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Lipoxygenase-mediated peroxidation of model plant extractives

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ABSTRACT

Three unsaturated fatty acids, namely 9-cis,12-*cis*-linoleic acid, 1,2,3-tri-cis, *cis*-9,12-octadecadienoyl (glycerol trilinolein) and 1,2,3-tri-*cis*-9-octadecenoyl (triolein) were selected as models of components of plant extractives to monitor the hydroperoxygenation induced by soybean lipoxygenase (LOX), which was applied as an oxidative catalyst at room temperature. The fatty acids were monitored in colloidal dispersions in relation to their molecular changes using $^{1}H/^{13}C$ nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR) and UV spectroscopies. The detection of the hydroperoxy group was limited due to its unstable nature. However, the reduction of protons associated with the diene groups and the substitution of hydroperoxy groups at the allylic positon in conjugated lipids were detected by the induced chemical shift of HOO-bearing ^{13}C and ^{1}H resonances and the oxygen absorption owing to changes in the molecule. Moreover, compared to the two other substrates, no oxygen substitution was observed in triolein, in accordance with its lower level of saturation and the absence of bis-allylic carbon. Our results are of relevance to plant fiber processing, since fatty acids are major constituents of hydrophobic deposits that cause a range of manufacturing challenges.

1. Introduction

Soybean lipoxygenase (LOX) is a type of enzyme that is found widely in plants, fungi, and animals (Siedow, 1991; Yamamoto, 1992). It is a non-heme iron-containing enzyme mostly responsible for catalyzing the stereo-selective dioxidation of methyl-interrupted polyunsaturated fatty acids and their esters, such as linoleic, linolenic and arachidonic acids, which contain a 1,4-cis, *cis*-pentadiene system, to their corresponding hydroperoxy derivatives and giving *cis*-trans conjugated hydroperoxide (Kuhn and Thiele, 1999). The roles of LOX in plants include responses to wounding and senescence (Kadamne et al., 2011; Porta and Rocha-Sosa, 2002; Siedow, 1991), and linoleic and arachidonic acid are common substrates for LOX in plant and animal tissue, respectively. It was reported that the enzyme is involved in inflammatory processes (Samuelsson et al., 1987), cell membrane maturation (Schewe and Kuhn, 1991), atherogenesis, osteoporosis (Cathcart and Folcik, 2000; Cyrus et al., 1999; Klein et al., 2004) and has important role in plant wilt resistance (Mhaske et al., 2013).

Lipid peroxidation catalyzed by LOX is a two-step process. In the first step, an oxidant Fe^{+3} from active lipoxygenase attacks unsaturated fatty acids (containing carbon–carbon double bound(s)) to abstract one allylic hydrogen from carbon, forming the carbon-centered lipid radical (L·). In the next step, the radicalized lipid reacts with the oxygen molecule (oxygen insertion) leading to a lipid peroxy radical (LOO·) that can abstract a hydrogen molecule from a neighboring lipid molecule and form a new lipid radical and a hydroperoxide (LOOH) (Girotti, 1998; Kanner et al., 1987; Yin et al., 2011).

Many studies indicate the ability of LOX to accept simple chain lipids; however, there is some evidence showing that the enzyme is even able to catalyze the oxygenation of more complex oils in living system and to produce monohydroperoxy derivatives (Feussner et al., 1998). Besides, LOX has been used to oxygenate not only fatty acids, but also ester lipids such as phospholipids or even bio-membranes (Brash et al., 1987; Maccarrone et al., 1994). Plant oils such as soy bean oil are important sources of unsaturated fatty acids such as oleic and linoleic acyl groups, which make them more prone to enzymatic oxidation (Chin et al., 1992).

Even though the oxidation of oils is usually an indicator of oil's poor quality and generates unpleasant taste in food stuff, in other instances it can be advantageous. For example, the reduction of fouling (tacky) compounds in process waters has been reported (Tayeb et al., 2017). Likewise, the modification of fatty acid fractions derived from wood extractives or other sources, through oxidation and radical formation from the unsaturated fatty acids, can be used for coupling onto cellulosic surfaces, and in turn, surface hydrophobization can be achieved (Cusola et al., 2014; Garcia-Ubasart et al., 2013).

Among the many analytical techniques, NMR spectroscopy is very useful to characterize plant oils and for functional group identification

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(Belury, 2002; Cao et al., 2007; Evans et al., 2002; Gunstone, 1993; Hämäläinen et al., 2001, 2002; Jie and Mustafa, 1997; Knothe and Kenar, 2004; Lie Ken Jie, 2001; Pajunen et al., 2008; Prema et al., 2013; Siciliano et al., 2013).

NMR was also used to determine the proportion of acyl group in the sesame oil throughout oxidation under high temperature, and to follow the formation of primary and secondary oxidation compounds in the oil (Guillén and Ruiz, 2004). Analyses of pure model lipids have adopted NMR for understanding the chemical composition of lipids such as soybean, fish, and vegetable oils. More importantly, ^{1}H NMR with its short testing time and high accuracy, has been useful to acquire detailed chemical information of lipid mixtures. NMR has been used to study low-density lipoprotein (LDL) peroxidation compounds as well as thermal stressing of fish oils (Claxson et al., 1994; Haywood et al., 1995; Lodge et al., 1995; Medina et al., 1998; Silwood and Grootveld, 1999).

However, despite the number of studies available on lipid oxidation, less information exists on the peroxidation of wood extractives catalyzed by soybean lipoxygenase. This might be due to the fact that the oxidation and peroxidation reaction products are usually mixtures that are quite difficult to separate. Besides, most of the derived components, in particular lipid hydroperoxides, are very unstable, having a short life time, and they are usually studied as hydroxy derivatives, which are expected to be more stable (Pajunen et al., 2008).

Pertinent studies highlighted the biological importance and the possible application of LOX in modifying and degrading unsaturated fatty acids in wood extractives (Gutiérrez et al., 2009; Marques et al., 2011; Zhang et al., 2007), Here, we further examine the effect of LOX on the molecular structure of three simple unsaturated oils present in wood extractives. This was accomplished in the presence of active enzyme, and the separation of the modified lipid was achieved by solvent phase separation. The ^{1}H and ^{13}C NMR of the conjugated diene allylic hydroperoxides were assigned to determine the influence of the peroxy radical on the chemical structure of the oils.

In order to obtain direct evidence and compare the effect of LOX on lipid radicalization and oxidation, the three substrates considered in this study were characterized by ^{13}C/^{1}H NMR before and after the enzymatic treatment upon dissolution in deuterated solvent. The study was complemented by measurements via Fourier transform infrared (FTIR) and UV spectroscopy. The obtained molecular level analysis is essential to understanding the structural changes that lipids undergo after treatment with the enzyme and to determining the effect of oxidation on water solubility, hydrophobicity, molecular weight, etc.

2. Materials and methods

Soybean lipoxygenase was obtained from Sigma Aldrich as glycine type I-B with 150,000 U/mg activity and 108 kDa molecular mass. Pure linoleic acid, glycerol trilinolein and triolein were also purchased from Sigma Aldrich and used as received. All the reagents and buffers were prepared with Millipore Milli-Q water. Boric acid and potassium hydroxide were obtained from Fisher Scientific for preparation of the buffer solutions.

2.1. Lipid oxidation by lipoxygenase

The lipid substrates were subjected to oxidation with lipoxygenase. The enzyme/substrate ratio, temperature, and pH were kept constant throughout the experiments. Experiments were carried out in an ambient atmosphere of air with magnetic stirring in an open reaction vial (inner volume 20 mL). 400 mg of lipid was dispersed in 20 mL of 0.2 M boric buffer (pH 9.0) under agitation at 250 rpm (magnetic stirring). In order to fully disperse the substrate, 10 min sonication was applied. Separately, 20 mg (185 nM) of soybean lipoxygenase was dissolved in 0.2 M boric buffer (pH 9.0) vial (20 mL) using a magnetic stirrer and was then added to the vial containing the dispersed lipid and stirred at 250 rpm. The reaction time for linoleic acid was 0.5, 2, 24 h and 1 week. However, 2 h reaction time was used for the other two lipids. After the completion of the reaction time, the dispersion was acidified with 1.0 M HCl solution to pH 4 to stop the enzymatic reaction. The treated lipid was then extracted through phase separation using three times chloroform extraction in a 100 mL separation funnel. Subsequently, the organic solvent was dried with $NaSO_4$, evaporated under reduced pressure and the separation yield was determined. For complete drying, the extracted lipid was left in the vacuum oven for 24 h. Finally, the residue was dissolved in deuterated solvent for subsequent NMR spectroscopy.

2.2. LOX reaction followed by UV spectrophotometry

LOX reaction with linoleic acid substrate was determined by UV spectroscopy using a Single-Beam UV/Visible Spectrophotometer (Holman et al., 1969). UV absorption at 234 nm was measured to detect enzyme activity. The production of hydroperoxide groups and conjugated diene systems in the solution induces UV absorbance at 234 nm for the *trans–trans* isomers and 236 nm for the *cis*-trans isomers (Chan and Levett, 1977).

2.3. ^{1}H and ^{13}C analyses of the lipids

The lipids were analyzed using proton NMR; carbon NMR was also used for the linoleic acid. The measurements were acquired on a Bruker 300 MHz spectrometer equipped with a quad probe dedicated to ^{31}P, ^{13}C, ^{19}F, and ^{1}H acquisition at 28 °C. NMR spectra were recorded when the NMR sample was prepared and placed into a normal 5 mm NMR tube by dissolving 20 mg of extracted lipid in 1 mL deuterated solvent. The tube was sealed with a Teflon cap and secured with paraffin film. The total number of scans for all the experiments was 256 with an acquisition time of 1.6 s. Linoleic acid samples were analyzed using a 500 MHz ^{13}C NMR spectrometer operating with topspin 3.2 software. For each test 100 mg of pure oil was dissolved in 0.8 mL DMSO in a 5 mm NMR tube. All the spectra were collected with a 1.6 s delay time and 5000 scans and signals phase/baseline were corrected.

2.4. Fourier transform infrared spectroscopy (FTIR)

The infrared absorption spectra of the oxidized and unmodified samples were obtained by using a Perkin Elmer Frontier FTIR spectrometer at a wavelength resolution of 4 cm^{-1} and using 64 scans per sample. Extracted lipid samples were dried overnight in a vacuum oven at 40 °C before subsequent FTIR analysis, which used the same as that NMR analysis.

3. Results and discussion

The experimental conditions were optimized in preliminary experiments. The main variables included the substrate type and the time used in the enzymatic reaction. Soybean lipoxygenase in plants has a single polypeptide chain with a molecular mass of 94–104 kDa, and it was used in our work because, compared to other types of enzymes, it is easier to purify and has been characterized already (Shibata et al., 1987). The goal of this study was to monitor the effect of LOX on different unsaturated fatty acids through multiple techniques. In addition, we address the question whether the enzyme is able to produce hydroperoxide groups in other substrates.

3.1. Characterization of pure lipids

After LOX treatment, modified lipids were successfully separated (yield 90%) using a nonpolar solvent (chloroform). Fig. 1 shows the ^{1}H NMR spectra of the three model lipids before enzymatic oxidative treatment. These experiments were performed to provide reference