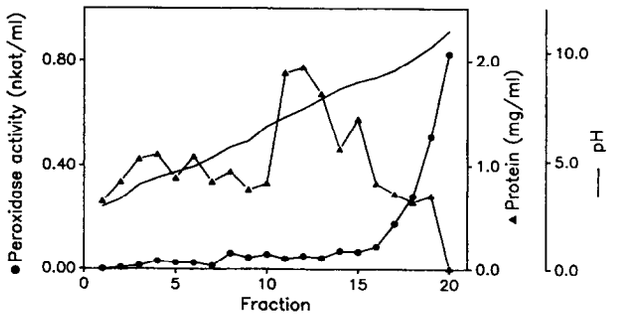

Fig. 1. Preparative isoelectric focusing of peroxidase from processing-ripe strawberries in glycerol-stabilized 3.0–10.0 pH gradients. At the end of the run, peroxidase (●), protein (▲) and pH values were determined in 20 fractions recovered from the focusing cell.

et al., 1994*b*), it is plausible that the group mediates browning reactions which appear in canned syrup strawberries since it remains active after appertization (Table 2). However, this does not preclude the possibility of the existence of other enzymatic and non-enzymatic browning reactions.

We therefore, attempted to purify and to characterize this peroxidase isoenzyme with respect to its catalytic properties during the oxidation of several phenols, initially by ammonium sulfate precipitation and preparative isoelectric focusing in glycerol-stabilized 3.5–10.0 pH gradients (Fig. 1). Preparative isoelectric focusing of the protein fractions from homogenates of processing-ripe strawberries (var. Oso Grande) shows this basic peroxidase isoenzyme in fractions 17–20 of the isoelectric focusing cell (Fig. 1). Using the two-step purification procedure, a purification factor of 8.25 was attained (Table 3).

Further purification was obtained by ion-exchange chromatography on CM-cellulose (Fig. 2). With this end-step in purification, a final purification factor of 48.32 was attained (Table 3), with a yield of 15%. Examination of the purified isoenzyme by analytical isoelectric focusing reveals that it migrates as one isoenzyme band during electrophoresis (Fig. 3).

From the profile of the CM-cellulose chromatography, a property emerges for this isoenzyme, which

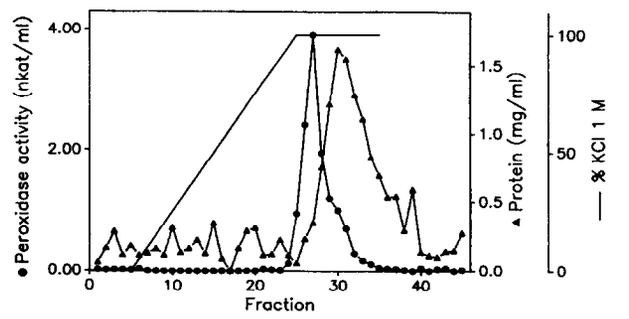

Fig. 2. CM-cellulose chromatography using a lineal gradient of KCl of peroxidase from processing-ripe strawberries previously purified by preparative isoelectric focusing in glycerol-stabilized 3.5–10.0 pH gradients. At the end of the run, peroxidase (●) and protein (▲) were determined in the fractions recovered from the ion-exchange chromatography.

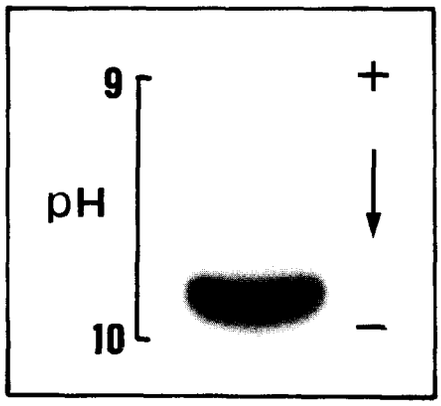

Fig. 3. Analytical isoelectric focusing on 3.5–10 pH gradients of the basic peroxidase isoenzyme purified by preparative isoelectric focusing and CM-cellulose chromatography from processing-ripe strawberries, stained for peroxidase activity with 4-methoxy-α-naphthol as substrate. Arrow indicates the direction of protein migration during focusing.

is frequently found in the BPrx HpI isoenzyme group. It is only removed from the cationic ion-exchange column at high ionic strength, i.e. when the KCl gradient reaches 1 M (Fig. 2). There is a strong affinity of the isoenzyme for the ion-exchange column similar to that found for the BPrx HpI isoenzyme group in its bond to negatively-charged surfaces such as the plant cell wall (Ferrer *et al.*, 1992). This explains the difficulties of solubilizing it from strawberries (López-Serrano & Ros Barceló, 1995), since it remains firmly bound to the soluble pectin fraction during extraction (López-Serrano & Ros Barceló, 1995).

In fact, this strong affinity of the basic isoenzyme for negatively-charged polymers such as pectins (López-Serrano & Ros Barceló, 1995), which is also illustrated in the case of CM-cellulose (Fig. 2), may be responsible for its partial thermostability (see Table 2), since it is well known that immobilization of peroxidase onto Sepharose polymers increases this property (Cremonesi & D'Angiuro, 1983). Furthermore, peroxidase binding to pectins increases the stability of the enzyme at acidic pH (Ros Barceló *et al.*, 1989), and such acidic pH values are recorded during the processing of canned syrup strawberries by appertization (see Tables 1 and 2).

Finally, the phenol oxidizing properties of purified basic peroxidase isoenzyme were studied. This is of particular interest since it has recently been reported that basic (cationic) peroxidases of mung bean hypocotyls show laccase-like (*p*-phenol oxidizing) activity (Chabenet *et al.*, 1993), and it has long been known that peroxidases also display catechol-oxidase (*o*-diphenol-oxidizing) and cresolase (capability to insert a hydroxyl group in *o*-position into a pre-existing hydroxyl group) activity (Srivastava & Van Huystee, 1977). This is due to the fact that some basic peroxidase isoenzymes exhibit a paraperoxidase character, i.e. an absorption spectrum similar to those of low-spin cytochrome b, and give rise to the formation of Compound III (CoIII) as the major steady-state form of the enzyme during activity (see Casella *et al.*, 1993). Thus, paraperoxidase

Table 4. Peroxidase, catecholase, cresolase and laccase specific activities of the basic peroxidase isoenzyme purified from processing-ripe strawberries (see Table 3) assayed using 4-methoxy-α-naphthol, 4-methylcatechol, tyramine-MBTH and syringaldazine, respectively, as substrates

Activity	Specific activity[a]
Peroxidase	19.23
Catecholase	nd[b]
Cresolase	nd
Laccase	nd

[a]Specific activity of basic peroxidase isoenzyme in nkat mg^{-1} protein.
[b]nd, not detectable.

isoenzymes are capable of exhibiting mixed peroxidase–oxidase activity. This is of great importance since the association of oxidase activities with this basic peroxidase isoenzyme fraction would imply that this isoenzyme is capable of oxidizing *o*-diphenols and *p*-diphenols in the absence of H_2O_2, these observations having being important as regards a possible control of browning reactions in strawberries. However, no catecholase (measured using 4-methylcatechol), cresolase (measured using tyramine-MBTH) or laccase (measured using syringaldazine) activity was associated with this basic peroxidase fraction (Table 4).

In fact, the oxidation of 4-methoxy-α-naphthol, a specific substrate for plant peroxidases (Ferrer *et al.*, 1990), was strictly dependent on H_2O_2, suggesting that this isoenzyme may be considered as a true peroxidase, needing H_2O_2 (the co-substrate) to act. This implies that catalytic cycles of this basic peroxidase isoenzyme arise only through Compound I (CoI) and Compound II (CoII), a previous reaction of the native enzyme (ferric enzyme) with H_2O_2 being necessary to give CoI, the true oxidizing agent in peroxidase-catalyzed oxidations.

ACKNOWLEDGEMENTS

This work was partially supported by a grant from the CICYT (project No. ALI 1018/95). M. López-Serrano holds a fellowship (BFPI) from the MEC (Spain). Thanks are given to Anukka Foods S.A. (Murcia, Spain) for providing the strawberries used in this study.

REFERENCES

Adams, J. N. Ongley, M. H. (1973). The degradation of anthocyanins in canned strawberries. I. The effect of various processing parameters on the retention of pelargonidin-3-glucoside. *J. Food Technol.*, **8**, 139–45.

Calderón, A. A., Pedreño, M. A., Ros Barceló, A. & Muñoz, R. (1990). Zymographic screening of plant peroxidase isoenzymes oxidizing 4-hydroxystilbenes. *Electrophoresis*, **11**, 507–8.

Calderón, A. A., Zapata, J. M., Muñoz, R. & Ros Barceló, A. (1993). Localization of peroxidase in grapes using nitrocellulose blotting of freezing/thawing fruits. *HortScience*, **28**, 38–40.

Calderón, A. A., Zapata, J. M. & Ros Barceló, A. (1994). Peroxidase-mediated formation of resveratrol oxidation products during the hypersensitive-like reaction of grapevine cells to an elicitor from *Trichoderma viride*. *Physiol. Mol. Plant Pathol.*, **44,** 289–99.

Casella, L., Gullotti, M., Poli, S., Ferrari, R. P., Laurenti, E. & Marchesini, A. (1993). Purification, characterization and catalytic activity of anionic zucchini peroxidase. *BioMetals*, **6,** 213–22.

Chabenet, A., Catesson, A. M. & Goldberg, R. (1993). Peroxidase and phenoloxidase activities in mung bean hypocotyl cell walls. *Phytochemistry*, **33,** 759–63.

Cremonesi, P. & D'Angiuro, L. (1983). Kinetic and thermal characteristics of enzyme-graft copolymers. *Biotechnol. Bioeng.*, **25,** 735–44.

Ferrer, M. A., Calderón, A. A., Muñoz, R. & Ros Barceló, A. (1990). 4-Methoxy-α-naphthol as a specific substrate for kinetic, zymographic and cytochemical studies on plant peroxidase activities. *Phytochem. Anal.*, **1,** 63–9.

Ferrer, M. A., Pedreño, M. A., Ros Barceló, A. & Muñoz, R (1992). The cell wall localization of two strongly basic isoperoxidases in etiolated *Lupinus albus* hypocotyls and its significance in coniferyl alcohol oxidation and indole-3-acetic acid catabolism. *J. Plant Physiol.*, **139,** 611–16.

López-Serrano, M. & Ros Barceló, A. (1995). Peroxidase in unripe and processing-ripe strawberries. *Food Chem.*, **52,** 157–60.

Lowry, O., Rosebrough, W., Farr, A. & Randall, R. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265–75.

Morales, M., Pedreño, M. A., Muñoz, R., Ros Barceló, A. & Calderón, A. A. (1993). Purification and kinetic characterization of a basic peroxidase isoenzyme responsible for lignification in Gamay rouge grape (*Vitis vinifera*) berries. *Food Chem.*, **48,** 391–4.

Pedreño, M. A., Bernal, M. A., Calderón, A. A., Ferrer, M. A., López-Serrano, M., Merino de Cáceres, F., Muñoz, R. & Ros Barceló, A. (1993). A general pattern for peroxidase isoenzyme localization and function in Vitaceae, Solanaceae and Leguminosae. In *Plant Peroxidases: Biochemistry and Physiology*, eds. K. G. Welinder, S. K. Rasmussen, C. Penel & H. Greppin. University of Geneva, Geneva, pp. 307–14.

Ros Barceló, A., Muñoz, R. & Sabater, F. (1987). Lupin peroxidases. I. Isolation and characterization of cell wall-bound isoperoxidase activity. *Physiol. Plant.*, **71,** 448–54.

Ros Barceló, A., Pedreño, M. A., Muñoz, R. & Sabater, F. (1989). Physiological significance of the binding of acidic isoperoxidases to cell walls of lupin. *Physiol. Plant.*, **75,** 267–74.

Ros Barceló, A., Zapata, J. M. & Calderón, A. A. (1994*a*). Tissue-specific expression of peroxidase activity in seedless grapes (*Vitis vinifera* cv. Thompson and Flame). *Rev. Esp. Cienc. Technol. Alim.*, **34,** 110–16.

Ros Barceló, A., Zapata, J. M. & Calderón, A. A. (1994*b*). Towards a role for the vacuolar grapevine basic peroxidase isoenzyme B_5 in anthocyanin turnover and degradation. *Plant Peroxidase Newsl.*, **4,** 35–9.

Rodriguez-López, J. N., Escribano, J. & García-Canovas, F. (1994). A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. *Anal. Biochem.*, **216,** 205–12.

Spayd, S. E. & Morris, J. R. (1981). Physical and chemical characteristics of puree from once-over harvested strawberries. *J. Amer. Soc. Hort. Sci.*, **106,** 101–5.

Srivastava, O. P. & Van Huystee, R. B. (1977). IAA oxidase and polyphenol oxidase activities of peanut peroxidase isoenzymes. *Phytochemistry*, **16,** 1527–30.