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Potato lipoxygenase catalysed co-oxidation of β-carotene

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Abstract

Co-oxidation of β-carotene in the presence of linoleic acid was catalysed by potato lipoxygenases. The rate of co-oxidation was dependent on the concentration of both linoleic acid and β-carotene. The maximum rate of co-oxidation occurs at a molar ratio of linoleic acid to β-carotene of 16:1.

1. Introduction

Lipoxygenases (linoleate: oxygen reductase EC 1.13.11.12) are one of the most widely studied enzymes at the present time (Robinson, Wu, Domonry, & Casey, 1995) and occur in more than 60 species of plants and animals. Lipoxygenases catalyse the bioxygenation of polyunsaturated fatty acids (PUFA) containing a *cis*,*cis*-1,4-pentadiene unit to form conjugated hydroperoxydienoic acids. Soybean lipoxygenase is the most extensively studied and the molecular structure has been reported by Boyington, Gaffiney and Amzel, (1993). Potato lipoxygenase, although not well studied to date, is unusual as it is a plant enzyme, which resembles mammalian lipoxygenase by catalysing the oxidation of the 20 carbon atom PUFA, arachidonic acid, to form 15-hydroperoxyeicosatetraenoic acid as the major product. In animals this hydroperoxide is the precursor of biologically active compounds of considerable pharmaceutical interest. Therefore, potato lipoxygenase is of special interest because of its greater availability and its potential use as a model and alternative for the mammalian enzyme. Three isoenzymes of lipoxygenase have been recently isolated from potato by Royo, Vancanney, Perez, Sanz, Stormann, Roshahl and Sanchez-Serrano, (1996) and defined as LOX-1, -2 and -3. Linoleic acid has been claimed to be the preferred substrate for potato LOX-1 and the 9-hydroperoxide as the dominant product. On the other hand, linolenic acid has been claimed as the preferred substrate for both potato LOX-2 and -3 which produce the 13-hydroperoxide as the main product.

Some types of lipoxygenases can also catalyse the co-oxidation of carotenoids in the presence of PUFAs. Soybean lipoxygenase type-1 (LOX-1) has been used for the bleaching of wheat flour and also been shown to act as a bread improver and a valuable processing aid during dough development (Frazier, Leigh-Dugmore, Daniels, Russell Eggitt, & Coppock, 1973; Cumbee, Hilderbrand, & Addo, 1997). On the other hand, a bleached colour can also indicate deterioration in either fresh vegetables, such as yellow french beans or fruits and processed food products, where carotenoids are important natural colorants and antioxidants. It has been reported that Type-2 lipoxygenases (i.e. LOX-2 and -3) of soybean, pea and wheat are pigment bleachers in the presence of linoleic acid (Weber, Laskawy, & Grosh, 1974), but most of the reported studies for the co-oxidation of carotenoids have been for soybean LOX-1. It has been claimed that, under anaerobic conditions, this enzyme shows strong co-oxidising activities in the presence of PUFA or a corresponding acyl hydroperoxide (Klein, Grossman, King, Cohen, & Pinsky, 1984), whereas, under aerobic conditions, it is not an efficient catalyst for the bleaching reaction. Soybeans cannot be grown widely throughout Europe and therefore other plant species are potential alternatives. Our recent investigations have been directed towards the properties and use of pea lipoxygenases and the respective cloned isoenzymes (Wu, Robinson, Casey, Hughes, West, & Hardy, 1995; Robinson et al., 1995; Casey, 1998; Hughes, Robinson, Hardy, West, Fairhurst, & Casey, 1998). Here we report the potentially useful bleaching properties of potato lipoxygenases as

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an alternative and cheaper source of lipoxygenase activity. This work is also of interest because of the increasing realisation of the importance of carotenoids as antioxidants in foods and as protective dietary agents against free radicals (Donnelly & Robinson, 1995). We have used *all trans*-β-carotene, vitamin A (retinol), its aldehyde (retinal) and acid (retinoic acid) as test substrates.

2. Materials and methods

Linoleic acid, *all trans*-β-carotene, retinol, retinal, retinoic acid, Lowry method kit (including BSA, bovine serum albumin), Lowry reagent and Folin and Ciocalteu's phenol reagent) were purchased from Sigma. Ammonium sulphate, EDTA and other chemicals were from Fisher Scientific. Red potato tubers were obtained from a local supermarket.

Crude extract was prepared by homogenising, in a Waring blender, peeled red potato tubers (250 g) in twice the volume of 100 mM phosphate buffer (pH 6.3) containing 2 mM sodium metabisulphite, 2 mM ascorbic acid and 1 mM EDTA. The homogenate was filtered through two layers of cheese cloth and clarified by centrifugation at 10 000 ***g***. The lipoxygenase enzymes in the crude extract were enriched by ammonium sulphate precipitation (15–45% saturation) at 4°C. The pellet was re-suspended in 40 mM phosphate buffer, pH 6.3, which is referred to as the partially purified enzyme. Protein was assayed using the Lowry method.

2.1. Oxidation of linoleic acid

Oxygenated sodium phosphate buffer was prepared by bubbling oxygen for 1 min and adding Tween 20 (200 μl) followed by sonication and dilution of 0.5 ml to 50 ml of phosphate buffer. 0.3 mM linoleic acid was freshly prepared by adding 18.6 μl of the acid to oxygenated 1 M phosphate buffer (1.733 ml) at pH 6.5; this was stirred continuously and then diluted by a factor of 100 with the same phosphate buffer. Enzyme extract (5 μl) kept in ice was added to 1 M oxygenated phosphate buffer (2 ml) and 0.3 mM linoleic acid (1 ml) and measurements taken at 15 s intervals for 5 min. Enzymic activity was measured by the increase in absorbance at 234 nm and one unit of activity is defined as an increase in absorbance of 0.001 min^{-1}.

2.2. Co-oxidation of carotenoids

Either β-carotene, retinol, retinal or retinoic acid (1 mg) was held in closed flask under nitrogen and was dissolved in chloroform (1 ml) containing Tween 80 (40 μl) and evaporated to dryness and then dissolved in 1 mM EDTA (10 ml). The assay solution contained oxygenated phosphate buffer (2 ml) at pH 6, 0.3 mM linoleic acid (1 ml) and 1 mM EDTA (50 μl) containing the carotenoid. Decreases in absorbance at 456, 328, 373 and 353 nm were measured for loss of β-carotene, retinol, retinal and retinoic acid, respectively. Optimum enzymic activity for the oxidation of linoleic acid and carotenoids was determined using phosphate buffers from pH 5 to 6.5.

3. Results and discussion

The specific activity of the partially purified potato lipoxygenase at pH 5.6 was 1.76×10^4 units/mg protein. The separate rates of oxidation of linoleic acid and β-carotene, are shown in Fig. 1 for certain concentrations of the substrates. The optimum pH value for the oxidation of linoleic acid by the partially purified potato lipoxygenase was pH 5.6 to 5.7, which is similar to that reported by Galliard and Phillips, (1971), but different from that reported by Sekiya, Aoshima, Kajiwara, and Hatanaka, (1977) for their purified potato enzyme. These small differences could be explained in a number

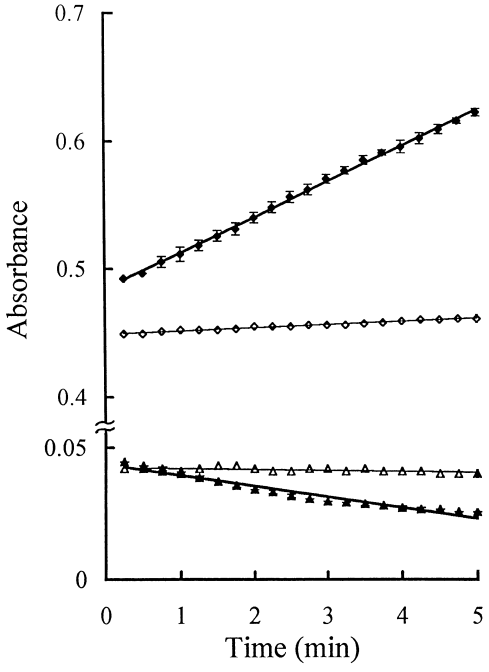

Fig. 1. Bi-oxygenation activity (-◆- and -◇-) and co-oxidation activity (-▲- and -Δ-) of potato lipoxygenases (from partially purified sample, i.e. ammonium sulphate fractions). -◆- and -▲-: with potato enzymes (error bars are given at the 95% confidence level with three repeated measurements); -◇- and -Δ-: without enzymes. (The conditions for the bi-oxygenation activity measurements were: linoleic acid 0.1 mM in 1 M phosphate buffer, pH 5.6, containing 5 μl partially purified potato lipoxgenases. The absorbance was measured at 234 nm. The conditions for the co-oxidation activity measurements were: linoleic acid 0.1 mM and carotene 3 μM, in 1 M phosphate buffer, pH 5.6, containing 5 μ partially purified potato lipoxygenases. The absorbance was measured at 456 nm.)

ways: first by the probable use of different cultivars, the segregation of different isoenzymes during the purification procedure of Sekiya et al. (1997) or perhaps the different composition and size of micelles present in the emulsified substrates. The optimum pH for the co-oxidation of β-carotene by our potato extract was pH 6.0, which is slightly higher than the optimum pH for the oxidation of linoleic acid. Hsieh and McDonald (1984) have reported a much higher value (pH 10.2) for both oxidation of PUFAs and the co-oxidation of carotenoids by wheat lipoxygenases, which might suggest that the wheat enzyme is similar to the soybean type-I enzyme, for which maximum activity has been observed at approximately pH 9.0.

Comparatively, the rate of co-oxidation of β-carotene is much lower than that of linoleic acid oxidation as shown in Fig. 1. However, the smaller decrease in absorbance at 456 nm with time may have been due to some of the oxidised products still absorbing at this wavelength. Such products may be similar to those tentatively identified in other unpublished work (Wu et al., 1998), where the number of conjugated double bonds remains unchanged. Therefore, the real rate of oxidation of β-carotene may have been much faster than the observed rate of co-oxidation measured at 456 nm. The concentrations of the reactants β-carotene, linoleic acid and the enzyme, can affect the co-oxidation rate. For both the β-carotene and linoleic acid there was an optimum concentration of each substrate for bleaching of the carotenoid (Fig. 2)— for β-carotene this was 6.0 μM (at the concentration of linoleic acid 0.1 mM), while that of linoleic acid was 0.05 mM (at the concentration of β-carotene of 3 μM). Furthermore, the ratio of concentrations of linoleic acid and β-carotene has also been found to affect the co-oxidation rate. Fig. 3 shows that when [linoleic acid]/[β-carotene] equalled 16, the co-oxidation rate was faster than at any other concentration ratios. On the other hand, the dependence of co-oxidation on the concentration of the enzyme was linear in the range studied from 5.9 to 40.8 units/ml.

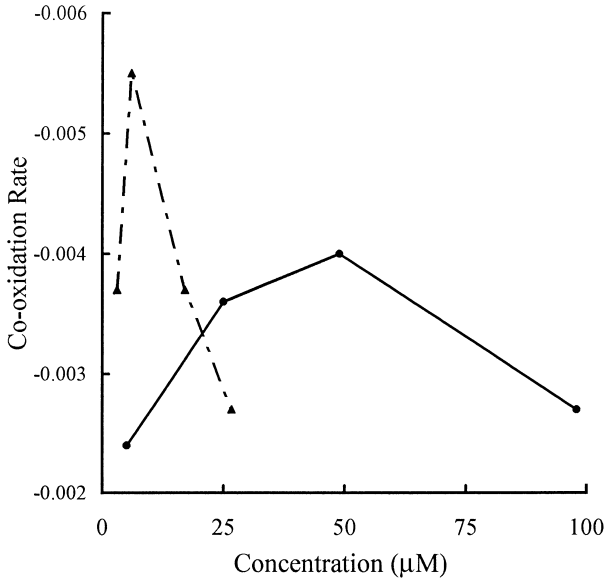

Fig. 2. The effect of concentrations of linoleic acid (while the concentration of β-carotene was fixed at 3 μM) (-●-) and β-carotene (while the concentration of linoleic acid was at 98 μM) (-▲-) on the co-oxidation rate.

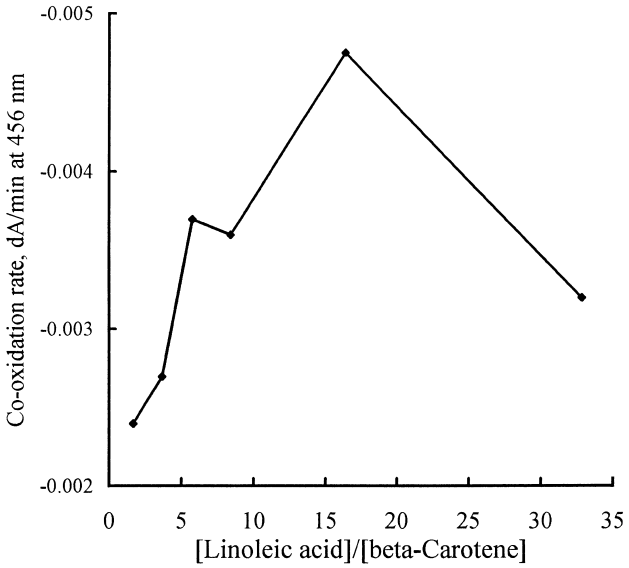

Fig. 3. The effect of concentration ratio (mole ratio) ([linoleic acid]/[β-carotene]) on the co-oxidation rate.

It is widely accepted that co-oxidation of β-carotene involves the simultaneous oxidation of the two substrates, namely the PUFA and the carotenoid. As the maximum rate of co-oxidation occurred at a molar ratio of [linoleic acid]/[β-carotene] of 16 under the experimental conditions used (Fig. 3), it is suggested that this may be due to either a requirement for an optimum molar ratio of linoleic acid to carotenoid in the emulsified micelles, or alternatively a requirement for a greater concentration of linoleic acid radicals for coupled oxidation of the carotenoid molecule. It is suggested by Hughes et al. (1998) that the peroxyl radical may be released more readily from some isoenzymes, enhancing the formation of carbonyl derivatives by chemical dismutation. It is suggested here that such a released peroxyl radical would be capable of bleaching and degrading solubilised β-carotene. No significant co-oxidation was found when retinol, retinal and retinoic acid were used as substrates, which indicates that these more polar carotenoids were not accessible to the enzyme.

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