Effect of Calcium on the Oxidation of Linoleic Acid by Potato (*Solanum tuberosum* Var. Desiree) Tuber 5-Lipoxygenase

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When the effect of calcium on the oxidation of linoleic acid by potato tuber 5-lipoxygenase (LOX) was investigated, it was seen to promote the enzyme's activity at pH values higher than the optimum pH of 6.3, resulting in an enzyme activation at alkaline pH. Kinetic analysis of calcium activation at different pH values revealed that the cation abolished the inhibition by high substrate concentration, which occurs in the absence of Ca^{2+}, thus leading to activation at high substrate concentration. Studies were conducted to investigate the influence of Ca^{2+} on the physicochemical nature of the substrate and its effect on the LOX activity expression. It was concluded that the aggregation mode rather than the aggregation state of linoleic acid is responsible for potato 5-LOX changes.

Keywords: *Lipoxygenase; calcium; linoleic acid; fluorescence; scattering*

INTRODUCTION

Lipoxygenases (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a group of enzymes that catalyze the dioxygenation of polyunsaturated fatty acids (PUFAs) containing one or more 1,4-*cis*,*cis*-pentadiene systems, such as linoleic (LA), linolenic, or arachidonic acid, to conjugated hydroperoxy fatty acids (Veldink and Vliegenthart, 1984; Schewe et al., 1986; Kühn et al., 1986). Calcium has been shown to affect the LOX-catalyzed reaction in both mammals (Jakschick et al., 1980; Percival et al., 1992) and plants (Zimmerman and Snyder, 1974; Aharony and Stein, 1986; Avdiushko et al., 1994). The mechanism by which Ca^{2+} activates certain mammalian LOXs is well established, its being associated with the translocation, in leukocytes, of 5-LOX from the cytosol to the plasma membrane (Rouzer and Sammuelsson, 1987; Rouzer and Kargman, 1988), where it is activated by the specific protein FLAP (Coffey et al., 1992).

In plants, the important role of Ca^{2+} has been recognized in physiological processes such as senescence and defense against pathogens, in which LOXs are also involved. This has led to suggestions that it may be involved in the oxidation of PUFAs by plant LOXs (Rosahl, 1996), although this role is far from clear. What is clear, though, is that the way in which Ca^{2+} affects plant LOXs must be very different from that which has been depicted in mammalian cells because its effects are observed in the absence of membrane systems.

Zimmerman and Snyder (1974) and Douillard and Bergeron (1979) postulated a role for Ca^{2+} as an activator of LOX-2 from soybeans, although it acted as an inhibitor for LOX-1 from the same plant material. Such an activation, it was suggested, is mediated by the interaction of the cation with the LOX substrate LA, allowing the access of the enzyme to the substrate. On the other hand, Pinsky et al. (1973), Mulliez et al. (1987), and Bostock et al. (1992) were unable to observe any effects of Ca^{2+} on 5-LOX from potato tubers.

Because of this continuing uncertainty concerning the role of Ca^{2+} in the oxidation of PUFA by plant LOXs and because we found conditions in which potato 5-LOX may be affected by Ca^{2+}, we investigated the effect of Ca^{2+} on this enzyme and its possible mechanism of action.

MATERIALS AND METHODS

LA was purchased from Cayman Chemical Co. (Paris, France). Dehydrated calcium chloride was obtained from Fluka (Madrid, Spain). Potato 5-LOX was purified according to the method of Mulliez et al. (1987) and had a specific activity of 27 μmol of O_2 min^{-1} (mg of protein)$^{-1}$. Diphenylhexatriene (DPHT) was from Fluka (Madrid), and tetrahydrofuran (THF) was from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity.

Fluorometric Determination of Critical Micelle Concentration (cmc). The cmc of LA in the absence or presence of calcium was determined at different pH values by means of a fluorescence spectroscopy method described by Chattopadhyay and London (1984) and adapted to fatty acids by Serth et al. (1991). The 2 mL samples contained 0.1 M phosphate or borate buffer, 0.88 μM DPHT (supplied in 2 μL of THF), 1% (v/v) ethanol, and the indicated concentrations of Ca^{2+} and LA. The samples were flushed with N_2 and incubated for 30 min in the dark at 25 °C for equilibration and to reverse the photoisomerizaation of the fluorescent probe.

Fluorescence intensity was measured at 430 nm (excitation wavelength = 358 nm) by means of a Kontron SFM-25 spectrofluorometer thermostated at 25 °C. The relative values of the fluorescence were plotted against the LA concentration, and the cmc was taken to be the value represented at the intersection between the lines defining the fluorescence tendency in the premicellar and postmicellar regions.

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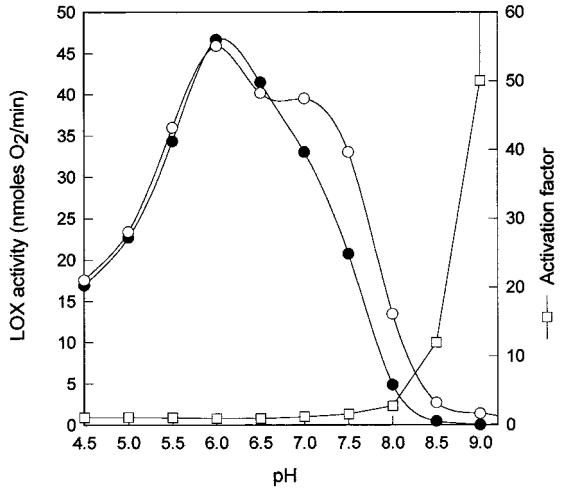

Figure 1. Dependence of potato 5-LOX activity on the pH in the presence of 0.8 mM Ca^{2+} (○) or in the absence of calcium (●). The oxygen consumption rate was determined at 25 °C as indicated under Materials and Methods. The LA concentration was 1.2 mM. The buffers used were 0.1 M sodium acetate from pH 3.0 to pH 5.0, 0.1 M Mopso K^+ from pH 5.0 to pH 7.5, and 0.05 M Tris-HCl + 0.05 M KCl from pH 7.5 to pH 9.5. Dependence of the activation factor of potato 5-LOX on pH in the presence of Ca^{2+} is shown (□). The LA concentration was 1.2 mM. The Ca^{2+} concentration was 0.8 mM.

Polarographic Determination of LOX. LOX activity was determined by monitoring O_2 consumption with a Clark-type electrode. Samples of 1 mL were prepared as for fluorescence spectroscopy but with the fluorescent probe and the solvent omitted. They were then shaken vigorously to become air-saturated and finally transferred to the stirred, thermostated oxygraph chamber (Hansatech Ltd., Norfolk, U.K.). The reaction was started by injection of 10 μL of potato 5-LOX. The millivolts recording scale was transformed into oxygen concentration as described elsewhere (Bru and García-Carmona, 1997). Activity was expressed as the maximal amount of O_2 consumed per minute (maximal slope of the reaction progress curves).

Determination of Scatter by LA Suspensions. The 2 mL samples contained 0.05 M Tris-HCl buffer (pH 8.5), 0.05 M KCl, 1% (v/v) ethanol, and the indicated concentrations of Ca^{2+} and LA. To avoid possible oxidation of the LA, the samples were flushed with N_2. The intensity of scattered light at an angle of 90° was determined in a Kontron SFM-25 spectrofluorometer thermostated at 25 °C. Dispersed light intensity was measured at 358 nm (incident wavelength = 358 nm) (López-Nicolás et al., 1995).

RESULTS AND DISCUSSION

Effect of Calcium on Potato 5-LOX Activity. The effect of pH on potato 5-LOX activity was determined in both the absence and presence of Ca^{2+} (Figure 1). In both cases, the LOX activity had a slightly acid optimum pH of 6.3, the activity decreasing sharply at other pH values. The presence of Ca^{2+} in the reaction medium resulted in substantial variations of the pH curve, particularly at basic pH values, at which the enzymes became more active in the presence of the cation, whereas in the neutral and acid regions the presence of Ca^{2+} did not lead to changes of enzymatic activity. These results agree, in part, with those presented by Pinsky et al. (1973), Mulliez et al. (1987), and Bostock et al. (1992), who showed that Ca^{2+} had no effect on potato 5-LOX activity at a pH around neutrality, although they did not study the effect of the cation at alkaline pH. Koch et al. (1971) found that the pH curve of navy bean was substancially modified by Ca^{2+}, as optimum pH shifted from 6.4 to 7.2 and the enzyme displayed activity from pH 7.0 to 9.0. To observe more clearly the effect of Ca^{2+} on the enzymatic activity, Figure 1 also shows the activation factor at each pH, defined as the ratio between LOX activity in the presence and in the absence of Ca^{2+} for a fixed substrate concentration. The activation factor increased sharply in the basic region of pH, although the absolute value of LOX activity decreased with respect to that obtained at the optimum pH. At pH 9.0, the activity was very low in the absence of Ca^{2+}, although it could still be accurately measured in appropriate conditions.

This Ca^{2+}-mediated activation of potato 5-LOX was further analyzed. Figure 2 shows the substrate-dependent kinetics of potato 5-LOX at different Ca^{2+} concentrations and at three different alkaline pH values. In all cases, for fixed LA and Ca^{2+} concentrations, the activity decreased when the pH was increased. However, the presence of Ca^{2+} at each pH caused dramatic changes in the enzyme kinetics, which led to the abolishment of the inhibition by high substrate concentration that occurs in its absence.

As a consequence of the Ca^{2+}-induced change in potato 5-LOX kinetics, an enzyme activation effect was observed when a suitable LA concentration was used, and the extent of activation—the activation factor—was dependent not only on the activator concentration but also on the substrate concentration, as seen in Figure 3.

For comparison, the substrate-dependent kinetics of potato 5-LOX at optimum pH (6.3) is also shown in the inset of Figure 2. In this case, Ca^{2+} has almost no effect on the LOX activity or on its kinetics. Moreover, this result agrees with those studies which conclude that Ca^{2+} has no effect on potato 5-LOX working in optimum pH conditions (Pinsky et al., 1973; Mulliez et al., 1987; Bostock et al., 1992).

An analysis of the data of Figure 2 at alkaline pH by Lineweaver–Burk transform reveals a complex kinetics that does not correspond to the typical patterns of enzyme activation (data not shown).

Other authors have suggested that Ca^{2+} might affect plant LOXs through its interaction with its fatty acid substrate (Zimmerman and Snyder, 1974; Douillard and Bergeron, 1979). To investigate the mechanism by which Ca^{2+} modifies potato 5-LOX kinetics at basic pH but not at neutral or acid pH values, we studied the possible effect of the cation on the physicochemical nature of the LA substrate. Thus, we investigated the effect of Ca^{2+} on both the aggregation state, that is, whether LA is aggregated or not, and the aggregation mode, that is, which type of aggregate it forms.

Effect of Calcium on the Aggregation State of PUFAs. The cmc values of LA were determined in the absence of Ca^{2+} and in its presence at different concentrations at different pH values to test the cation's effect on the fatty acid aggregation state. As can be seen from Table 1, Ca^{2+} lowered the cmc, which was stronger at alkaline (8.5) than at acid (6.3) pH. This difference might be significant with respect to the different effects of Ca^{2+} at these pH values on the enzyme's activity: no effect at pH 6.3 and a strong effect on the enzyme's kinetics at pH 8.5. However, the cmc, that is, the aggregation state, does not seem to be related to inhibition by high substrate concentration because this phenomenon begins at LA concentrations that are well below the cmc at pH 9 and 8.5, whereas at pH 8.0 it

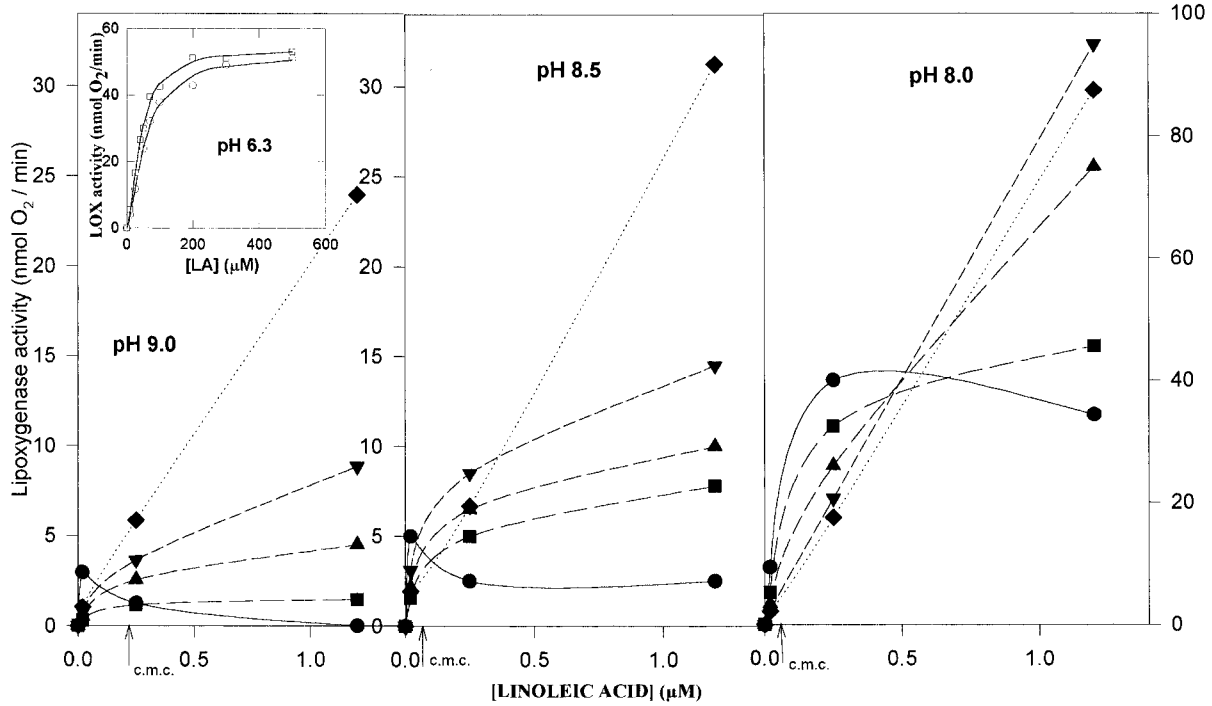

Figure 2. Potato 5-LOX-catalyzed oxidation of LA in the presence of different Ca^{2+} concentrations at three basic pH values: (●) no Ca^{2+}; (■) 0.1 mM Ca^{2+}; (▲) 0.3 mM Ca^{2+}; (▼) 0.6 mM Ca^{2+}; (◆) 0.8 mM Ca^{2+}. The arrows indicate the cmc values of linoleic acid in the absence of Ca^{2+} at each pH. (**Inset**) Potato 5-LOX-catalyzed oxidation of LA at pH 6.3 in the presence (○) and in the presence (□) of Ca^{2+} (0.25 mM).

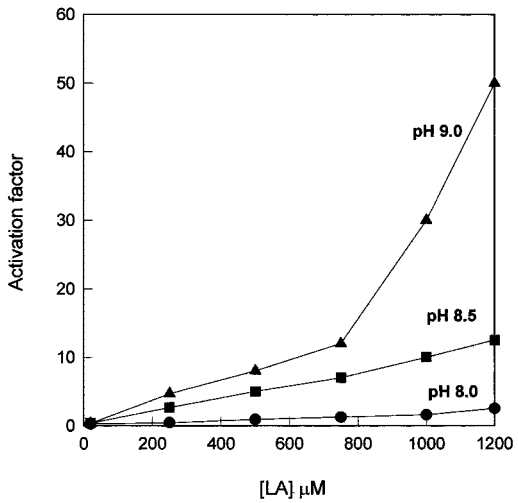

Figure 3. Effect of LA concentration on the activation factor of potato 5-LOX at basic pH values. The concentration of calcium used was 0.8 mM. The values of pH were (●) pH 8.0, (■) pH 8.5, and (▲) pH 9.0.

Table 1. Effect of Ca^{2+} on the Critical Micelle Concentration of Linoleic Acid at Different pH Values

	cmc (μM) at			
Ca (mM)	pH 6.3	pH 8.0	pH 8.5	pH 9.0
0	11.92	55	78.57	190
0.25	10.83		43.01	
0.5	9.00		32.90	

begins at concentrations that are well above the cmc. cmc values in the absence of Ca^{2+} are indicated in Figure 2 at each pH to better illustrate this reasoning. Therefore, it does not seem plausible that a Ca^{2+}-mediated decrease in cmc is the main reason for the radical changes observed in LOX kinetics in the presence of Ca^{2+}.

Effect of Calcium on the Aggregation Mode of LA. The presence of Ca^{2+} in the reaction medium may induce changes in other physicochemical parameters of LA such as the size of the aggregates. The size variations of the LA aggregates can be estimated by monitoring the scattered light intensity at an angle with respect to the incident beam direction, for instance, at 90° in a spectrofluorometer. Large aggregate suspensions (turbid) produce a dispersion of the incident light beam and thus a high intensity of scattering; on the other hand, neither true molecular solutions of monomeric LA nor micellar aggregates of small and monodisperse size (both transparent) have the capacity to scatter visible light.

As can be seen in Figure 4, at pH 8.5 and in the absence of Ca^{2+} no light scattering was detected up to 200 μM LA, which is a concentration well above the cmc (78.57 μM, see Table 1); that is to say, in these conditions, small micellar aggregates are formed above the cmc. According to Cistola et al. (1988) at that pH and above, long-chain fatty acids would form micellar solutions, which are transparent by virtue of the small and monodisperse size of the micelles.

In the presence of Ca^{2+}, changes in light scattering intensity can be clearly appreciated only at LA concentrations that are above the cmc at each particular Ca^{2+} concentration. This demonstrates that LA aggregates formed above the cmc in the presence of Ca^{2+} are different from those formed in the absence of the cation. In particular, the former are large enough to produce light scattering.

On the other hand, at pH 6.3 and in the absence of Ca^{2+}, LA aggregates are large because solutions are turbid. Although the present data do not confirm that the LA aggregates observed at pH 8.5 in the presence of Ca^{2+} are the same as those formed at pH 6.3 (conditions in which LOX expresses high activity), it can

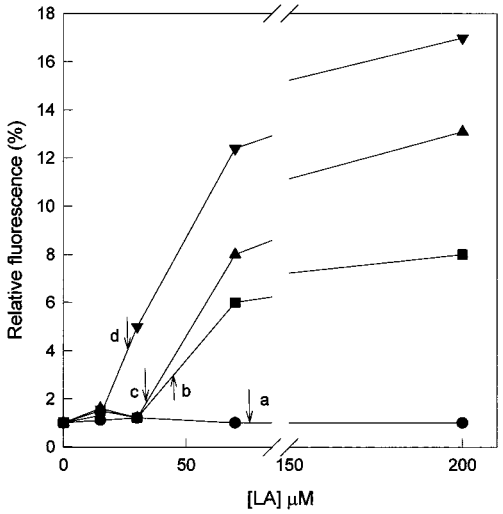

Figure 4. Effect of LA concentration on the dispersed light intensity at 358 nm at pH 8.5 at different Ca^{2+} concentrations: (●) no Ca^{2+}; (■) 0.25 mM Ca^{2+}; (▲) 0.5 mM Ca^{2+}; (▼) 0.8 mM Ca^{2+}. The arrows indicate the cmc values of linoleic acid at different Ca^{2+} concentrations: (a) no Ca^{2+}; (b) 0.25 M Ca^{2+}; (c) 0.5 mM Ca^{2+}; (d) 0.8 mM Ca^{2+}.

be concluded that they share the property of being large and are not like the small ones formed in the absence of Ca^{2+} at alkaline pH, at which LOX is inhibited by high substrate concentration. Thus, the aggregation mode of the LA substrate seems to be related with the expression of activity and kinetic behavior of potato 5-LOX.

In a previous study (Bru and García-Carmona, 1997), we demonstrated that at pH 6.3 the inhibition of large LA aggregate formation by cyclodextrins paralleled the inhibition of potato 5-LOX by these LA complexing agents and concluded that an aggregated substrate is needed to express greatest activity. The results obtained in the present study indicate that, in addition, these LA aggregates must be large to react optimally with potato 5-LOX at any pH.

Tatulian et al. (1998) have provided evidence that the Ca^{2+} ion supported binding of soybean LOX L-1 to negatively charged phospholipid membranes. In addition, these authors demonstrated that Ca^{2+} ions affect both the structure and function of the enzyme. In particular, moderate concentrations of Ca^{2+} stabilize the enzyme structure and stimulate hydroperoxidation of monodisperse fatty acids, whereas >1 mM Ca^{2+} exerts a destabilizing effect on L-1 structure and promotes the conversion of PUFA to conjugated ketodienes.

We determined the effect of Ca^{2+} on potato LOX activity by measuring O_2 consumption. The results obtained are in agreement with those presented by Tatulian et al. (1998) for soybean LOX L-1. The possibility that the calcium-mediated changes of the kinetic behavior of potato LOX are related with changes of reaction products is currently under investigation.

ABBREVIATIONS USED

LOX, lipoxygenase; PUFA, polyunsaturated fatty acids; LA, linoleic acid; cmc, critical micelle concentration; THF, tetrahydrofuran; DPHT, diphenylhexatriene.

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