JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Lipoxygenase Activity in Plant Extracts Using a Modified Ferrous Oxidation–Xylenol Orange Assay

Maria del Carmen Pinto, Adrián Tejeda, Antonio L. Duque, and Pedro Macías*

Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain

The ferrous oxidation−xylenol orange (FOX) assay method for determination of lipid hydroperoxides is based on that under acidic conditions Fe^{2+} is oxidized to Fe^{3+}, which then oxidizes xylenol orange to a product that absorb at 550 nm. The procedure has been adapted for determination of lipoxygenase activity in plant extracts. This enzyme is responsible for generation of off-flavors in vegetal foods, bleaching of pigments, and a lot of oxidative degradations. It is of interest to check the initial lipoxygenase activity in vegetal foods before the processing, using an assay that is rapid, reproducible, and easily adaptable to high throughput format. The enzymatic assay is based on a discontinuous determination of lipoxygenase activity using the FOX reagent for colorimetric determination of hydroperoxides accumulated in the medium by a period of incubation that is established by the addition of the extract (start of the reaction) and the addition of FOX reagent (finish of the reaction). The procedure is capable of detecting lipoxygenase activity in a number of vegetable homogenates, being especially useful for a rapid visual evaluation of this enzymatic activity.

KEYWORDS: Lipoxygenase; xylenol orange; enzymatic activity determination

INTRODUCTION

Lipoxygenase is a non-heme iron dioxygenase that is ubiquitous in plants and animals and catalyzes the dioxygenation of polyunsaturated fatty acids (PUFAs) containing a (1*Z*,4*Z*)-pentadiene system such as, for instance, linoleic acid, α-linolenic acid, or arachidonic acid. The enzyme may have a specific location in plants and produces the formation of hydroperoxy PUFAs with a temporal differentiation of activity, the involvement in storage lipid metabolism and the formation of jasmonic acid being their main physiological functions (*1*). In vegetables, lipoxygenase possesses, in addition to dioxygenase activity, a hydroperoxidase activity that produces the cooxidation of suitable substrates (*2*). With the aim to prevent oxidative degradations or pigment bleaching produced by peroxidase activity, it is of great interest to check lipoxygenase activity before or during the industrial processing of vegetal foods. In addition, the measurement of lipoxygenase activity may constitute a valid procedure to determine the efficiency of thermal treatments during vegetal foods preparations.

Typically, the methodologies used for the measurement of lipoxygenase activity are the spectrophotometric assay based on the measurement of the absorbance at 234 nm produced by hydroperoxy lipid product or the oxygraphic assay based on the evaluation of oxygen consumption using an oxygen electrode (*3*). Both methods, although accurate, are time-consuming, need specialized equipment, and are unsuitable for rapid screening of multiple samples. Alternatively, other methods have been described for determination of plant lipoxygenase activity; for instance, the use of MBTH (3-methyl-2-benzothiazolinone), coupled with DMAB [3-(dimethylamino)benzoic acid] in the presence of hemoglobin, which catalyzes the reaction of detection of linoleic acid hydroperoxide, constitutes an assay method capable of detecting lipoxygenase activity in vegetable homogenates, but the quasi-lipoxygenase activity of hemoglobin needs precise control of the experimental conditions (*4*).

A colorimetric method for determination of lipoxygenase activity applicable to high throughput assays, based on that a lipid hydroperoxide can oxidize F^{2+} to Fe^{3+}, which in acidic medium oxidized xylenol orange to a colored forms that absorbs in the region of 500−600 nm has been reported (*5*). Although the procedure was validate by the use of a solubilized preparation of platelet 12-lipoxygenase, the method could result in false positive and requires a complete adaptation for their use in plant extracts. A modified ferrous oxidation−xylenol orange (FOX) assay has been used for detection of lipid hydroperoxides in plant tissues (*6*). Recently, the FOX assay, with some modifications, has been applied to the screening of 5-lipoxygenase, obtaining rapid and precise results with a high level of sensitivity using insect cell lysates or commercial human 5-lipoxygenase as source of enzyme (*7*).

On the other hand, has been reported that the assay FOX for peroxides determination is optimized if sulfuric acid is substituted by perchloric acid, resulting in an increase in the sensitivity and reproducibility, due principally to a better maintenance of

* Corresponding author. E-mail: pedrom@unex.es.

10.1021/jf070537x CCC: $37.00 © 2007 American Chemical Society
Published on Web 06/30/2007

the correct pH and an increase in the molar absorption coefficient of the ferric−xylenol orange complex (*8*). The substitution of classical sulfuric acid by perchloric acid in the FOX assay protocol has been successfully applied to the measurement of protein and lipid hydroperoxides in cultured cells and human blood serum, being especially efficient for measurements of lipid hydroperoxides in complex samples (*9*).

In this study, we describe for the first time the use of a modified FOX peroxide assay for determination of lipoxygenase activity in crude vegetal extracts samples.

MATERIALS AND METHODS

All reagents used in this work were of the purest analytical grade available. Soybean lipoxygenase, linoleic acid, and 3,3′-bis [*N*,*N*-bis-(carboxyethyl)aminomethyl]-*o*-cresolsulfonepthalein sodium salt (xylenol orange) were from Sigma.

Spectrophotometric Assay. Lipoxygenase activity was measured by determination of the increase in absorbance at 234 nm upon incubation of the enzyme with linoleic acid. Reaction was started by the addition of enzyme being measured at room temperature in 50 mM Tris-HCl buffer, pH 7.5. The rate of the reaction was determined from the rate of diene conjugated formation (A_{234}) using an $\epsilon = 25\,000\ M^{-1}\ cm^{-1}$ (*3*).

FOX Assay. The FOX assay was carried out with some modifications of the previously described method (*8*). The assay is based on the formation of the complex Fe^{3+}/xylenol orange with absorption at 550 nm. Lipoxygenase from soybean, tomato, or potato extract was incubated with linoleic acid for 10 min, unless otherwise stated, in the presence of 50 mM Tris-HCl, pH 7.5 to a final volume of 100 μL. The assay was finished by the addition of 500 μL of FOX reagent: perchloric acid (110 mM), xylenol orange (150 μM), ferrous sulfate (2 mM) in methanol:water (9:1). In all experiments a blank was carried out for determination of the possible activity in the absence of substrate during the incubation period.

Linoleic Acid Hydroperoxide Generation. For titration of FOX assay, linoleic acid hydroperoxide was generated according to Hamberg et al. (*10*): Briefly, 500 μL of 95% ethanol was added to 500 μL of linoleic acid, and this mixture was dissolved in 50 mL of 100 mM borate buffer, pH 9.0. Fatty acid was solubilized with 100 μL of Tween-20. This solution was diluted 4-fold with borate buffer, soybean lipoxygenase was added, and the mixture was slowly stirred at 4 °C until no increase in absorbance at 234 nm was detected. This solution was diluted, finally, 30-fold with methanol. Concentration of hydroperoxide of linoleic acid was determined from the $\epsilon = 25\,000\ M^{-1}\ cm^{-1}$.

Statistical Analysis. Results were expressed as means ± SE. Statistical significance was evaluated using Student's test, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In this study we evaluate if the FOX assay possesses the adequate sensitivity and reproducibility to check lipoxygenase activity in crude homogenate vegetables. It is known that ferrous ion (Fe^{2+}) in the presence of hydroperoxides is oxidized to the ferric state (Fe^{3+}), which then oxidizes xylenol orange to form a complex characterized by a peak of absorbance centered at 550 nm. This procedure has been validated for determination of hydroperoxide concentration using hydrogen peroxide, cumene hydroperoxide, butyl hydroperoxide, or 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (*5*).

With the aim to validate the method for determination of lipoxygenase activity, the product, hydroperoxy linoleic acid, was incubated in the presence of FOX reagent. The product of this incubation was scanned by spectrophotometric measurement in the range of 400−800 nm. The results obtained clearly show a shift of the absorbance maximum of the Fe^{3+}−xylenol complex, from 475 to 550 nm (Figure 1). This displacement to higher wavelengths is produced by the presence of the hydroperoxide, the color generated being stabilized in 10 min (see inset of Figure 1). The concentration of hydroperoxide obtained from the absorbance at 550 nm fit well with the concentration obtained from the absorbance value at 234 nm for the same lipid hydroperoxy solution. Figure 2 clearly shows the correlation between the absorbance values measured at 550 and 234 nm, for the range assayed for hydroperoxy linoleic acid concentrations. However, the linearity of the absorbance at 550 nm with the product concentration is lost for hydroperoxide concentrations over 25 μM. This behavior is coincident with that described by Waslidge et al. (*5*) for 12-HPTE.

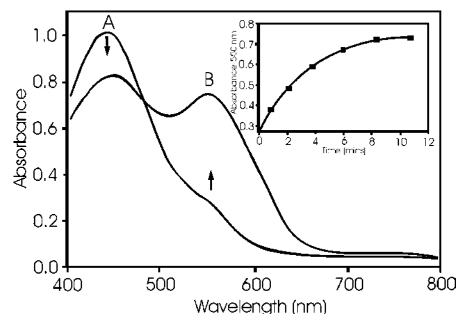

Figure 1. Absorption spectra of the complex Fe^{3+}–xylenol orange formed in the presence of hydroperoxy linoleic acid. Xylenol orange shows a peak centered at 475 nm (plot A) that decreases in the presence of hydroperoxy linoleic acid due to the formation of the complex xylenol–Fe^{3+}, characterized by a broad absorption band at 550 nm. (plot B). Plot A correspond to the FOX reagent spectra before the incubation in the presence of 10 μM hydroperoxy linoleic acid. Plot B is the spectra of the incubation mixture after 10 min of reaction.

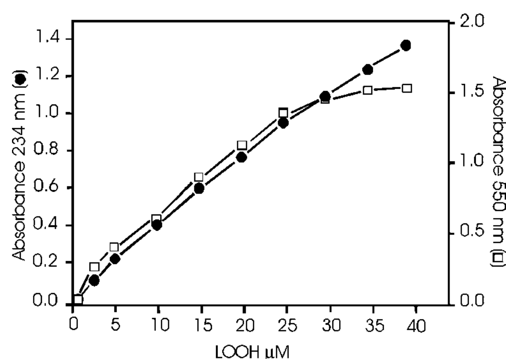

Figure 2. Comparison between the absorbance at 550 nm of Fe^{3+}–xylenol orange complex produced by hydroperoxy linoleic acid and the intrinsic absorbance of this compound at 234 nm in Tris–HCl, pH 7.5.

The main goal of this work is the adaptation of the FOX procedure to the measurement of lipoxygenase activity using a discontinuous method in which lipoxygenase is incubated in the presence of linoleic acid. At the end of each incubation period, FOX reagent was added to the medium and the enzymatic reaction stopped. The color developed by the generation of the FOX−hydroperoxide complex was measured at 550 nm, corresponding to a hydroperoxy linoleic acid concentration similar to that determined from the absorbance value at 234 nm under the same experimental conditions (Figure 3).

It has been described (*5*) that in samples containing high protein concentration, a false color may be generated by an unspecific binding hydroperoxide−protein or by an incomplete inactivation of enzyme when FOX reagent is added. To check for a possible interference of protein on the color generation, the effect of protein concentration on the determination of lipoxygenase activity using the FOX method was studied. The

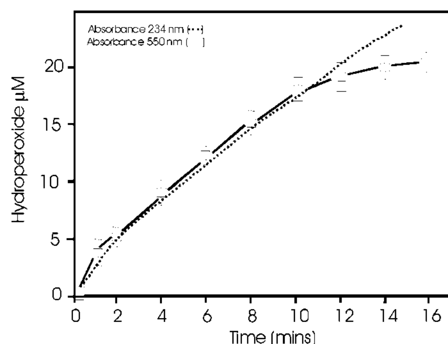

Figure 3. Time course of the hydroperoxy linoleic acid generation by lipoxygenase, using the FOX reagent (continuous line), and by the continuous measurement of the absorbance at 234 nm (dotted line). Incubation was carried out as is indicated in Materials and Methods for spectrophotometric assay. Samples of this mixture were taken at the times indicated by squares and hydroperoxy concentration was established by the FOX assay. Each plot is the mean of four experiments ± SE.

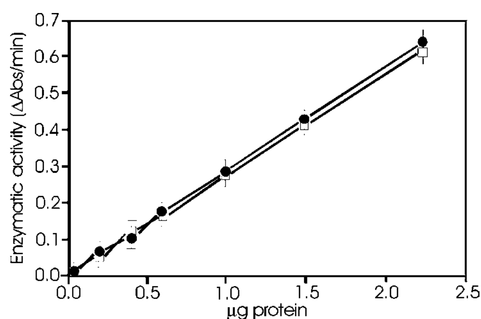

Figure 4. Effect of protein concentration on the enzymatic activity of lipoxygenase measured using the FOX reagent (squares) or by the determination of intrinsic absorbance of the product at 234 nm (circles).

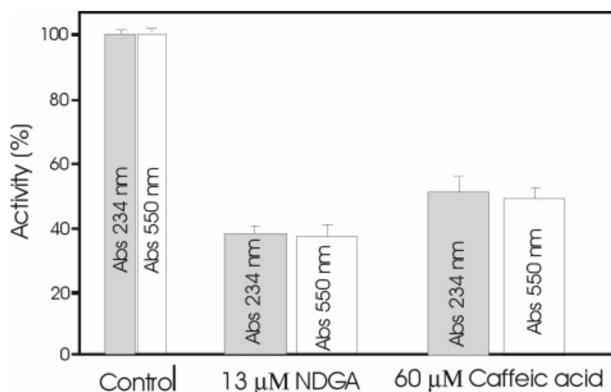

Figure 5. Effect of lipoxygenase inhibitors on enzymatic activity measured by FOX reagent and by measurement at 234 nm.

results obtained were compared with that obtained when lipoxygenase activity was determined by continuous measurement of absorbance at 234 nm. When protein concentration in the reaction mixture was plotted versus enzymatic activity value, a straight line was obtained (Figure 4). These results clearly indicate that no interference between FOX reagent and protein is produced.

To check if the proposed method for lipoxygenase activity measurement is suitable for detection of changes in enzymatic activity, the effect of lipoxygenase inhibitors was studied and the measurement of activity by FOX reagent was compared with the measurement based on the absorbance at 234 nm. Figure 5 shows that the effect of 13 μM NDGA or 60 μM caffeic acid on lipoxygenase activity produces an inhibition detected by the measurement of absorbance at 234 nm that is similar to the observed when FOX reagent was used. This pattern was maintained for several concentration of NDGA and caffeic acid (data not shown). These results reveal that the measurement of lipoxygenase activity based on the use of FOX reagent is suitable for the study of lipoxygenase inhibitors.

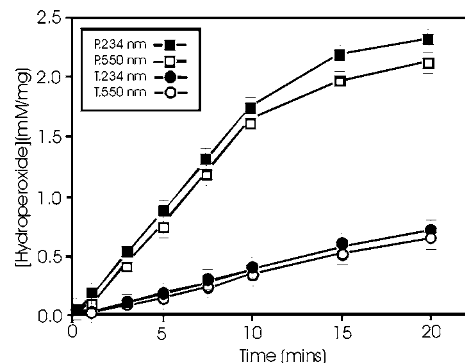

Figure 6. Time course of hydroperoxy linoleic acid generation by tomato (circles) or potato (squares) lipoxygenase, measured by the FOX reagent (open symbols) or by measurement of the absorbance at 234 nm (filled symbols).

Table 1. Inhibitors Effect on Potato and Tomato Lipoxygenase

enzyme	assay	control (% activity)	NDGA, 25 μM (% activity)	phenidone, 45 μM (% activity)
tomato	abs 234 nm	100	20	30
lipoxygenase	abs 550 nm (FOX)	100	14	45
potato	abs 234 nm	100	38	40
lipoxygenase	abs 550 nm (FOX)	100	40	45

To check if FOX assay may constitute a valid tool for the measurement of lipoxygenase activity from vegetal sources, a crude extract of tomato or potato was incubated with linoleic acid. The time course of the hydroperoxide generation was followed using the FOX method, and the results were compared with those obtained by the measurement of absorbance at 234 nm. Figure 6 shows the remarkably high level of concordance between the results obtained by both procedures. Likewise, when the effect of the protein concentration on the measurement of lipoxygenase activity was checked, the results obtained by both methods were similar (data not shown).

As for soybean lipoxygenase, the effect of inhibitors on the lipoxygenase activity of potato and tomato extracts was checked. Table 1 reveals the close results obtained by both procedures. The remarkable efficiency of the FOX method for lipoxygenase activity measurements on vegetal samples is due to the absence of interferences and the high correlation with the results obtained using the classical assay for lipoxygenase activity based on the measurement of absorbance at 234 nm.

In summary, our data reveal clearly that the FOX assay may constitute an easy, inexpensive, and sensitive procedure for the measurement of lipoxygenase activity that only needs generally available apparatus. In addition, all the reagents used are easy to prepare and their lifetimes are long. Finally, the method possesses high linearity and the obtained results are highly reproducibly.

LITERATURE CITED

(1) Feussner, I.; Wasternack, C. The lipoxygenase pathway. *Annu. Rev. Plant Biol.* **2002**, *53*, 275−297.

(2) Kühn, H.; Wiesner, R.; Rathman, J.; Schewe, T. Formation of ketodienoic fatty acid by the pure pea lipoxygenase-1. *Eicosanoids* **1991**, *4*, 9−14.
(3) Axelrod, B.; Chesebrough, T. M.; Laakso, S. Lipoxygenase from soybeans. *Methods Enzymol*. **1981**, *71*, 441−445.
(4) Khun, H.; Gotze, R.; Schewe, T.; Rapoport, S. M. Quasi-lipoxygenase activity of hemoglobin. A model for lipoxygenase. *Eur. J. Biochem*. **1981**, *120*, 161−168.
(5) Waslidge, N. B.; Hayes, D. J. A colorimetric method for the determination of lipoxygenase activity suitable for use in high throughput assay format. *Anal. Biochem.* **1995**, *231*, 354−358.
(6) DeLong, J. M.; Prange, R. K.; Hodges, D. M.; Forney, C. F.; Bishop, M. C.; Quilliam, M. Using a modified ferrous oxidation−xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. *J. Agric. Food Chem.* **2002**, *50*, 248−254.
(7) Cho, Y. S.; Kim, H. S.; Kim, C. H.; Cheon, H. G. Application of the ferrous oxidation−xylenol orange assay for the screening of 5-lipoxygenase inhibitors. *Anal. Biochem*. **2006**, *351*, 62−68.
(8) Gay, C. A.; Gebicki, J. M. Perchloric acid enhances sensitivity and reproducibility of the ferric−xylenol orange peroxide assay. *Anal. Biochem.* **2002**, *304*, 42−46.
(9) Gay, C. A.; Gebicki, J. M. Measurement of protein and lipid hydroperoxides in biological systems by the ferric−xylenol orange method. *Anal. Biochem*. **2003**, *315*, 29−35.
(10) Hamberg, M.; Samuelsson, B. On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxydase. *J. Biol. Chem.* **1967**, *242*, 5329−5335.

Received for review February 23, 2007. Accepted May 31, 2007. This work has been supported by the grant 2PR02A040 and 2PR04A050 from the Junta de Extremadura.

JF070537X