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Simultaneous determination of the lipoxygenase and hydroperoxide lyase specificity in olive fruit pulp

By **Joaquín J. Salas**

Instituto de la Grasa. Av. Padre García Tejero, 4
Sevilla 41012, Spain.

RESUMEN

Determinación simultánea de la especificidad de la lipoxigenasa y de la hidroperóxido liasa en pulpa de aceituna.

La regioespecificidad de la lipoxigenasa y la especificidad del sustrato hidroperóxido liasa de pulpa de aceituna son parámetros importantes en la justificación de la composición en volátiles del aceite de oliva. En este trabajo se describe un nuevo método de marcaje radioactivo para determinar simultáneamente estas propiedades, usando sólo etapas de cromatografía en capa fina. El método implica la incubación de una preparación enzimática de pulpa de aceituna con linoleato marcado, seguido del fraccionamiento de los productos lipídicos resultantes, previamente tratados con 2,4-dinitrofenil hidrazina, sobre placas de cromatografía en capa fina soportadas con polietilenglicol 400. Los resultados obtenidos están de acuerdo con estudios previos llevados a cabo con otros métodos.

PALABRAS-CLAVE: Aceite de oliva - Aceituna - Aroma - Especificidad - Hidroperóxido liasa - Lipoxigenasa.

SUMMARY

Simultaneous determination of the lipoxygenase and hydroperoxide lyase specificity in olive fruit pulp.

Olive pulp lipoxygenase regiospecificity and hydroperoxide lyase substrate specificity are important parameters in order to justify the volatile composition of olive oil. A new radiolabelling method to determine simultaneously these properties using only thin layer chromatography steps is described in the present work. The method involves incubation of an enzyme preparation from olive pulp with radiolabelled linoleate, followed by the fractionation of the resulting lipid products, previously treated with 2,4-dinitrophenyl hydrazine, on thin layer chromatography plates coated with polyethylenglycol 400. The results obtained are in agreement with previous studies carried out by other methods.

KEY-WORDS: Flavor - Hydroperoxide lyase - Lipoxygenase - Olive - Olive oil - Specificity.

1. INTRODUCTION

Among the different plant metabolic pathways involved in food technology the so called «lipoxygenase pathway» plays an important role in processes such as synthesis of volatile components of aroma, lipid oxidation and production of off-flavours (Robinson *et al.*, 1995).

The term «lipoxygenase pathway» involves a complex cascade of biochemical reactions, which produces a variety of compounds like hydroperoxides, ketols, oxoacids, jasmonates and volatile aldehydes, most of them of potential industrial interest (Gardner, 1996). This pathway starts with the oxidation of a polyunsaturated fatty acid, catalysed by the enzyme lipoxygenase, to produce the corresponding fatty acid hydroperoxides (Siedow, 1991). These compounds are not accumulated but they are quickly metabolised through different branches of this pathway (Blée, 1998). Among them, the hydroperoxide lyase branch is linked to the biosynthesis of the volatile aldehyde components of the aroma of some fruits and vegetables (Hatanaka *et al.*, 1987). This enzyme catalyses the cleavage of fatty acid hydroperoxides to yield a non-volatile oxoacid and a volatile aldehyde. Depending on the positional hydroperoxide isomer used, C6 aldehydes (hexanal and 3(Z)-hexenal from 13-hydroperoxides) or C9 ones (3(Z)-nonenal and 3(Z),6(Z)-nonadienal from 9-hydroperoxides) are formed (Galliard and Phillips, 1976; Vick and Zimmerman, 1976). These aldehydes can be subsequently modified to produce a great variety of volatile compounds. The most direct transformation is the isomerisation of 3(Z)-enals to 2(E)-enals, catalysed by an enal-isomerase or by a non-proteic isomerisation factor (Phillips *et al.*, 1979; Hatanaka *et al.*, 1989). Moreover, these aldehydes can be reduced by alcohol dehydrogenase to their corresponding alcohols (Salas & Sánchez, 1998), which can be subsequently esterified by acyl-CoAs by alcohol acyltransferase to produce volatile esters (Pérez *et al.*, 1993).

The important role of the enzymes lipoxygenase and hydroperoxide lyase in aroma biosynthesis has stimulated the interest on these enzymes in the field of food technology in the last years (Hatanaka, 1993; Shibata *et al.*, 1995). Among the catalytic properties of these enzymes the regiospecificity of lipoxygenase and the substrate specificity of the hydroperoxide lyase are especially interesting. The term regiospecificity is defined as the ability of lipoxygenase to produce either 13- or 9-hydroperoxides. This parameter depends on the source of the enzyme as well as on

the conditions at which the oxidation reaction takes place (Galliard and Chan, 1980; Gardner, 1989). Furthermore, the substrate specificity of hydroperoxide lyase refers to its capacity to cleave the 13-ZE(Z) or 9-EZ(Z) fatty acid hydroperoxide. In previous works it has been reported that plant hydroperoxide lyases have strict specificity, so they can only cleave one of those isomers (Matsui *et al.*, 1989). The 13-hydroperoxide lyases are the most common in the plant kingdom, catalysing the formation of C6 aldehydes responsible for green odour (Hatanaka, 1993). On the other hand 9-hydroperoxide lyases, producing the C9 aldehydes, which yield the characteristic cucumber-like flavour, are less common (Galliard and Phillips, 1976).

The study of lipoxygenase and hydroperoxide lyase specificities usually involves the incubation of enzyme extracts with their specific substrates (polyunsaturated fatty acids in the case of lipoxygenase or their corresponding hydroperoxides in the case of hydroperoxide lyase), followed by lipid extraction, derivatisation (methylation of carboxyl groups and formation of the 2,4-dinitrophenylhydrazones of aldehydes), and HPLC analysis (Olías *et al.*, 1990; Sanz *et al.*, 1992). These procedures require the use and optimisation of complex equipment, as well as time-consuming steps which could make a study extended to a large number of samples difficult. Moreover, an additional difficulty is the possible interaction of these enzymes. Thus, when the lipoxygenase specificity is being determined in a crude fraction that possesses, as usual, hydroperoxide lyase activity, the final regiospecificity result could be misled if the metabolised hydroperoxides are not considered.

In the present work a straightforward and fast radiolabelling method for simultaneous determination both the regiospecificity of lipoxygenase and substrate specificity of hydroperoxide lyase in olive fruit pulp is described. This method, involving only thin layer chromatography steps, overcomes some of the difficulties described above and reproduces previous results obtained by conventional methods in that species. The application of this method to other biological systems is discussed.

2. EXPERIMENTAL

Plant material and preparation

Developing olives (20 weeks after flowering) were harvested from 30-year old trees endowed with drop irrigation located in an orchard near Sevilla (Spain). Once the fruits were washed and the endocarps removed, a mass of 100 g of the fleshy pulp was homogenised in a domestic blender with 600 mL of cold (-20°C) acetone. This process was carried out in three steps, involving the successive homogenisation with 200 mL of acetone and filtration under vacuum of the resulting solids. Finally the filtrate was washed with diethyl ether to remove the rests of oil, and the resulting whitish powder was stored in flasks containing silica gel dissicator at -20°C (Salas and Sánchez, 1998).

Preparation of standard compounds

Fatty acid hydroperoxides were prepared by oxidation of linoleic acid with soybean lipoxygenase (Sigma) as described in Salas and Sánchez (1998b). The 12-oxo-9(Z)-dodecenoic acid was prepared from vernolic acid by opening of the epoxy ring as reported by Vioque (1969) and cleaving the resulting diol with $NaIO_4$ (Olías *et al.*, 1990). The 9-oxononanoic acid was prepared from the 9,10-epoxyestearic acid in a similar manner. Both vernolic acid and 9, 10-epoxyestearic acid were kindly provided by Dr. Francisco Millán (Instituto de la Grasa, Sevilla).

TLC plates

Silica gel TLC plates were purchased from Whahtman (SiG60, 25 mm). For the separation of oxoacid-2,4-dinitrophenylhydrazone silica gel plates impregnated with polyethylenglycol 400 (PEG 400, Merk) were used. This was accomplished by developing the plate with a solution of PEG 400 and acetone (20:80). Afterwards the plate was activated by heating at 80°C for 24 h.

General procedure

A suspension was prepared by homogenisation in a glass manual homogeniser of 100 mg of olive pulp acetone powder and 3 mL of 50 mM MES pH 6.0 containing 7 mM 2-mercaptoethanol and 0.5% Tritón X-100, which had been previously saturated of oxygen by air bubbling. A volume of 0.25 mL of this suspension was added to 0.5 mM [1-^{14}C]-linoleate (130 kBeq/μmol, Dupont), in a final incubation volume of 0.3 mL. This mixture was maintained at room temperature for 30 min, then 25 μL of 0.2% 2,4-dinitrophenylhydrazine, in 5 M HCl were added and the resulting solution was maintained at 80°C for 10 min. Thereafter, lipids were extracted using the method reported by Hara and Radin (1978). The resulting organic phase was evaporated under N_2 and redisolved in 50 μL of chloroform. This lipid fraction was chromatographed on a PEG 400-coated silica gel plate using hexane-diethyl ether-formic acid (20:80:1) as the solvent. Radioactive bands were

located by using a betascope Instant Imager from Packard, and the relative incorporation of the label into hydroperoxides, 12-oxoacids and 9-oxoacids was calculated. Afterwards, the band corresponding to the hydroperoxide fraction was scrapped off and the hydroperoxides were eluted with 10 mL of diethyl ether and derivatised to their methyl esters with diazomethane (Schlenk & Gellerman, 1960). The resulting hydroperoxide methyl esters were then reduced to their hydroxy-derivatives with $NaBH_4$, by a protocol involving the evaporation of the diethyl-ether solvent under N_2, resuspension in 1 mL of dry methanol and the addition of 1 mg of the reductant. Once the reduction process finished, which took about 30 min, a volume of 2 mL of 5% NaCl was added and the hydroxymethylester derivatives were extracted three times with 1 mL of hexane. These compounds were then chromatographed on silica gel plates using hexane-diethyl ether (3:2) as the solvent and the resulting radioactive bands were located in a betascope Instant Imager. The relative incorporation into 9-and 13-hydroperoxydes was then calculated.

3. RESULTS

Linoleate metabolism into hydroperoxides and oxoacids

The TLC chromatograms of the lipid fraction resulting from incubation of labelled linoleate with olive pulp extracts showed two bands corresponding to the hydroperoxide and oxoacid fractions (Figure 1), which are produced by the action of lipoxygenase and hydroperoxide lyase on the labelled precursor. However, this system was unable to separate either the different positional hydroperoxide isomers or the oxoacids with different chain length, so it was not suitable to determine the specificity of the enzymes involved in their biosynthesis. The experimental protocol described in the present work, involving oxoacids derivatisation to their corresponding 2,4-dinitrophenylhydrazones plus fractionation of the resulting lipid fraction in plates coated with PEG 400, allowed the separation of the labelled precursor, the hydroperoxide fraction and the hydrazones of both 12-oxo-9(Z)- dodecenoic acid and 9-oxononanoic acid (Figure 2), thus providing a more complete information about the specificity of the hydroperoxide lyase present in the system under investigation. In the case of olive fruit, only a band corresponding to the 2,4-dinitrophenyl- hydrazone of the 12-oxo-9(Z)-dodecenoic acid was detected (Figure 2). Furthermore, the separation of the positional isomers of fatty acid hydroperoxides was not possible using this TLC system, so it was necessary to carry out a further analysis of this fraction to determine the regiospecificity of olive lipoxygenase.

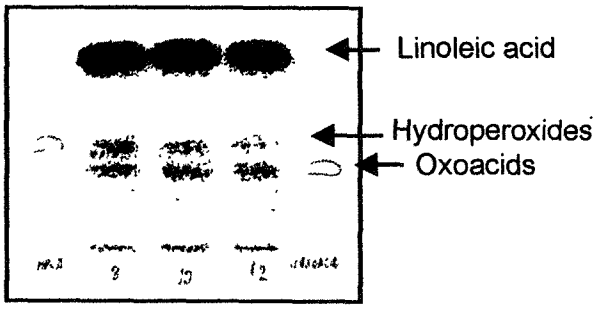

Figure 1
Separation of products of the metabolism of radiolabelled linoleate by olive acetone powder on silica gel plate. The solvent used was hexane-diethylether-formic acid (50:50:1).

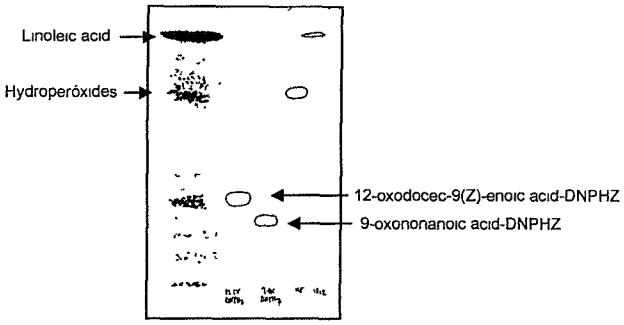

Figure 2
Radiochromatogram of products from the incubation of radiolabelled linoleate with olive pulp acetone powder after the treatment with 2,4-dinitrophenylhydrazine on polyethylenglycol 400-coated silica gel plate

Determination of the positional isomer composition of hydroperoxides

Unlike fatty acid hydroperoxides and their corresponding methyl esters, the different positional isomers of fatty acid methyl-hydroxyesters are separable by TLC on silica gel (Gardner, 1975). Therefore, the labelled linoleic acid hydroperoxide fraction isolated in the previous step was methylated and reduced as described in Experimental to produce their methyl-hydroxyesters derivatives. These compounds were chromatographed again on silica gel plates, resulting in the separation of two radioactive bands corresponding to the 9-and 13-positional isomers (Figure 3), which allowed to determine the composition of the initial fatty acid hydroperoxide fraction.

On the basis of these separations, it was possible to determine the composition of both the fatty acid

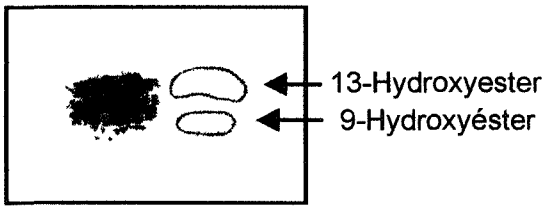

Figure 3
Radiochromatogram of the methyl-hydroxyesters of the hydroperoxide fraction isolated in the chromatographic step shown in Figure 2. The methyl-hydroxyester standards were prepared from hydroperoxides produced by soybean lipoxygenase (Sigma).

hydroperoxides and oxoacids produced in olive fruit by the action of lipoxygenase and hydroperoxide lyase on radiolabelled linoleic acid (TABLE I), and hence to establish the specificity of these enzymes.

4. DISCUSSION

The lipoxygenase pathway is the main fatty acid degradative pathway involved in olive oil processing and quality (Salas and Sánchez, 1999). This is because the volatile fraction of this oil, which is responsible for its flavor and aroma, contains a high proportion (>80%) of aldehydes, alcohols and esters of alcohols of six carbon atoms (Morales *et al.*, 1995), typically produced via hydroperoxide lyase branch of the lipoxygenase pathway (Hatanaka, 1993). Hence, the characterisation of the enzymes involved in this pathway in olive fruit pulp constitutes an important point that determines volatile composition of olive oil.

The separation of the products formed from the metabolism of linoleic acid (Figure 1, TABLE I) showed that the oxoacid fraction contained 12-oxo-9(Z)-dodecenoic acid only (TABLE II), which resulted from the cleavage of 13-hydroperoxylinoleic acid. This result reproduces the previous studies carried out on hydroperoxide lyase from olive fruit (Olías *et al.*, 1993; Salas & Sánchez, 1998b), in which a strict specificity of this enzyme, for 13-ZE(Z)-hydroperoxides was reported. This result explains the absence of nonenals (produced from 9-hydroperoxides) in the volatile composition of olive oil.

Table I
Relative composition of the hydroperoxide and oxoacid fractions produced by an olive acetone powder resuspension from linoleic acid. Results are the means of two independent determinations

Product	Relative Incorporation (%)
13-ZE-Hydroperoxylinoleic acid	21 ± 2
9-EZ-Hydroperoxylinoleic acid	23 ± 5
12-Oxododec-9(Z)-enoic acid	56 ± 3
9-oxononanoic acid	0

Table II
Specificity parameters of olive lipoxygenase and hydroperoxide lyase resulting from the analysis of the metabolic products yielded from radiolabelled linoleate

Product/substrate	Lipoxygenase regiospecificity (%)	Hydroperoxide lyase substrate specificity (%)
13-hydroperoxylinoleic acid	77	100
9-hydroperoxylinoleic acid	23	0

Regarding the regiospecificity of olive lipoxygenase, a first examination of the composition of the hydroperoxide fraction could bring us to the conclusion that olive lipoxygenase produces 9- and 13-hydroperoxide isomers in the same proportion (TABLE I). However, to determine the actual regiospecificity of olive lipoxygenase both non-metabolised and metabolised hydroperoxides produced by lipoxygenase have to be considered. Since the only product of hydroperoxide degradation detected was the 12-oxo-9(Z)-dodecenoic acid, which is produced from the 13-hydroperoxylinoleic acid, the label metabolised into this oxoacid has to be accounted as its precursor in the determination of regiospecificity. Hence, a clear preference (77%) to produce 13-hydroperoxides was found in olive pulp lipoxygenase (TABLE II). This is in a good agreement with both previous results (Salas and Sánchez, 1998c) and the composition of the olive oil volatile fraction, in which the C6 aldehydes and alcohols synthesised from 13-hydroperoxides are the most abundant components (Morales *et al.*, 1995). Moreover the absence of other products of lipoxygenase, like ketodienes, found in the chromatograms shown in Figures 1 and 2 has also been confirmed by HPLC analysis (Salas and Sánchez, 1998c).

Moreover, a form of lipoxygenase with a similar regiospecificity has been described to be present in non-filtered virgin olive oil (Georgalaki *et al.*, 1998). Although this enzyme has been found to be associated to a form bound to the lipid bodies of olive endosperm, the lipoxygenase present in olive fruit pulp, where olive oil is actually extracted from, could also contribute to the above phenomenon.

In summary, a method for simultaneous determination of lipoxygenase regiospecificity and hydroperoxide lyase substrate specificity based only on TLC separations is described in the present work.

The specificity parameters obtained using crude preparations of olive pulp reproduced previous results carried out using standard methods (Salas and Sánchez, 1998b; 1998c). The present method has, however, some limitations like the impossibility of determining geometrical and optical isomers of the hydroperoxides and oxoacids produced. However, it provides valuable information about the lipoxygenase and hydroperoxide lyase, which could be useful either as a fast approach to study a new biological system or to confirm previous specificity results. Moreover, the fact that this method involves only TLC steps could make both easier and cheaper a specificity study extended to a large number of samples, aimed to, for example, the selection of mutant species.

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