Enzymatic Oxidation of Linoleic Acid by Lipoxygenase Forming Inclusion Complexes with Cyclodextrins as Starch Model Molecules

José M. López-Nicolás, Roque Bru, and Francisco García-Carmona*

Departamento de Bioquímica y Biología Molecular A, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, E-30001 Murcia, Spain

The structural resemblance between cyclodextrins (CDs) and starch in its helical conformation makes the former a suitable model system to study the oxidation of polyunsaturated fatty acids such as linoleic acid (LA) which naturally occur associated with amylose as inclusion complexes in different plant storage tissues. In the presence of β-cyclodextrin (β-CD), LA forms inclusion complexes of 1:2 stoichiometry which are in equilibrium with free LA and free β-CD. For the oxidation of LA by LOX in the presence of β-CD, we propose a model in which free LA is the only effective substrate; thus the oxidation of the complexed substrate requires the previous dissociation of the complex. Consistently, β-CD was shown to slow down the reaction rate of LOX oxidation, which was especially due to the increase of K_m, V_{max} remaining unchanged. The apparent inhibition produced by β-CD (increase of K_m) is due to removal of effective substrate in the form of inclusion complexes. This "sequestered" substrate can, however, be converted since it is in equilibrium with the free.

Keywords: *Linoleic acid; cyclodextrin; soybean lipoxygenase; cyclodextrin assay*

INTRODUCTION

Lipoxygenases (linoleate:oxygen reductase, EC 1.13.11.12) are iron-containing dioxygenases that converted polyunsaturated fatty acids possessing one or more 1(*Z*),4(*Z*)-pentadiene systems into *Z*,*E* conjugated hydroperoxy fatty acids (Veldkin and Vliegenthart, 1984; Schewe et al., 1986; Kühn et al., 1986). Lipoxygenases play a fundamental role in PUFA metabolism in plants and animals. The products of mammalian LOX are directly involved in the immune response and in inflammatory processes (Yamamoto, 1992). The physiological role of plant lipoxygenases is less clear. LOX are postulated to be involved in senescence stimulating the intracellular free radical production (Fobel et al., 1987) that meets directly with membrane deterioration (Dhindsa et al., 1982). LOX is also involved in the response of plants to wounding as hydroperoxides of linoleic acid (LOOH) are precursors of traumatic acid (Hitchcock and Nichols, 1971).

LOX is found in high levels in cotyledons of mature seeds [wheat (Guss et al., 1968), maize (Sekiya et al., 1983), rice (Ohta et al., 1986)] and in other storage organs such as potato tubers (Mulliez et al., 1987). In these, LOX and also the oxidation products of PUFA have been postulated to play a role in germination and early development of stems (Hildebrand and Hymowitz, 1981).

In storage tissues starch appears associated with lipids, of which free fatty acids may account for 0.5% of starch weight, more than 60% being unsaturated (Swinkels, 1992).

The type of association of lipids with starch has been well-known for several decades (Mikus et al., 1946). The lipid forms molecular complexes with amylose in the helical configuration, the lipid occupying the central longitudinal space left by the helix. The metabolism of the entrapped fatty acid (FA) by LOX must involve a previous step of release either by change of amylose conformation or by destruction of amylose by hydrolysis or dissociation. Molecular models of the specific interactions involved in the amylose complexation by fatty acids have been proposed to describe the starch−fatty acid interaction (Godet et al., 1993). These modelings have also been shown to be an efficient tool to predict the interactions between cyclodextrins (CDs) and guest molecules.

Cyclodextrins can be thought of as model molecules of starch (Abadie et al., 1994; Szejtli and Banky-Elöd, 1975) as they are cyclic oligosaccharides formed by the enzymatic hydrolysis of starch (Szejtli, 1988) and composed of six to eight residues of glucopyranose. Likewise they have the property of forming inclusion complexes with fatty acids. Complexes of CD with PUFA were previously characterized in our laboratory and found to have a stoichiometry 1FA:2CD and stability constants which were influenced by pH, temperature, and the type of CD (López-Nicolás et al., 1995; Bru et al., 1995)

Oxidation of PUFA forming inclusion complexes with CD by LOX can thus be regarded as a model system of their oxidation in storage tissues complexed with starch. In this study the oxidation of linoleic acid entrapped in β-CD by soybean LOX-1 has been characterized and a reaction scheme that supports the complexing agent oxidation-preventing properties has been proposed.

MATERIALS AND METHODS

Linoleic acid was purchased from Cayman Chemical Co. (Paris, France). β-CD and lipoxygenase (type V prepared by affinity chromatography; specific activity, 630.000 units/mg of protein) from soybean were obtained from Sigma (Madrid, Spain). Diphenylhexatriene (DPHT) was a product from Fluka (Madrid), and tetrahydrofuran (THF) was from Merck (Darmstadt, Germany). All the other chemicals used were of the highest purity.

Preparation of LA−β-CD Complexes. LA−β-CD complex solution was prepared as described previously (López-Nicolás et al., 1995). Briefly, β-CD was dissolved in 0.1 M potassium borate buffer, pH 9, containing 1% v/v ethanol, followed by the addition of the fatty acid prepared in the same buffer. The samples were flushed with N_2 to prevent oxidation

* Corresponding author (telephone +34 68 30 71 00, ext. 2923; fax + 34 68 36 41 47; e-mail bru@fcu.um.es).

S0021-8561(96)00553-5 CCC: $14.00

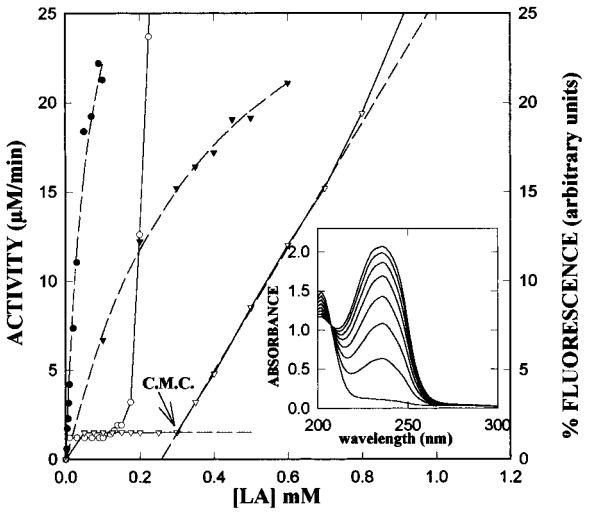


Figure 1. Soybean LOX-catalyzed oxidation of LA in the presence of β-CD. Effect of LA concentration on LOX activity in the pre-micellar region of LA: (●) no β-CD; (▼) β-CD 0.5 mM. Fluorescence intensity: (○) no β-CD; (▽) β-CD 0.5 mM. Inset: scan of LOX-catalyzed oxidation of LA in β-CD medium. The conditions are $S_T = 160\ \mu$M, [β-CD] = 150 μM. The spectra were recorded every two minutes in a Hewlett-Packard 8452A diode array spectrophotometer.

of LA during preparation. Just before using, samples were vigorously shaken to supply O_2 for the enzymatic reaction.

Fluorometric Determination of cmc. The critical micellar concentration (cmc) value of LA was determined by means of a fluorescence spectroscopy method as described elsewhere (López-Nicolás et al., 1995; Chattopadhyay and London, 1984). Two-milliliter samples contained 0.1 M borate buffer (pH 9), 0.88 μM DPHT (supplied in 2 μL of THF), 1% v/v ethanol, and the indicated concentration of β-CD and LA. The samples were flushed with N_2 and incubated for 30 min in the dark at the desired temperature for equilibration and to reverse the photoisomerization of the fluorescent probe.

Fluorescence intensity measurements were made at 25 °C at 430 nm (358 nm excitation wavelength) in a Kontron SFM-25 (Milano, Italy) spectrofluorimeter equipped with thermostated cells. The cmc was determined graphically from a plot of fluorescence relative values versus LA concentration (see Figure 1) as the intersection between the lines defining the fluorescence tendency in the pre- and postmicellar regions.

This determination makes it possible to set the conditions in which LA is molecularly dispersed and does not form aggregates.

Enzyme Assay. LOX activity was assayed by monitoring the increase in absorbance at 234 nm ($\epsilon_{234} = 25\ 000\ M^{-1}\ cm^{-1}$) of the forming hydroperoxides in a Kontron Uvikon 940 spectrophotometer at 25 °C equipped with thermostated cells. The reaction was started by adding 5 μL of enzyme (60−240 units as defined in the Sigma catalog; one unit caused an increase in A_{234} of 0.001/min at pH 9 at 25 °C when linoleic acid is the substrate in a 3.0 mL volume) to 1 mL of complex LA−β-CD preparation. Spectra were recorded in a Hewlett-Packard 8452A (Madrid, Spain) diode array spectrophotometer.

RESULTS AND DISCUSSION

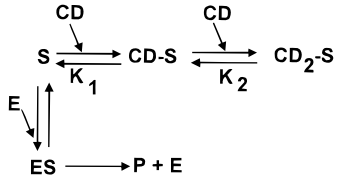
Long-chain fatty acids in aqueous solution may form a micellar phase above their cmc at pH 9 or higher (Cistola et al., 1988). As shown in Figure 1, the formation of micellar aggregates of LA can be monitored by the increase in fluorescence of the probe DPHT (López-Nicolás et al., 1995; Chattopadhyay and London, 1984). The cmc increases in the presence of β-CD, reflecting the formation of inclusion complexes and resulting in the extension of the range of monomeric

Table 1. Kinetic Parameters of Soybean LOX-1 in 0.1 M Borate Buffer, pH 9, in the Absence or Presence of 0.5 mM β-CD

parameter (units)	−	+
V_{max} (μM/min)	34.7 ± 3.8	35.2 ± 2.5
K_m (μM)	62 ± 5.2	400.1 ± 4.2[a]

[a] This is an apparent K_m in the presence of β-CD.

Scheme 1. Scheme of an Enzymatic Reaction in the Presence of CD Forming 1:2 Complexes with the Substrate



LA. In a previous study (López-Nicolás et al., 1995) these complexes were shown to have a stoichiometry of 2:1 (β-CD:LA), being in equilibrium with free LA. The equilibrium, as shown in Scheme 1, was characterized by two equilibrium constants, K_1 and K_2, which were determined in a series of conditions. The equilibrium constants permit us to know the concentration of each species in Scheme 1 once both the total concentration of CD and LA have been set. As shown in Figure 1, LA is transformed by LOX in this system, and both the transparency of the inclusion complex solution and the low absorption of β-CD, even at very low wavelengths, permit the UV spectroscopic characterization of the reaction.

Scheme 1 gives rise to an important question concerning the enzyme reaction: Which species of substrate, if any, in addition to the free LA are being utilized by LOX? Below we will show how this point is clarified.

In order to explore the role of β-CD in the enzymatic oxidation of LA, we studied the dependence of LOX activity on LA concentration in the absence and presence of β-CD (Figure 1). As indicated by the low fluorescence emission, the LA concentration region explored consists of no aggregates and only free or complexed monomers. CD produced an increase in K_m (from 60 to 400 μM) and had almost no effect on V_{max}, as can also be seen from Table 1, suggesting that β-CD behaves apparently as a competitive inhibitor, although its structure is very different from that of LA.

The K_m value obtained without β-CD is 2-fold greater than values reported in the literature (Scheller et al., 1995). As we have not utilized Tween 20 (the substrate is in pure state) for the solubilization of LA, this fact may contribue to the differences observed in K_m values with those of the literature.

The type of inhibition by β-CD was further studied by changing β-CD concentration at constant LA concentration. As shown in Figure 2, the LOX activity is inhibited by increasing concentrations of β-CD, at a fixed LA concentration. The inhibition of LOX by β-CD has been qualitatively described in a medium containing Tween 20, the explanation given being the possible formation of inclusion complexes (Laakso, 1984). Our interpretation of the observed inhibition also points to the formation of inclusion complexes that most likely cannot be utilized by LOX, unless they dissociate. This interpretation is based, in principle, on the fact that the activity drops practically to zero when sufficient CD is

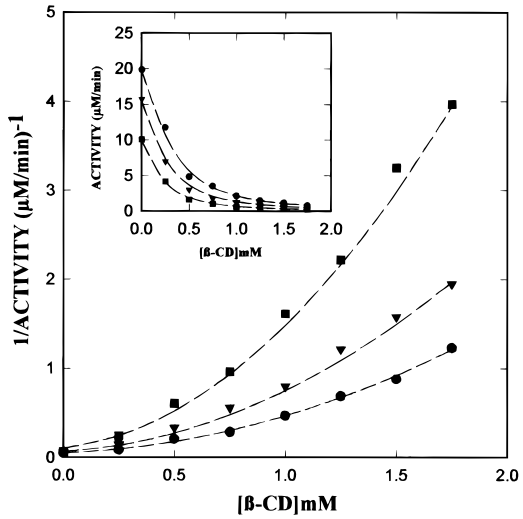


Figure 2. Analysis of β-CD effect on LOX-catalyzed oxidation of LA. Dixon plot and direct plot (inset) of effect of β-CD concentration on the reaction rate of soybean-LOX catalyzed LA oxidation at different LA concentrations: (■) 24.8 μM; (▼) 50 μM; (●) 82.3 μM.

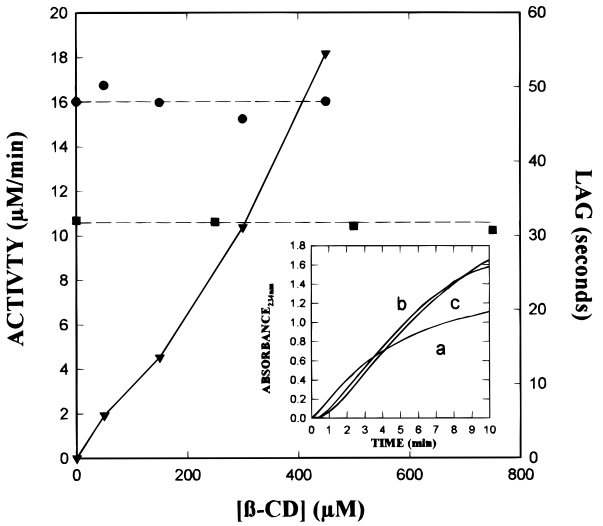


Figure 3. Cyclodextrin assay. Soybean LOX-catalyzed oxidation of LA reaction rates at two different S_{free} concentrations: (●) 82.3 μM; (■) 50 μM, and induction period at (▼) 82.3 μM concentration of S_{free}. Inset: time course of the soybean LOX-catalyzed oxidation of LA in the absence (a) and presence (b, c) of β-CD at pH 9. In (a) a 82.3 μM LA concentration was used. In (b) the medium contained 0.3mM β-CD and 295 μM LA, which yields 82.3 μM free LA, and in (c) the medium contained 0.45 mM β-CD and 485 μM LA, which yields 82.3 μM free LA.

added (see Figure 2). In accordance with Scheme 1, free LA would be totally sequestered at high concentrations of β-CD. The total inhibition of LOX could not be seen in the presence of Tween 20 (Laakso, 1984) since the detergent competes with CD for the solubilization of LA in the form of a mixed micelle that is used by soybean LOX (López-Nicolás et al., 1994). It should be possible that some interaction between β-CD and LOX would lead to enzyme inhibition or denaturation thus being the cause of the observed activity decay. A kinetic experiment to determine whether the preincubation of LOX with β-CD induces time-dependent activity losses with respect to a control incubated without β-CD has been carried out. As a result, there are no significant differences between the enzyme incubated for 2 h with β-CD (5.3 $\pm$ 0.3 μM/min) and the control (5.6 $\pm$ 0.3 μM/min); thus we conclude that the β-CD−LOX interaction, if it even exists, is not the cause of the activity decrease observed in Figure 2.

Considering that free LA is the only form of substrate LOX can use, the reaction rate in the presence of β-CD can easily be quantitatively described through the equilibrium constants. If the concentration of complex is negligible with respect to the total concentration of β-CD (CD_T), the amount of free, noncomplexed substrate can be expressed as

$$S_{free} = \frac{S_T}{1 + K_1 CD_T + K_1 K_2 CD_T^2} \quad (1)$$

Applying this to the Michaelian reaction rate equation, we have

$$V = \frac{V_{max} S_T}{K_m(1 + K_1 CD_T + K_1 K_2 CD_T^2) + S_T} \quad (2)$$

or, in reciprocal form the equation

$$\frac{1}{V} = \frac{K_m K_1 K_2}{V_{max} S_T} CD_T^2 + \frac{K_m K_1}{V_{max} S_T} CD_T + \frac{1}{V_{max}} + \frac{K_m}{V_{max} S_T} \quad (3)$$

Equation 3 predicts that a Dixon-type plot is not linear but quadratic, as shown in Figure 2. Since K_m and V_{max} are known in the absence of β-CD (see Table 1), inhibition data can be fit by nonlinear regression to the above equation to estimate the equilibrium constants.

The parameters which best fit the data in Figure 2 are $K_1 = 4.1 \pm 1.5$ and $K_2 = 3.7 \pm 1.6$ mM^{-1}. The equilibrium constants determined by this enzymatic method procedure are quite similar to those determined previously by the cmc procedure (López-Nicolás et al., 1995), although they are slightly different here. This may be a consequence of the sensitivity of the enzymatic method, since information concerning S_{free} is obtained from every S_T tested, while with the cmc method, information is obtained only when one particular S_T concentration, namely the cmc, is reached, and sometimes there is no sharp change in fluorescence intensity.

Our study contrasts with that of Jyothirmayi and Ramadoss (1991) since they maintain that complexes can be used by LOX and that the inhibition observed in the presence of β-CD is due to a direct interaction of β-CD with LOX. These differences may be due to the pH used since they observed an activity enhancement in the presence of CDs only at pH 7.5 but not at pH 9. We suggest that, at least at pH 9, complexes are not substrates for LOX and that there is no such direct inhibition, but simply the effective substrate concentration is diminished by β-CD when forming the inclusion complexes and, as a consequence, the inhibition observed is only apparent.

Thus, it is expected that when the concentration of effective free substrate is constant, the enzyme activity is constant too, independent of the total substrate concentration. The stored substrate is released as it is being transformed into product, obeying the equilibria depicted in Scheme 1.

To confirm this, an experiment called the "cyclodextrin assay" was designed in which, by using the equilibrium constants, S_T is calculated so that S_{free} remains constant, independent of the β-CD concentration used (see eq 1). As shown in Figure 3, activity is independent

of the total LA concentration, thus proving our expectations concerning the mechanism of inhibition.

Concerning the reaction progress in conditions of the cyclodextrin assay (Figure 3), it can be observed that while the effective substrate is rapidly exhausted without β-CD, the reaction proceeds at a constant rate for a long time in the presence of β-CD, producing an amount of total product which is much greater than the amount of effective substrate. The reason for this must be that the complexed substrate is released as it is being transformed into product, obeying the equilibria depicted in Scheme 1 and the release of substrate from the β-CD cavity is not a limiting step, at least at the concentration of enzyme used in our assays.

As shown in Figure 3 the kinetic lag phase of LOX increased with increasing concentration of β-CD probably because the reaction product, LOOH, which is the activator of the enzyme (Pistorius and Axelrod, 1976), is also sequestered by the excess free β-CD, and so, a larger amount has to be produced in order to activate the enzyme.

CONCLUSION

In this model system, CD competes with LOX to bind LA, the complexed LA not being a suitable substrate. Therefore, the oxidation of LA by LOX proceeds after a committed step of LA−CD inclusion complex dissociation, so the CD behaves as a competitive inhibitor. Moreover, the complex dissociation is not a rate-limiting step since the equilibrium is very fast. As a result, CD prevents the oxidation of LA by removal of effective substrate. The ability of starch to include FA should have an effect as that of CD concerning the enzymatic oxidation of LA. However, the complexes of starch and LA may not be as readily dissociable as those of CD, and thus the slow release of LA from starch could limit the oxidation rate and so effectively protecting LA from oxidation in the storage tissue. If the release of LA from amylose occurs through the open extremes of the helix, the exchange of LA is practically nil, and only when the amylose is hydrolyzed or its helical structure destroyed can there be a massive release of fatty acid readily oxidizable by the abundant LOX. This fact would conceal the apparent paradox of the coexistence of LOX and free PUFA in seeds and tubers without being oxidized (Lojkowska, 1988).

ABBREVIATIONS USED

LOX, lipoxygenase; FA, fatty acid; PUFA, polyunsaturated fatty acids; CD, cyclodextrin; β-CD, β-cyclodextrin; LA, linoleic acid; DPHT, diphenylhexatriene; THF, tetrahydrofuran; cmc, critical micellar concentration; S_{free}, free substrate; S_T, total substrate; CD_T, total CD; LOOH, linoleic acid hydroperoxide.

LITERATURE CITED

Abadie, C.; Hug, M.; Kübli, C.; Gains, N. Effect of cyclodextrins and undigested starch on the loss of chenodeoxycholate. *Biochem. J.* **1994**, *299*, 725−730.

Bru, R.; López-Nicolás, J. M.; García-Carmona, F. Aggregation of polyunsaturated fatty acids in the presence of cyclodextrins. *Colloids Surf.* **1995**, *97*, 263−269.

Chattopadhyay, A.; London, E. Fluorimetric determination of critical micelle concentration avoiding interference from detergent charge. *Anal. Biochem.* **1984**, *139*, 408−412.

Cistola, D. P.; Hamilton, J. A.; Jackson, D.; Small, D. M. Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule. *Biochemistry* **1988**, *27*, 1881−1888.

Dhindsa, R. S.; Dhindsa, P. P.; Reid, D. M. Leaf senescence and lipid peroxidation: effects on some phytochromes and scavengers of free radicals and singlet oxygen. *Physiol. Plant.* **1982**, *56*, 453−457.

Fobel, M.; Lynch, D. V.; John, E. T. Membrane deterioration in senescing carnation flowers: Coordinated effects of phospholipid degradation and the action of membranous lipoxygenase. *Plant Physiol.* **1987**, *85*, 204−211.

Godet, M. C.; Tran, V.; Delage, M. M.; Buléon, A. Molecular modelling of the specific interactions involved in the amylose complexation by fatty acids. *Int. J. Biol. Macromol.* **1993**, *15*, 11−16.

Guss, P. L.; Macko, V.; Richardson, T.; Stahmann, M. A. Lipoxidase in early growth of wheat. *Plant Cell Physiol.* **1968**, *9*, 415−422.

Hildebrand, D. F.; Hymowitz, T. Two soybean genotypes lacking lipoxygenase-1. *J. Am. Oil Chem. Soc.* **1981**, *58*, 583−586.

Hitchcock, C.; Nichols, B. W. In *Plant Lipid Biochemistry (Experimental Botany)*; Academic Press: New York, 1971; Vol. IV, pp 388.

Jyothirmayi, N.; Ramadoss, C. S. Soybean lipoxygenase catalysed oxygenation of unsaturated fatty acid encapsulated in cyclodextrin. *Biochim. Biophys. Acta* **1991**, *1083*, 193−200.

Kühn, H.; Schewe, T.; Rapoport, S. M. The stereochemistry of the reactions of lipoxygenase and their metabolites. Proposed nomenclature of lipoxygenases and related enzymes. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1986**, *58*, 273−311.

Laakso, S. Inhibition of lipid peroxidation by casein evidence of molecular encapsulation of 1,4-pentadiene fatty acids. *Biochim. Biophys. Acta* **1984**, *792*, 11−15.

Lojkowska, E. Lipid composition and post-wounding degradation in potato slices from cultivars differing in susceptibility to autolysis. *Potato Res.* **1988**, *31*, 541−549.

López-Nicolás, J. M.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. An octhaethylene glycol monododecyl ether-based mixed micellar assay for lipoxygenase acting at neutral pH. *Anal. Biochem.* **1994**, *221*, 410−415.

López-Nicolás, J. M.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. Use of "soluble lipids" for biochemical processes: linoleic acid-cyclodextrin inclusion complexes in aqueous solutions. *Biochem. J.* **1995**, *308*, 151−154.

Mikus, F. F.; Hixon, R. M.; Rundle, R. E. Complexes of fatty acids with amylose. *J. Am. Chem. Soc.* **1946**, *68*, 1115−1123.

Mulliez, E.; Leblanc, J. P.; Girerd, J. J.; Rigaud, M.; Chottard, J. C. 5-Lipoxygenase from potato tubers. Improved purification and physicochemical characteristics. *Biochim. Biophys. Acta* **1987**, *916*, 13−23.

Ohta, H.; Ida, S.; Mikami, B.; Morita, Y. Changes in lipoxygenase components of rice seedlings during germination. *Plant Cell Physiol.* **1986**, *27*, 911−918.

Pistorius, P. K.; Axelrod, B. Evidence for participation of iron in lipoxygenase reaction from optical and electron spin resonance studies. *J. Biol. Chem.* **1976**, *251*, 7144−7148.

Scheller, G.; Jäger, E.; Hoffmann, B.; Schmitt, M.; Schreier, P. Soybean lipoxygenase: substrate structure and product selectivity. *J. Agric. Food Chem.* **1995**, *43*, 1768−1774.

Schewe, T.; Rapoport, S. M.; Kühn, H. Enzymology and physiology of reticulocyte lipoxygenase: comparison with other lipoxygenases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1986**, *58*, 191−272.

Sekiya, J.; Kajiwara, T.; Munechika, T. Distribution of lipoxygenase and hydroperoxide lyase in the leaves of various plant species. *Phytochemistry* **1983**, *22*, 1867−1869.

Swinkels, J. J. M. Differences between commercial native starches. *AVEBE, General Information.* **1992**, Ref. no: 05.00.02.012 EF.

Szejtli, J. *Cyclodextrin Technology*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1988.

Szejtli, J.; Banky-Elöd, E. Inclusion complexes of unsaturated fatty acids with amylose and cyclodextrin. *Starch/Staerke* **1975**, *27*, 368−376.

Veldkin, G. A.; Vliegenthart, J. F. G. In *Advances in Inorganic Biochemistry;* Eichhorn, G. L., Marzili, L. G., Eds.; Elsevier: Amsterdam, 1984; Vol. VI, pp 139−161.

Yamamoto, S. Mammalian lipoxygenases: molecular structures and functions. *Biochim. Biophys. Acta* **1992**, *1128*, 117−131.

Received for review July 22, 1996. Revised manuscript received December 16, 1996. Accepted January 2, 1997.[⊗] This work has been supported in part by research grants from CICYT (Proyecto BIO94-0541) and INTAS (no. 93-2223 ext.). J.M.L.N. is a holder of a grant of CajaMurcia. R.B. holds a contract for Doctores Reincorporados.

JF960553M

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.