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Characterisation of lipoxygenase isoforms from olive callus cultures

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ABSTRACT

Two lipoxygenase isoforms from olive callus cultures were separated from each other. Acetone powders were made to stabilise activity and remove lipids. Separation was then achieved by salt precipitation and ion-exchange chromatography. Both isoforms had comparable activity with linoleic and α-linolenic acid substrates, a basic pH optimum and had molecular masses of around 95 kDa. The callus extracts preferentially formed the 13-hydroperoxy products, in keeping with the pattern of volatile derivatives found in olive tissues and oils derived therefrom.

1. Introduction

Lipoxygenases (LOXs) (EC 1.13.11.12) are a class of non-haem, iron-containing dioxygenases that use molecular oxygen for the oxygenation of polyunsaturated fatty acids with a 1,4-*cis, cis* pentadiene moiety. LOXs are ubiquitous in the plant and animal kingdoms. In plants, the products of the reaction are conjugated *cis, trans* hydroperoxy derivatives, which are highly reactive and toxic to cells. They are rapidly metabolised to non-toxic but physiologically-active components (see Hildebrand, 1989; Gardner, 1991; Siedow, 1991; Porta and Rocha-Sosa, 2002; Wasternack, 2007). There are two distinct pathways in higher plants which lead to either traumatin or jasmonic acid production, both of which are known to have regulatory roles. By-products of traumatin formation include short chain aldehydes which can be further metabolised to form numerous volatile oxylipins (Blée, 1998; Feussner and Wasternack, 1998). In addition to the above mentioned pathways for fatty acid hydroperoxide metabolism (catalysed by allene oxide synthase and hydroperoxide lyase) there are several other potential catabolic routes for utilisation (Hildebrand et al., 2000; Stumpe et al., 2008).

In animals, the LOX pathway is responsible for the production of the physiologically-active leukotrienes and lipoxins. Whereas the physiological role of mammalian LOXs have been clearly delineated (Funk, 1996; Yamamoto et al., 1997; Brash, 1999), the role of these enzymes in higher plants is more complex. LOXs are involved in a number of functions, including defence (Porta et al., 1999; González-Aquilar et al., 2004; Prost et al., 2005; Nemchenko et al., 2006; Mita et al., 2007), signal transduction (Blokhina et al., 2003; Kourtchenko et al., 2007), plant growth and development (Hildebrand et al., 2000: Kolomiets et al., 2001; Santino et al., 2003; Vellosillo et al., 2007) and fruit ripening (Zhang et al., 2006). Major roles for products of the lipoxygenase pathways are in defence against pathogen attack and wounding (Croft et al., 1993; Fournier et al., 1993; Vick, 1993; Creelman and Mullet, 1995; Blée, 1998; Weber et al., 1999) and senescence (Siedow, 1991; Paliyath and Droillard, 1992; Page et al., 2001; Leverentz et al., 2002). LOXs may also function as vegetable storage proteins in soybean leaves and developing organs (Tranbarger et al., 1991; Fischer et al., 1999). In addition, they have been reported to be involved in lipid reserve mobilisation from lipid bodies (Feussner and Kindl, 1992; Feussner et al., 1996, 2001).

An understanding of the cellular and subcellular localisation of LOXs can often help to determine their physiological functions. The majority of LOXs which have been characterised are, in fact, soluble (Siedow, 1991) but there is increasing evidence to suggest that LOXs are diversely distributed in subcellular organelles as well as being membrane-bound (Todd et al., 1990; Bowsher et al., 1992; Feussner and Kindl, 1992; Nellen et al., 1995; Stephenson et al., 1998). Further confusion occurs because LOXs often exist as multiple isoforms (Domoney et al., 1990; Todd et al., 1990; Siedow, 1991; Chen et al., 2004). The compartmentalisation of the isoforms may well be associated with distinct physiological functions.

There is considerable interest in the food industry concerning plant LOXs because the oxylipin by-products have characteristic tastes and smells (desirable and undesirable). LOX activity in

Abbreviations: LOX, lipoxygenase; HPO, hydroperoxy.

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tomatoes (Smith et al., 1997), barley (Doderer et al., 1992; Holtman et al., 2000), leek (Nielsen et al., 2004), potato leaves (Salas et al., 2005), oilseed rape (Terp and Brandt, 2000) and soybean (Saravitz and Siedow, 1995) have been investigated, amongst other important food crops. Virgin olive oil is recognised as one of the finest vegetable oils because of its delicious taste (Aparicio, 2000). It is also particularly appreciated for its organoleptic properties which are derived from the by-products of the LOX pathway. Moreover, sensory analysis of the volatiles in olive oil has led to the identification of many of the constituent components including (*Z*)-2-hexenal, 3-hexenal and 3-methylbutanal which each contribute to virgin olive oil's sweet and fruity aroma (Morales et al., 1994) and are derived directly from activity of the LOX pathway. LOX activity has been found in virgin olive oil (Georgalaki et al., 1998a, b) as well as in olive fruit (*Olea europaea*) (Salas and Sanchez, 1998a; Georgalaki et al., 1998b; Ridofi et al., 2002). LOX activity in olive fruits (Salas et al., 1999) and callus has been previously reported by the authors with a number of different isoforms being identified, both soluble and membrane-bound (Williams and Harwood, 1998; Williams et al., 2000). Such LOXs are thought to pay a pivotal role in the generation of important sensory components of olive oil during extraction and processing (Salas and Sanchez, 2000). Thus, a better knowledge of the properties of olive LOXs has clear relevance to industry.

In order to gain more information about such enzymes, we have used tissue culture systems which not only provide reliable plant material to use in enzymological and metabolic studies (Williams and Harwood, 2000) but also appear to have low levels of proteases and enzyme inhibitors (Rutter et al., 1997) thus making enzyme purification easier. Previous work (Williams et al., 2000) had shown that the highest olive callus LOX activity towards both linoleic and α-linolenic acids was soluble and so we concentrated on this fraction for further characterisation. Here we report information on two isoforms which both have comparable activities toward the two main endogenous substrates, linoleic and α-linolenic acids.

2. Results and discussion

2.1. LOX purification

Acetone powders were found to be necessary for optimisation of LOX extraction from olive callus and preservation of activity. Enzyme activities in olive fruit (Salas and Sanchez, 1998b) and olive callus (Williams et al., 2000) have been shown previously to be maintained by this extraction method. Solubilised acetone powders were subject to ammonium sulphate precipitation as a first step of LOX purification. A 30-60% cut contained the majority of the activity and this was used for ion-exchange chromatography (Table 1). After testing a number of methods to preserve activity, it was found eventually that, following ammonium sulphate precipitation and ion-exchange purification, introducing 30% (v/v) glycerol into the final storage solution provided the best conditions for stabilisation of LOX activity.

LOXs bound to a column of DEAE Sephadex A50 were eluted with a gradient of 0–0.5 M sodium chloride (Fig. 1). Two distinct peaks of LOX activity were detected (designated Peaks 1 and 2) which were purified 48- and 55-fold from the olive acetone preparation when linoleic acid (pH 8.8) was used as substrate (Table 1). Similar purification values were found when α-linolenic acid was used at pH 8.0 (data not shown). Recovered activity was also distributed evenly between the two peaks and the pH optima were unchanged for the two substrates, remaining at 8.8 and 8.0 for linoleic and α-linolenic acids, respectively (Fig. 2) which were in agreement with previously published data for crude acetone powders of olive tissue cultures (Williams et al., 2000). At pH 8.0 there was a minor peak of activity with linoleic acid which we suggest may be as a result of some activity when the enzyme is in a conformation that favours activity with α-linolenate. Indeed, because the pK values for the two substrates are very similar, one presumes that the subtle differences in pH optima for the two substrates is due to alteration in the 3-dimensional architecture of the LOX protein(s). The total recovery of activity of the partially purified LOX fractions was 10–19% in different preparations which was expected, due to the unstable nature of many plant LOXs. These values are similar to recoveries reported for other plant tissues such as tomato fruit (Smith et al., 1997) and barley (Doderer et al., 1992).

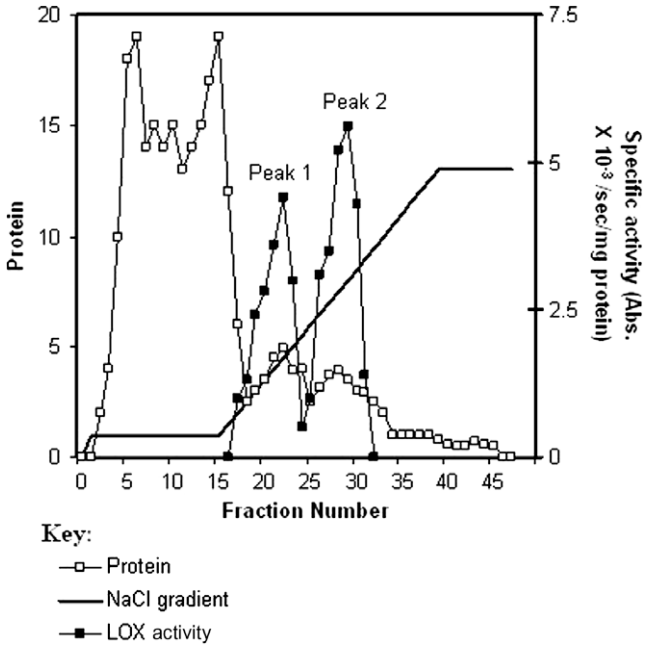

Fig. 1. Elution profile of soluble lipoxygenase isoforms from olive callus on a DEAE Sephadex A50 anion-exchange column. The column was loaded with a 30–60% ammonium sulphate cut as detailed under Section 4.3 and assayed with linoleic acid at pH 8.8. The profile is typical of three separate experiments.

Table 1
Purification of LOX isoforms from acetone powders of olive callus

Fraction	Protein (mg)	Total activity (μmoles/s)	Specific activity (μmoles/s/mg protein)	Recovery (%)	Purification (-fold)	K_m values (μM)
Crude extract	883	151 (177)	0.17 (0.20)	100 (100)	1 (1)	n.m
30% $(NH_4)_2SO_4$ supernatant	217	75 (89)	0.34 (0.40)	50 (50)	2 (2)	n.m.
30-60% $(NH_4)_2$ SO_4 cut	23	47 (53)	2.0 (2.3)	31 (30)	12 (11)	n.m
ANION DEAE A50 (Peak 1)	1.2	9.8 (10.7)	8.2 (8.9)	6.5 (6)	48 (46)	80
ANION DEAE A50 (Peak 2)	0.9	8.5 (7.1)	9.4 (7.9)	5.6 (4)	55 (39)	43

Data represent means (n = 3). Fractions were assayed at pH 8.8, the optimal value for linoleic acid. Numbers in parenthesis represent incubations for α-linolenic acid at pH 8.0.

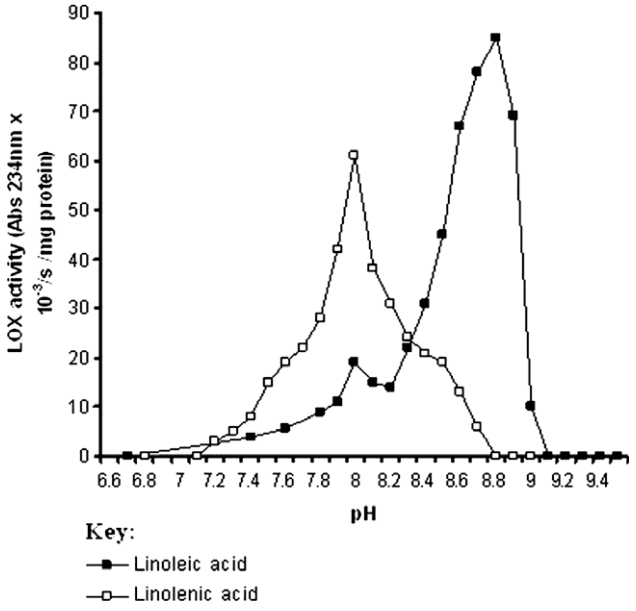

Fig. 2. pH profile of Peak 2 for LOX activity from a DEAE Sephadex A50 anion column assayed with linoleic or α-linolenic acid substrates.

When linoleic acid was used as substrate, Peak 1 had an apparent K_m value of 80 μM at pH 8.8 whereas Peak 2 had a K_m value of 43 μM (Table 1). Together with their effective separation, the distinct K_m values suggested that these two peaks might represent isoforms. In contrast to data with linoleic acid (data not shown), Michaelis–Menton kinetics were never obeyed when α-linolenic acid was used as substrate and sigmoidal curves were repeatedly observed. The Hill equation was used with Chemstation Micromath Scientist Software (Fig. 3) and suggested a co-operative effect (Hill constant = 3.15 ± 0.06). We have no explanation for this phenomenon and, without detailed knowledge of any olive lipoxygenase, it would seem premature to speculate further. Nevertheless, the sigmoidal kinetics, even though interesting, precluded any meaningful calculation of K_m values for α-linolenic acid.

2.2. Further properties of the LOX isoforms

The purpose of this study was not to purify any olive LOX isoform to homogeneity but to achieve sufficient purification to allow characterisation of some of the basic properties. Accordingly, the two peaks were examined and both were found to contain LOXs of around 95 kDa, as judged by Western blotting (Fig. 4) and gel filtration (Fig. 5). These results were in good agreement with previously published data for other plant LOXs such as soybean (Shibata et al., 1988), pea (Ealing and Casey, 1989), tomato (Bowsher et al., 1992), cucumber (Feussner and Kindl, 1994), parsley (Noehringer et al., 2000) and oilseed rape (Terp and Brandt, 2000).

However, Western blots using polyclonal antisera raised against pea LOX showed cross-reactivity with a 50 kDa peptide (Fig. 4) even though the crude acetone powder showed little cross-reactivity except at 95 kDa. Peptides smaller than 90–95 kDa plant LOXs have been reported before and, because they were inactive, were assumed to be degradation products. Thus, van Aarle et al. (1991) found two fragments of around 63 kDa in addition to a 90 kDa LOX in barley seeds, Doderer et al. (1992) detected a 65 kDa protein in barley leaves and Terp and Brandt (2000) found an 80 kDa polypeptide in oilseed rape. However, gel filtration of olive LOX isoforms (Fig. 5) showed that the band corresponding to 50 kDa proteins retained some, albeit low, activity. Unfortunately, this activity was highly unstable and attempts to purify or analyse it further were unsuccessful. It has been shown previously that soybean LOXs, for instance, contain an active site which is embedded deep within the molecular structure (Boyington et al., 1993) which might account for retained but reduced activity in the 50 kDa polypeptide. Therefore, in the absence of further evidence

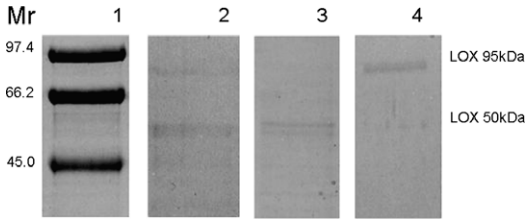

Fig. 4. SDS-PAGE and immunological identification of olive callus lipoxygenases. Lane 1, molecular mass markers: phosphorylase B 97.4 kDa, albumin (bovine serum) 66.2 kDa, ovalbumin 45 kDa; lane 2, soluble LOX fraction (peak 1) eluted from anion exchange chromatography; lane 3, soluble LOX fraction (50 kDa) eluted from Sephadex G150 column.; lane 4, immunological identification of LOXs from solubilised acetone powders.

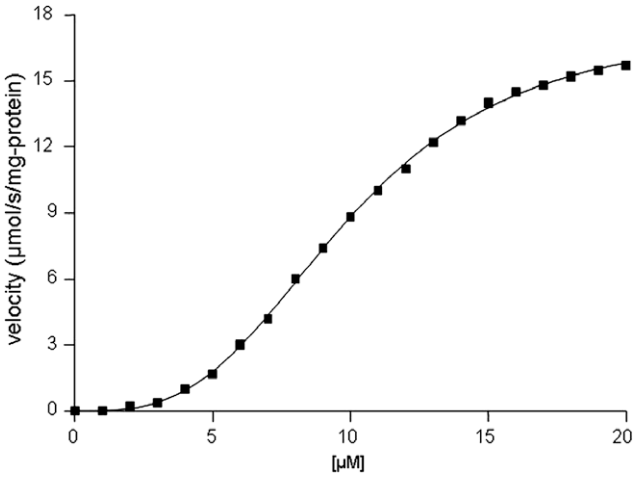

Fig. 3. Determination of enzyme activity for Peak 2 for LOX activity from a DEAE Sephadex A50 anion column and assayed with α-linolenic acid at pH 8.0 (n = 6).

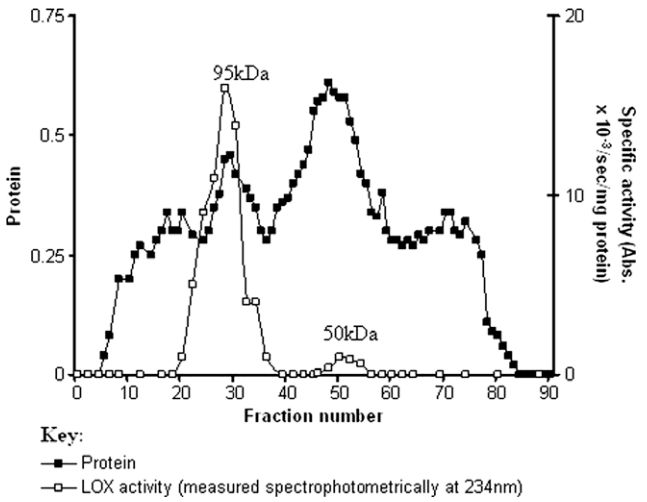

Fig. 5. Elution profile of 30–60% ammonium sulphate fraction of olive callus lipoxygenase activity from a Sephadex G-150 column. The profile shown is representative of three separate purifications. The column was calibrated with standards of known molecular weight. Assay was made with linoleic acid.

Table 2
Radiolabelled hydroperoxy fatty acid and aldehyde production following *in vitro* incubations of solubilised LOX fractions from resuspended acetone powders of olive callus with either [U-^{14}C]linoleate or [U-^{14}C]linolenate at optimal pHs

	Radioactive HPOs (%)			
Substrate	Radiolabelled hexanal d.p.m. ($\times 10^{-3}$/mg protein)	Radiolabelled HPO d.p.m. ($\times 10^{-3}$/mg protein)	13-HPO	9-HPO
[U-^{14}C]Linoleate	1.91 ± 0.21	2.62 ± 0.31	75.1 ± 3.1	24.9 ± 2.4
[U-^{14}C]Linolenate	n.d.	3.27 ± 0.37	72.6 ± 4.2	27.4 ± 2.7

Analyses of HPOs was by TLC and HPLC as detailed under **Experimental 3.6.**
Data represent means ± S.D. (where $n = 3$) for separate experiments. Abbreviation: HPO = hydroperoxy fatty acid; n.d. = not detected.

to the contrary, we assume that this polypeptide fragment represents a degradation product of one or more of the 95 kDa LOXs but which still retains its active site.

2.3. Reaction products of LOX activity

The immediate products of the LOX reaction are hydroperoxy (HPO) fatty acids (Siedow, 1991). We analysed HPO fatty acid production using solubilised acetone powders at short incubation times. Analysis of the HPO products showed that they consisted of a mixture of 13-HPO and 9-HPO derivatives, with the former being favoured (approximately 3:1 ratio) (Table 2). The ratio was consistent throughout the linear accumulation of products in the 10 min incubation period confirming that further metabolism in the LOX pathway, which could have influenced the ratio, was not significant. The results are consistent with those obtained from olive fruit (Salas and Sanchez, 1998a) and with preliminary experiments with acetone powders of calli (Williams and Harwood, 1998) where the ZE/EE ratios were reported. The production of 9- and 13-HPO fatty acids no doubt contributes to the array of volatile oxylipin by-products that have been detected in virgin olive oil (Morales et al., 1994) and in olive callus (Williams et al., 1998). Moreover, the preferential production of 13-HPO fatty acids is consistent with data from Georgalaki et al. (1998b) and with the high amounts of short chain C6 compounds which characterise virgin olive oil (Morales et al., 1994) and which would be formed following HPO lyase activity in the LOX pathway.

HPO lyases are responsible for the next step in the LOX pathway, producing short-chain C6 aldehydes and 12-oxo-(*Z*)-dodecenoic acid. Changing the pH to that optimal for HPO lyases (Salas and Sanchez, 1998b) and prolonging the incubations (see Section 4.5) allowed further metabolism with solubilised acetone powders. When [U-^{14}C]linoleate was used in the incubation mixture, [^{14}C]hexanal was detected in the products which was indicative of hydroperoxy lyase activity deriving from the cultures (Table 2). Apart from hexanal, small amounts of radioactivity were also found in nonanal, isoamyl alcohol, ethyl acetate and hexylacetate. When [U-^{14}C]linolenate was substrate, radiolabelled nonenal and hexenal were also detected. These results were qualitatively similar to those obtained for tomato fruits by Stone et al. (1975).

As a generalisation, 13-hydroperoxy products are favoured by most plant tissues (Hildebrand et al., 2000). Plant LOXs differ in their regioselectivity and catalyse the oxidation of unsaturated 18C fatty acids into either 9- or 13-hydroperoxyoctadecadi (tri) enoic acids, or a mixture of both, depending on the source of enzyme. Often the same tissues can yield both 9- and 13-specific LOXs. For example, parsley cell cultures have been shown to contain a constitutive 9-LOX and to yield a 13-LOX also when challenged with a fungal elicitor (Noehringer et al., 2000). Likewise, in barley seedlings, two isoforms have been described which are specific for the formation of 9S- and 13S-hydroperoxide derivatives, respectively (Holtman et al., 2000). Moreover, mutagenesis involving single point mutations can be used to convert a soya bean 13-LOX into a 9-LOX (Hornung et al., 1999).

For olive tissues, whether they be cultures (Table 2) or maturing fruits (Georgalaki et al., 1998b; Salas et al., 1999) the balance of 9-LOX to 13-LOX activities fits well with the balance of volatile products which can be detected in virgin olive oil (Morales et al., 1994) and olive tissue cultures (Williams et al., 1998). Thus, we believe that the pattern of sensory products in olive oils, which are so important to the latter's quality (Salas and Sanchez, 2000) is largely influenced by the regiospecificity of the olive LOX activity.

3. Conclusions

We have succeeded in separating two soluble LOX fractions from olive callus cultures. Both of the partially purified LOXs had alkaline, but distinct pH optima for either linoleic acid or α-linolenic acid as substrates and had molecular masses of about 95 kDa. They were clearly different from other olive LOX isoforms which have acidic pH optima (Salas et al., 1999). Regiospecificity studies showed that acetone powders of cultures yielded preferentially 13-hydroperoxy products in agreement with experiments for maturing olive fruits and consistent with the main products of the LOX pathway detected in tissues or in olive oils derived therefrom. The simple (2-step) method of LOX purification used here and the demonstrated possibility of using gel permeation chromatography (Fig. 5) further, mean that more work on these important olive enzymes should be easily accomplished in the future. Moreover, the apparent sigmoidal kinetics observed when α-linolenic acid was incubated at pH 8.0 with Peak 2 LOX isoform, was novel and merits further investigation.

4. Experimental

4.1. General experimental procedures

LOX activity was determined spectrophotometrically according to a method described by Axelrod et al. (1981) using a Hewlett Packard Diode Array Spectrophotometer incorporating a Hewlett Packard 8452 UV–vis Chemstation Micromath Software programme. Protein concentration was determined as described by Bradford (1976). [1-^{14}C]Acetate was purchased from GE Healthcare Ltd. Radioactivity was measured using a 1209 Rackbeta Liquid Scintillation Counter. Radiolabelled hydroperoxy (HPO) fatty acid products were analysed by reverse-phase HPLC on a Hypersil-C18 column (5 μm, 4.6 × 100 mm) (Hewlett Packard). Linoleic acid, α-linolenic acid, hexanal and soybean lipoxygenase were purchased from Sigma–Aldrich (Poole, Dorset, UK).

4.2. Plant materials

Olive callus cultures (cv. Coratina) were established from sterile olive pericarps as previously described (Williams et al., 1993). The calli were routinely subcultured at 30-day intervals onto Murashige and Skoog medium (Murashige and Skoog, 1962) at 25 °C with a 12 h light/dark cycle. The light was provided by cool white fluorescent tubes (20 μmol m^{-2} s^{-1}). The growth medium was supplemented with an auxin-cytokinin combination consisting of 2,4-dichlorophenoxyacetic acid (12 μM) and benzylaminopurine riboside (0.56 μM) (Williams et al., 1998).

4.3. Lipoxygenase extraction and purification

Olive callus (50 g) was ground and extracted three times in 500 ml acetone (total volume) at −20 °C using a polytron blender. The acetone powders were filtered under vacuum, rinsed with diethyl ether, air-dried and stored desiccated at −70 °C until required, essentially according to a method of Salas and Sanchez (1998b). LOX activity has been previously shown to be preserved completely under these conditions (Williams et al., 2000). Acetone powders were resuspended using a glass homogenizer in 50 mM Hepes buffer (pH 7.2) containing dithioerythritol (4 mM) and magnesium chloride (10 mM). Protein was extracted from the homogenate by gentle stirring of the suspension for 1 h at 4 °C, followed by centrifugation at 10,000*g* for 10 min in order to remove solid debris. The supernatant was taken to 30% saturation with solid ammonium sulphate and stirred for 60 min. The suspension was centrifuged (10000*g* for 10 min) and the pellet discarded. The supernatant was then taken to 60% $(NH_4)_2SO_4$ saturation. After stirring for another 30 min the suspension was centrifuged as above. During addition of ammonium sulphate, the pH was carefully monitored and adjusted to ensure that it remained at 7.2. The protein pellet was resuspended in a minimum volume of 20 mM potassium phosphate buffer (pH 7.2).

Any traces of ammonium sulphate were removed from samples by eluting through a column of Sephadex G-25 previously equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The sample was then concentrated by ultra filtration in a Centricon C-30 concentrator and loaded on to a 5 × 60 cm column of DEAE Sephadex A50 anion column previously equilibrated with 20 mM phosphate buffer (pH 7.2). LOXs were eluted with a linear gradient of 0–0.5 M sodium chloride in phosphate buffer with a flow rate of 0.5 ml min^{-1}. Gel permeation chromatography (gel filtration) used a Sephadex G-150 column (5 × 60 cm) equilibrated with 20 mM phosphate buffer (pH 7.2). The active fractions were pooled, concentrated as described above, then glycerol was added to 30% (v/v) and stored at −70 °C.

4.4. LOX assays

LOX activity was determined spectrophotometrically at 234 nm by measuring conjugated diene formation using either sodium linoleate or linoleic acid (at pH 8.8) or α-linolenic acid (at pH 8.0) as substrates at 25 °C as described by Axelrod et al. (1981). The reaction mixture, which had a total volume of 1 ml, consisted of either 20 mM borate buffer pH 8.8 or pH 8.0 (800 μl), 10 mM linoleic acid/linolenic acid (50 μl) and 150 μl of enzyme preparation. A sodium borate buffer system (pH 8.0–9.5) and a sodium phosphate buffer system were used for establishing pH profiles of olive callus.

4.5. Electrophoresis and Western blotting

Western blotting was as reported previously (Towbin et al., 1979). SDS-PAGE of the protein preparation was on 7.5% acrylamide gels (Laemmli, 1970). The gels were stained with Coomassie Blue (0.1%, w/v in 40% methanol, 10% acetic acid) and destained in 10% methanol, 7.5% acetic acid (v/v). Polyclonal antibodies were raised against purified pea LOX as a primary antibody and alkaline phosphatase conjugated anti-rabbit IgG was used as a secondary antibody.

4.6. Analysis of reaction products

[U-^{14}C]Linoleate and [U-^{14}C]α-linolenate were isolated from plant leaves incubated with [1-^{14}C]acetate. Aliquots of solubilised acetone powders (500 μl) were incubated with either [U-^{14}C]linoleate (pH 8.8) or [U-^{14}C]α-linolenate (7.54 KBq) (pH 8.0) in oxygen-saturated (0.2 M) sodium borate buffer for 10 min in order to analyse LOX products. The pH values were optimal for soluble olive LOX isoforms with each substrate (see Results and Discussion). Air was gently bubbled through the mixture (flow rate of 5 ml min^{-1}). Autoxidation of the radiolabelled substrate was monitored by using controls containing heat-denatured LOX preparations. The reaction was stopped with 2 M HCl. The radiolabelled HPO fatty acid products of the reaction were extracted from the incubation mixture using diethyl ether and then separated by TLC on silica gel Merck 60 F_{254} plates at 4 °C using a solvent system consisting of hexane/diethyl ether/formic acid (60:40:1, by vol.). The bands were visualised by UV and were further purified and identified by HPLC as described by Doderer et al. (1992). The reaction products were then analysed by reverse-phase HPLC on a Hypersil-C18 column (5 μm, 4.6 × 100 mm) (Hewlett Packard) using isocratic conditions. Column elution was with tetrahydrofuran/methanol/water/acetic acid (25:30:44.9:0.1, by vol.) at a flow rate of 0.9 ml min^{-1}. The HPO fatty acid products were detected by a UV detector set at 237 nm. The identity of the HPO fatty acids was confirmed by co-chromatography with standards as follows. 9-HPO standards were prepared by incubating sodium linoleate (1 ml, 10 mM) with a potato tuber LOX preparation (1 ml) in potassium phosphate buffer (5 ml, 50 mM, pH 7.0) (Schmizu et al., 1984). The potato tuber LOX was prepared using the same method as that for the olive LOX extract (see Section 4.2). 13-HPO fatty acids were prepared enzymatically by incubating sodium linoleate (500 μl, 10 mM) with soybean LOX (Sigma, lipoxidase type 1-S) (Gardner, 1997). LOX activity was monitored spectrophotometrically at 234 nm, as described in Section 4.3.

In order to examine further products of the LOX pathway, solubilised acetone powders (500 μl) were incubated with [U-^{14}C]linoleate (pH 8.8) in oxygen-saturated (0.2 M) sodium borate buffer for 10 min as before. Thereafter, the pH was reduced to 7.0 which is regarded as the optimum pH for hydroperoxide lyase in olive fruit (Salas and Sanchez, 1998b) and the incubation was allowed to continue for a further 60 min. The reaction was stopped by lowering the pH to 4.0 with 2 M HCl. Hydrazone aldehyde derivatives were prepared by adding 2.5 ml of 0.1% 2,4-dinitrophenylhydrazine to the mixture. The hydrazone derivatives were then extracted three times with hexane. These were then separated by TLC using silica gel Merck G60 plates impregnated with PEG 400 using a solvent system consisting of hexane/diethyl ether (3:2, by vol.). The derivatives were then analysed by reverse-phase HPLC using a Hypersil-C18 column with an elution solvent consisting of acetonitrile: water: tetrahydrofuran (45:24:1, by vol.) at a flow rate of 1 ml min^{-1} (Blée and Joyard, 1996). The compounds were detected using a UV detector set at 350 nm. The identity of the aldehydes was confirmed by co-chromatography with authentic standards (Sigma–Aldrich). Radiolabelled aldehydes and HPO fatty acids were quantified by means of scintillation counting.

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