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Pigment cooxidation activity by chickpea lipoxygenases

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Two lipoxygenase isozymes present in chickpea, CL-1 and CL-2, show cooxidation properties for β-carotene and retinyl acetate, with a higher activity for CL-1. Neither of them produces chlorophyll bleaching. Linoleic acid is necessary for the cooxidation reaction to take place. Besides pigment bleaching, CL-1 produces hydroperoxydienes and oxodienes, and CL-2 practically only hydroperoxydienes. No synergic effect of the two isozymes for the cooxidation activity was observed. CL-1 and CL-2 enzymatic activities are affected by β-carotene concentration, with total inactivation at concentration values equal to or higher than 14·0 μM.

INTRODUCTION

Lipoxygenase (linoleate : oxygen oxidoreductase, E.C. 1.13.11.12) is a dioxygenase that catalyses the hydroperoxidation of polyunsaturated fatty acids containing a Z-l, Z-4-pentadiene system. The enzyme owes its original name, carotene oxidase, to its capacity to oxidise carotenes and other pigments. This characteristic was discovered prior to its properties in oxidising polyunsaturated fatty acids, for which it is currently better known (Galliard & Chan, 1980). Today it is known that lipoxygenase (LOX) is an integral part of the pigment cooxidation process, and that the presence of both polyunsaturated acid and enzyme is necessary for this to take place. The results obtained by Klein *et al.* (1984) suggest that the enzyme-pentadienyl radical of the anaerobic cycle of LOX (Ludwig *et al.*, 1987) is the real pigment-oxidising agent and not the alkyl, alkoxy or peroxyl radicals proposed by other authors.

There are probably different cooxidation mechanisms, depending on the nature of the pigment. Hildebrand and Hymowitz (1982) suggested that the mechanisms bleaching chlorophylls and carotenes are different. Using antioxidants such as 2,6-di-*t*-butyl-4-hydroxytoluene, it has been possible to show that there is a first type of cooxidation by free radicals that are dissociated from the enzyme (as in the case of chlorophyll cooxidation), and a second type that carries out cooxidation by radicals linked to the enzyme at the catalytic site (Regdel *et al.* 1985).

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Different LOX isozymes have different efficiencies for catalysis of the cooxidative reaction. There is some confusion about whether they are more effective individually or in combination by a synergic process, as demonstrated by Ramadoss *et al.* (1978) for the soybean enzyme L-1 in combination with L-2 or L-3.

The bleaching of pigments by LOX has been described frequently but the products of such degradations have not usually been identified because of their complexity (Parry & Horgan, 1991). This bleaching property of LOX is usually studied by spectrophotometrically monitoring the loss in absorbance of β-carotene (460 nm), retinol (225 nm) or chlorophyll (668 nm) (Ben-Aziz *et al.*, 1971; Barimalaa & Gordon, 1988). The aim of this work is to show the differences between the chickpea LOX isozymes CL-1 and CL-2, regarding their capacity for pigment cooxidation.

MATERIALS AND METHODS

Enzymatic preparation

Certified seeds of chickpea (*Cicer arietinum*) var. Pedroxillano, Koipesol, S.A., Andújar, Jaén) were ground to a fine flour (0·4 mm mesh) and extracted with 50 mM sodium phosphate buffer pH 6·8, (1 : 7 (w/v)) by agitation at 0–4°C for 2 h, followed by filtration and centrifugation (48000*g* × 20 min). Proteins with LOX activity in this enzymatic extract were purified according to Sanz *et al.* (1992*a*), by fractionation with ammonium sulphate (40—60% sat.), and chromatography

on Sephacryl S-300, DEAE-cellulose and phenyl-sepharose. By this purification procedure, two proteins with LOX activity CL-1 and CL-2 were obtained pure and free of other contaminating proteins, verified by SDS electrophoresis followed by silver staining (Sanz *et al.*, 1992*a*), and chromatofocussing on a fast protein liquid chromatograph—Mono P column—in the pH range 3·90–6·35 (Sanz *et al.*, 1992*b*). Proteins had specific activities, in terms of LOX activity, of 61·12 U mg^{-1} for CL-1, and 81·5 U mg^{-1} for CL-2.

Substrate

For analysis of the pigment, cooxidation power of both isozymes of chickpea LOX, pigment substrate solutions were prepared as follows (Ben-Aziz *et al.*, 1971): 1 mg of β-carotene (1·525 mg of retinyl acetate or 1 mg of chlorophyll) was dissolved in 1 ml of chloroform to which 36 μl of Triton X-100 had been previously added. From this stock solution, 0·1 ml was taken; the solvent was evaporated in nitrogen flow and dissolved in 1 ml of 50 mM sodium phosphate buffer, pH 5·5 (or 6·0 for the studies with CL-2), AEDT 0·25% (w/v). To this solution was added 2·5 ml of a solution of linoleic acid 10 mM, prepared according to Axelrod *et al.* (1981), and 16·5 ml of sodium phosphate buffer 50 mM, pH 5·5 (or 6·0 for CL-2).

Cooxidation of pigments and lipoxygenase activity

The cooxidation reaction was carried out by incubating 2 ml of the substrate solution with different amounts of purified LOX isozymes for 10 min at 20°C. Bleaching of the pigments was followed spectrophotometrically at 460 nm for β-carotene, 330 nm for retinyl acetate and 668 nm for chlorophyll.

LOX activity was measured according to the method of Axelrod *et al.* (1981), by following the increase of absorbance at 234 nm due to the conjugated diene formed in the enzymatic hydroperoxidation. The molar extinction coefficient for these compounds is 25000 M^{-1} cm^{-1} (Verhagen *et al.*, 1977). At the same time, the enzymatic activity forming ketodienes was measured following the increase of absorbance at 280 nm; the chromophore corresponding to the conjugated ketodiene (Vioque & Holman, 1962) has a molar extinction coefficient of 22000 M^{-1} cm^{-1} (Verhagen *et al.*, 1977).

RESULTS AND DISCUSSION

The ability of some LOX isozymes to catalyse reactions of pigment decoloration by cooxidation has long been known, and used both for qualitative assays of LOX activity and in commercial applications—for example

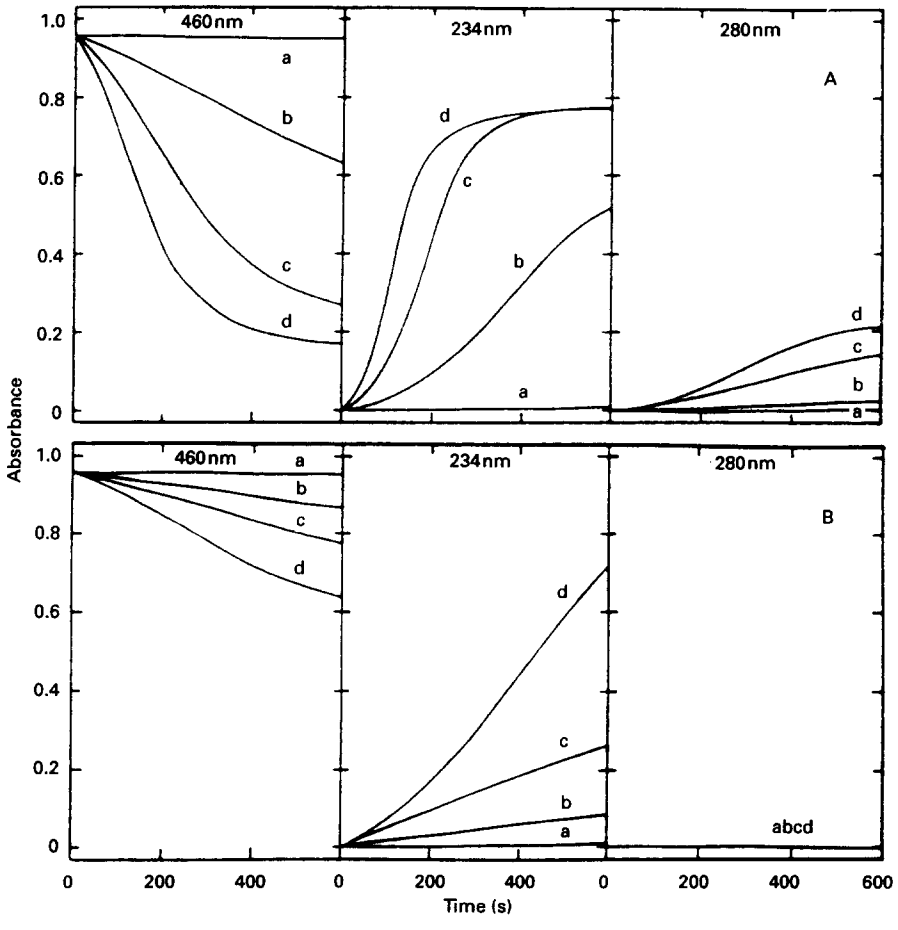

Fig. 1. Kinetics of cooxidation of β-carotene (460 nm), formation of hydroperoxydienes (234 nm) and ketodienes (280 nm) for different amounts of CL-1 (A): (a)—(b) 20·66 mU, (c) 61·98 mU, (d) 103·29 mU; and of CL-2 (B): (a)—(b) 61·15 mU, (c) 101·91 mU, (d) 305·74 mU.

in the manufacture of white bread by addition of soybean or bean flour (both rich in LOX activity) to that of wheat (Haas & Bohn, 1934). LOXs from different sources differ in their cooxidative activities (Grosch *et al.*, 1976; Ramadoss *et al.*, 1978; Reynolds and Klein, 1982; Zamora *et al.*, 1988), and neither the reaction mechanisms of this decoloration nor the relationship between the LOX–linoleate system and the decoloration of pigments is clear. The resulting oxidised products have not been fully identified by chromatographic methods. According to some authors, pigment decoloration may be associated with the formation of secondary carbonyl products. Galliard and Chan (1980) suggested an association of carotene oxidase activity with that of type II LOX isozymes, which are characterised (among other properties) by having an optimum pH of action between 5·5 and 7·0, and a low regiospecificity.

Chlorophyll and β-carotene are the most widely used pigments in studies of LOX cooxidation. Chickpea LOX isozymes cooxidise, to a different degree, both β-carotene and retinyl acetate, and have no effect at all on chlorophylls. This may be explained by the difference between the mechanisms of cooxidation of the different pigments, as described by Hildebrand and Hymowitz (1982).

Figure 1 shows the effect of different concentrations of chickpea LOX isozymes on the cooxidation of β-carotene, measuring the decrease of absorbance at 460 nm—the absorption maximum of this compound (under the experimental conditions β-carotene has a molar extinction coefficient of 102150 M^{-1} cm^{-1}). β-Carotene did not undergo autoxidation reactions during the enzymatic reaction (10 min), as shown by the stability of absorbance at 460 nm of the reaction mixture in the absence of any of the isozymes. CL-1 showed a higher cooxidative power for β-carotene than CL-2 (approximately double in the first minute of reaction). The kinetics of hydroperoxide formation (234 nm) showed an induction period not observed in the enzymatic reactions of hydroperoxidation of linoleic acid in the absence of β-carotene (data not shown). This could be due to an immediate deviation of the oxidising power of the molecular oxygen obtained during the enzymatic reaction towards cooxidation of the pigment. The formation of ketodienes also shows an induction period as in the absence of pigment, but in the presence of β-carotene this induction period is longer.

Ramadoss *et al.* (1978) demonstrated a synergic effect on the cooxidation of pigments by two soybean LOX isozymes. The decrease of absorbance at 460 nm after adding 1·014 μg (61·98 mU) of CL-1 and 0·750 μg (61·15 mU) of CL-2 jointly to the substrate did not show a significant increase in the cooxidation of β-

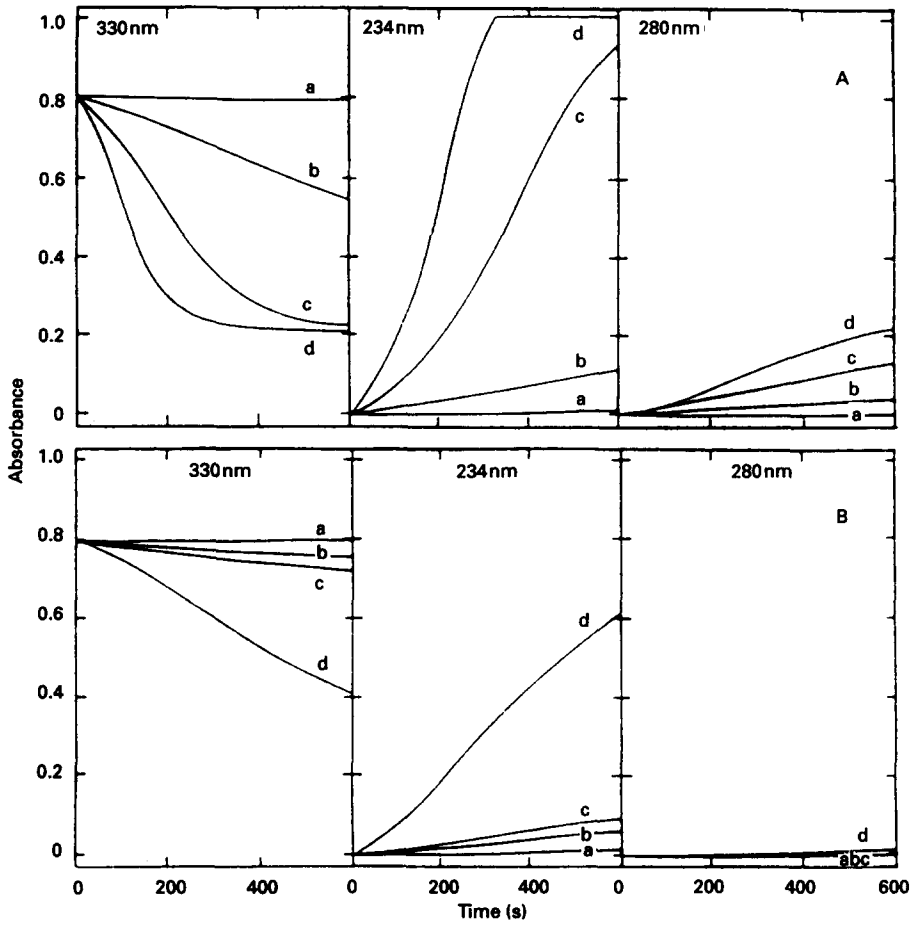

Fig. 2. Kinetics of cooxidation of retinyl acetate (330 nm), formation of hydroperoxydienes (234 nm) and ketodienes (280 nm) for different amounts of CL-1 (A): (a)—(b) 20·66 mU; (c) 61·98 mU; (d) 103·29 mU; and of CL-2 (B): (a)—(b) 61·15 mU, (c) 101·91 mU; (d) 305·74 mU.

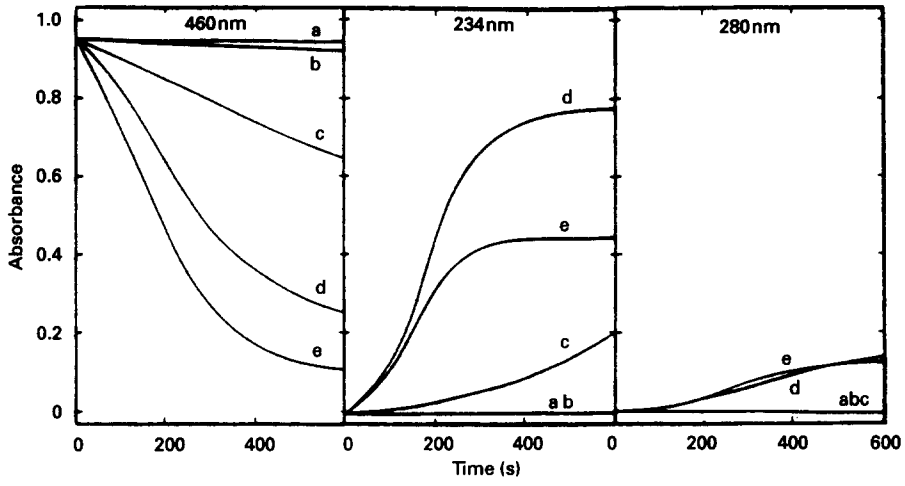

Fig. 3. Effect of the concentration of linoleic acid on the kinetics of cooxidation of β-carotene (460 nm), formation of hydroperoxydienes (234 nm) and ketodienes (280 nm) by CL-1. The reaction mixture contained 61·98 mU of CL-1, β-carotene 9·3 μM and different concentrations of linoleic acid: (a)—(b) 0·25 mM, (c) 0·75 mM, (d) 1·25 mM, (e) 1·75 mM.

carotene. This implies the non-existence of synergic action between the isozymes of chickpea lipoxygenase.

Vitamin A or retinol also acts as substrate for the cooxidation reaction of chickpea LOX isozymes (Fig. 2). The substrate for the cooxidation reaction contained 23·2 mM retinyl acetate and 1·25 mM linoleic acid as described in the experimental section. In our experimental conditions, the molar extinction coefficient for retinyl acetate at 330 nm was 34483 M^{-1} cm^{-1}. Barimalaa and Gordon (1988) found a molar absortivity of 37 313 M^{-1} cm^{-1} at 325 nm for retinyl acetate in their experimental conditions. The kinetics of retinyl acetate cooxidation were carried out by following the decrease of absorbance at 330 nm. These are very similar to those obtained using β-carotene as substrate, but with a higher production of hydroperoxydienes for CL-1 quantities larger than 0·338 μg in the reaction mixture. This could be due to a lower inhibition of hydroperoxydiene production by retinyl acetate than by β-carotene, or to a lower need of oxidising power by the retinol molecule than by that of β-carotene.

CL-1 needs the presence of linoleic acid for the reaction of bleaching of β-carotene to take place, and the latter increases with the concentration of acid (Fig. 3). In contrast, this effect is not parallel to the formation of hydroperoxydienes and ketodienes. Thus, at concentrations exceeding 1·25 mM of linoleic acid, there is an inhibition of the production (mainly) of hydroperoxydienes and (less so) of ketodienes. This inhibition by excess of substrate (linoleic acid) has previously been demonstrated for CL-1 in the calculation of its K_M value (Sanz *et al.*, 1992*b*). An inhibitory effect of β-carotene on the formation of hydroperoxydienes has also been shown for both CL-1 and CL-2, with a practically complete inactivation at values equal to or higher than 14·0 μM of β-carotene in the reaction mixture. Cohen *et al.* (1985) suggested the formation, in type II soybean LOX isozymes, of an inactive enzyme–pigment complex, which prevents the polyunsaturated acid from entering the catalytic site and thereby forming an active complex with the enzyme.

The data obtained in this and previous work (Sanz *et al.*, 1992*a,b*) seem to suggest the existence of two catalytic sites on chickpea LOX isozymes. One could give rise to acid hydroperoxydienes and is the classic catalytic site of LOX activity, (which we can denominate as principal). The other could be exclusively for the cooxidation of substrates. This cooxidation site would be occupied mainly by a pigment with polyenic structure (in this case, type poly-E) giving rise to the pigment oxidation and bleaching, and there would be competition between the pigment and linoleic acid to occupy the site. If the acid occupies the cooxidation site, the reaction product could be a ketodiene. On the other hand, the occupation of the principal site by a pigment molecule would completely block the catalytic activity of the enzyme, as Cohen *et al.* (1985) suggested in studies with type II soybean LOXs.

Chickpea CL-1 is a LOX with all the characteristics of the type-II LOX of Galliard and Chan (1980), as it possesses a high capacity for cooxidation of β-carotene and retinyl acetate (Figs 1(A) and 2(A)). In contrast, CL-2 has a lower cooxidative capacity (Figs 1(B) and 2(B)), and can be considered the type-I LOX of Galliard and Chan. This is confirmed by its high regiospecificity; CL-2 formed, almost exclusively, 13-hydroperoxides from linoleic acid (Sanz *et al.*, 1992*b*), also characteristic of soybean LOX-1 (Van Os *et al.*, 1979).

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