Chemoenzymatic Production of (+)-Coriolic Acid from Trilinolein: Coupled Synthesis and Extraction

Mohamed Gargouri* and Marie Dominique Legoy

Laboratoire de Génie Protéique et Cellulaire, Pôle Sciences et Technologie, Université de La Rochelle, 17042 La Rochelle Cédex 1, France

ABSTRACT: Chemoenzymatic conversion of trilinolein to (+)-coriolic acid was investigated in this work. Lipase-catalyzed hydrolysis of trilinolein and lipoxygenation of liberated linoleic acid were coupled in a two-phase medium that consisted of a pH 9 borate buffer and a water-immiscible organic solvent (octane). High concentrations of trilinolein could be dissolved in the organic phase (up to 340 mM). Linoleic acid, liberated after hydrolysis, transferred to the aqueous phase and was enzymatically converted to the preferred 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid with soybean lipoxygenase-1. This product, which remained in the aqueous phase, could be recovered by centrifugation and then chemically reduced to (+)-coriolic acid (purity >95%). Recovery of this compound by liquid–liquid extraction was easy. The structure of (+)-coriolic acid has been confirmed by ^{1}H nuclear magnetic resonance spectroscopy, mass spectrometry, and infrared spectroscopy. High yields were obtained with pure trilinolein or sunflower oil as initial substrates.

JAOCS 74, 641–645 (1997).

KEY WORDS: Bienzymatic bioreactor, biphasic media, chemoenzymatic synthesis, (+)-coriolic acid, hydroperoxide, linoleic acid, liquid–liquid extraction, *Pseudomonas* sp. lipase, soybean lipoxygenase-1, trilinolein.

(+)-Coriolic acid (13*S*-hydroxy-9Z,11*E*-octadecadienoic acid) is a metabolite of the lipoxygenase (LOX) pathway of linoleic acid (LA). This acid plays a physiological role in plant and animal cells. It is present in heart mitochondria (1) and takes part in the defense against rice blast disease (2) and in the synthesis mechanism of prostacyclin PGI_2 (3). It has also been shown to act as a chemorepellant that influences platelets, leukocytes, or malignant cells (4–6).

The synthesis of (+)-coriolic acid has been already achieved chemically (7,8) and chemoenzymatically (9). Martini *et al.* (10) have produced (+)-coriolic acid from LA by lipoxygenation under oxygen pressure (2.5 bar), followed by reduction of the hydroperoxide (HPOD).

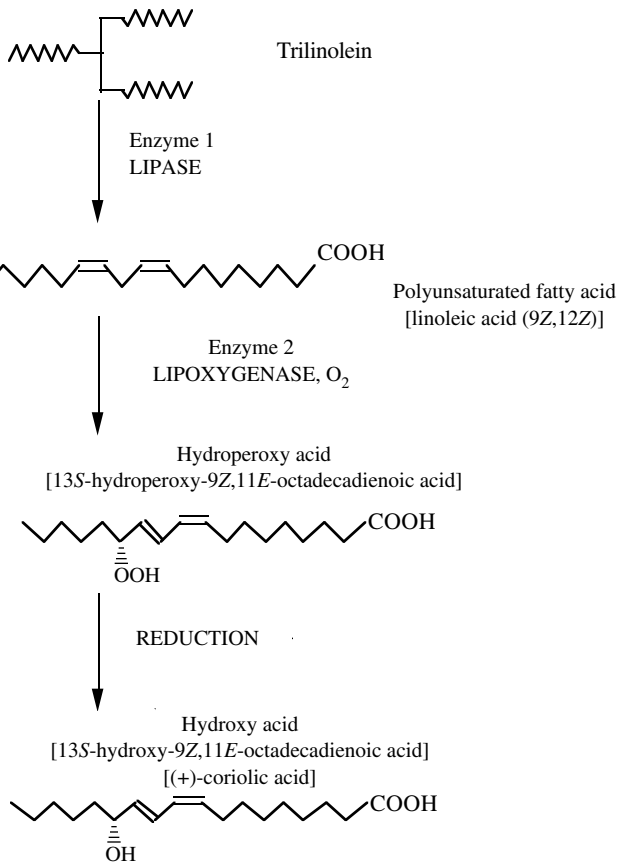
*To whom correspondence should be addressed at Laboratoire de Génie Protéique et Cellulaire, Pôle Sciences et Technologie, Université de La Rochelle, Avenue Marillac, 17042 La Rochelle Cédex 1, France.
E-mail: mgargour@bio.univ-lr.fr.

In plants, HPOD is an intermediate compound in the LOX pathway (11,12). Lipase (E.C.3.1.1.3)-catalyzed hydrolysis of trilinolein (TL) produces LA, which is oxygenated in the presence of LOX (E.C.1.13.11.12) and molecular oxygen. The HPOD obtained are highly reactive compounds (13).

The majority of intracellular enzymes use the products of other enzymes as substrates, and the biocatalysis does not take place in a homogeneous medium but rather in a compartmentalized cellular structure. A number of multienzymatic systems, consisting essentially of membrane- (14) or matrix- (15,16) bound enzymes, have been studied. These enzymes catalyze a set of consecutive chemical reactions and are then close to their respective substrates.

Bienzymatic reactions have been studied in low-water media and show interesting aspects regarding the transfer and partition of reaction components (17). Recently (18), we have studied a bienzymatic system that consisted of lipase and LOX in a water-organic biphasic medium. Polyunsaturated fatty acid triacylglycerols were converted to HPOD with high yields.

In this work, we present a chemoenzymatic conversion of TL to (+)-coriolic acid in two steps. The use of TL as starting substrate is a new approach. The first step is enzymatic and consists of coupled hydrolysis and lipoxygenation in a two-phase medium (borate buffer/octane). The first substrate (TL) is dissolved in octane and is hydrolyzed in the presence of lipase at the octane-water interface. The produced free fatty acid, being poorly water-soluble, transfers slowly to the aqueous phase, which contains the LOX (19). Soybean lipoxygenase-1 (LOX-1), used in this work, has the advantage of high selectivity. It converts LA preferentially to 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPOD) (20). The lipoxygenation takes place in the bulk of the aqueous phase. The produced HPOD is hydrophilic and therefore remains in the aqueous phase (21). The kinetic behavior of this bienzymatic system in a two-phase bioreactor was previously studied (22). Interactions between bioconversion and mass transfer were observed. In the second step, the aqueous phase is separated from the organic phase. 13*S*-HPOD is then chemically reduced to (+)-coriolic acid (Scheme 1).



SCHEME 1

EXPERIMENTAL PROCEDURES

Materials. LA, TL, and LOX-1 type 1-*S* (60% protein) were purchased from Sigma Chemical Co. (Paris, France). Solvents [high-performance liquid chromatography (HPLC) grade] were obtained from Aldrich (Paris, France). Crude powder lipase from *Pseudomonas* sp. (LPS) was kindly provided by Amano (Nagoya, Japan). Enzymes were used without further purification. Water was MilliQ grade (18.2 MΩcm; Millipore, Paris, France). Sunflower oil was obtained from Huilerie Lezay (Région Poitou-Charentes, France). Infrared (IR) spectra were recorded on a Perkin-Elmer Paragon 1000PC instrument (Paris, France). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM300WB spectrometer (Paris, France). Mass spectra were realized by "Centre Régional de Mesures Physiques de l'Ouest, Université de Rennes 1."

Coupling of LPS and LOX-1. Coupled hydrolysis and lipoxygenation were carried out in 50-mL flasks with a working volume of 12 mL (2 mL octane and 10 mL borate buffer, pH 9). Flasks were incubated in rotary shakers at 250 rpm under constant temperature control (18). TL was initially dissolved in the organic phase, and enzymes were dissolved in the aqueous phase. Oxygen (30 mL/min) was continuously flushed through the medium. Typical reaction times were of the order of 5 h.

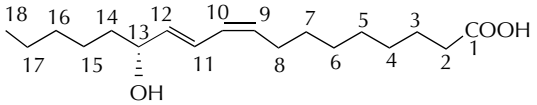
Chemical reduction of HPOD and extraction of hydroxy acids (HOD). At the end of the enzymatic step, the organic and aqueous phases were separated and proteins were eliminated with centrifugation. The aqueous phase, containing all the HPOD (98–99%) produced in the enzymatic step, was added to 2 mL ethanol and $NaBH_4$ (1 mg for 4 mg of initial TL). After 1 h at 25°C, the reaction was stopped by adding 1 M HCl. HOD was then extracted with diethyl ether (3× the same volume). The organic phase was dried with $MgSO_4$, and diethyl ether was evaporated under vacuum. HOD was diluted in ethanol and stored at −25°C.

Analysis. Separation and analysis of the different acylglycerols and fatty acids were carried out by HPLC equipped with an ultraviolet (UV) detector (Waters 486 at 210 nm, Paris, France) and a C_{18} reverse-phase column (μBondapak 3.9 mm × 300 mm; Waters). Samples (10 μL) were periodically withdrawn from the two-enzyme reactor and diluted 10 to 100 times before analysis. The mobile phase had the following composition: acetonitrile/acetone/acetic acid (7:4:0.02, vol/vol/vol). The pump rate was 1.8 mL/min.

Measurements of HPOD and HOD concentrations were performed at 234 nm on a Perkin-Elmer UV (Lambda16) spectrophotometer, assuming a molar extinction coefficient value of $\varepsilon = 25{,}000\ cm^{-1}\cdot M^{-1}$ for the two compounds. Sample withdrawal has been described elsewhere (19).

Regioselectivities of HOD and HPOD were analyzed by HPLC (UV detection at 234 nm) with a Hypersil-10 μ Sandon (Paris, France) silica column (4.6 mm × 300 mm). The elution phase (1.5 mL/min) was composed of hexane/diethyl ether/acetic acid (7.5:2.5:0.01, vol/vol/vol). Samples withdrawn from the reduction medium were diluted in water. To stop the reaction, 1 M HCl was added at 0°C.

Purification and characterization of (+)-coriolic acid. (+)-Coriolic acid was separated from the other isomers by liquid chromatography (20 mm × 300 mm) over silica gel 60 (Merck, Paris, France) with 2% (10 min) to 4% (increasing 0.25% per min) methanol in dichloromethane (vol/vol; 4 mL/min). Pure (+)-coriolic acid was then obtained (45 mg) as a colorless oil. Found: *m/z* 296.2346; $C_{18}H_{32}O_3$ requires M^+, 296.2351; ν_{max} (cm^{-1}), 3300, 3055, 2928, 2855, 1730, 1440, 1422, 995, and 896:



δ ^{1}H (300 MHz, $CDCl_3$): 0.85 (3H, *t*, *J* = 8 Hz, 18-CH_3), 1.20–1.35 (14H, *m*, 17, 16, 15, 7, 6, 5, 4-CH_2), 1.50–1.65 (4H, *br m*, 14, 3-CH_2), 2.15 (2H, *m*, 8-CH_2), 2.35 (2H, *t*, *J* = 9 Hz, 2-CH_2), 4.20 (1H, *m*, 13-CH), 5.42 (1H, *dt*, J_1 = 7 Hz, J_2 = 11 Hz, 9-CH), 5.65 (1H, *dd*, J_1 = 7 Hz, J_2 = 15Hz, 12-CH), 5.95 (1H, *t*, $J_1 = J_2$ = 11 Hz, 10-CH) and 6.47 (1H, *dd*, J_1 = 14 Hz, J_2 = 11 Hz, 11-CH); reference peaks: TMS, *m/z* 296 (M^+, 3%), 278 ($M^+ - H_2O$, 11), 236 (2), 221 (1), 207 (10), 189 (2), 179 (7), 164 (6), 147 (7), 135 (12), 125 (4), 121 (9), 109 (11), and 99 (100).

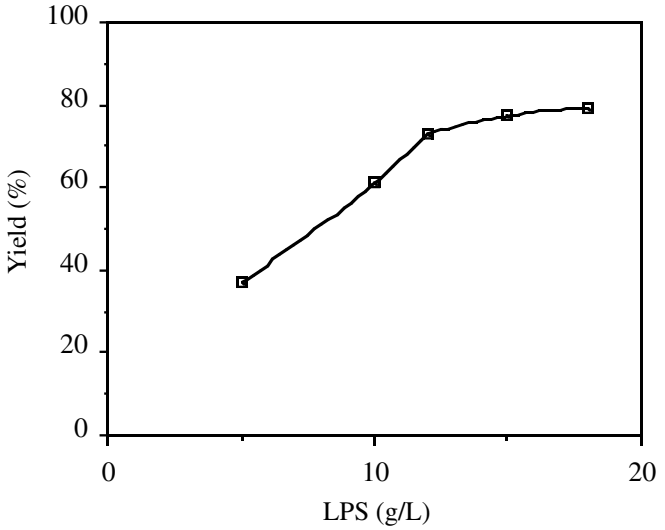


FIG. 1. Dependence of the yield of hydroperoxide (mM)/trilinolein (mM) on the concentration crude powder lipase from *Pseudomonas* sp. in the aqueous phase at 28°C. The same conditions were used as in Table 1.

TABLE 1
Yields (mM) of the Different Steps of Hydroxy Acid (HOD) Preparation from Trilinolein (TL) for Chemoenzymatic Synthesis Followed by Liquid–Liquid Extraction[a]

TL (g/L)	LA/TL (%)	HPOD/LA (%)	HPOD/TL (%)	HOD/TL (%)	HODext/TL (%)
20	95.1	91.6	87.1	87.1	78.4
50	90.5	80.6	72.9	72.9	62.7
70	89.4	64.7	57.8	57.8	50
100	87.9	56.3	49.5	49.5	36.9
120	79.8	55.8	44.5	44.5	33.9
150	79.4	52.7	41.8	41.8	29
200	47.5	55.2	26.2	26.2	18.5
300	44.2	55.7	24.6	24.6	17.2

[a]Conditions: 1.2 mg crude powder lipase from *Pseudomonas* sp. (LPS) and 0.25 mg soybean lipoxygenase-1 (LOX-1) were used per 1 mg TL at 28°C. Conversion of TL to linoleic acid (LA) (hydrolysis yield = LA/TL) was measured by reverse-phase high-performance liquid chromatography (HPLC). Syntheses of hydroperoxide (HPOD) and HOD were measured spectrophotometrically after 5 h. Lipoxygenation yield = HPOD/LA, and hydrolysis–lipoxygenation yield = HPOD/TL. Chemoenzymatic synthesis of HOD from TL before (hydrolysis–lipoxygenation–reduction yield = HOD/TL) and after extraction (total yield = HOD_{ext}/TL) was confirmed by HPLC (silica).

TABLE 2
Yields of the Different Steps of HOD Preparation from Crude Sunflower Oil by the Chemoenzymatic Synthesis Followed by Liquid–Liquid Extraction[a]

TL (g/L)	LA/TL (%)	HPOD/LA (%)	HPOD/oil (%)	HPOD/TL (%)	HOD/TL (%)	HOD_{ext}/TL (%)
20	89	89	47.5	79.2	79.2	70
50	69.2	90.5	37.6	62.6	62.6	52.6
100	70	75	31.5	52.5	52.5	39.5
200	47	75.1	21.2	35.3	35.3	25

[a]The same conditions as those in Table 1 were used. For abbreviations, see Table 1; HPOD/oil means yield of the bienzymatic system in relation to sunflower oil (%).

octane. Concentrations of LPS and LOX-1 in the aqueous phase were, respectively, 12 and 25 g/L. The reaction period was 5 h with a constant flow of O_2 (30 mL/min). The optimal temperature for the bienzymatic system LPS/LOX-1 was 28°C (yield = 72.9%). The yield changed significantly at either side of the optimal temperature (yield = 62% at 26°C and 51.5% at 30°C).

In the biphasic medium, crude powder LPS and LOX-1 were coupled by using different lipase concentrations. Five concentrations of LPS were tested. The yields of HPOD/TL are presented in Figure 1. These results indicate that the yield of HPOD synthesized from TL is better when an LPS concentration of 12 g/L is used in the aqueous phase for 50 g/L TL concentration in the organic phase. Higher concentrations of LPS enhance the yield only slightly. Logically, we chose 1.2 mg LPS for 1 mg TL for the following experiments in the biphasic system (ratio octane/borate buffer = 1:5).

Preparation of (+)-coriolic acid from TL. Intermediaries and final yields of the chemoenzymatic synthesis of HOD from TL were studied as a function of initial TL concentration (Table 1). High yields were obtained especially when using concentrations of TL below 100 g/L in the organic phase of the biphasic medium. The yield of extracted product decreased when the initial TL concentration was increased. The chemical reduction of HPOD to HOD is complete for all concentrations.

Preparation of (+)-coriolic acid from a crude vegetable oil. In the next section of work described here, we used a crude vegetable oil rich in LA (sunflower oil) rather than pure TL. This allows a more realistic scenario for industrial-scale manufacture of HOD and was the main reason for developing this chemoenzymatic approach. LA is the major fatty acid component (60% molar). Linolenic acid is present in negligible quantities (<0.02%) in the sunflower oil. This oil contains 6% palmitic acid, 4.5% stearic acid, and 20% oleic acid, which are not substrates of LOX-1. In the two-enzyme system, lipolysis of triglycerides liberates fatty acids, which transfer to the aqueous phase. Only LA, the single polyunsaturated fatty acid in sunflower oil, was oxidized in the presence of LOX. HPOD obtained in the bienzymatic reactor were reduced chemically. Yields were studied as a function of the initial oil concentration (Table 2). They were calculated in relation to TL present in the sunflower oil after every step. A second yield for the two-enzyme system was calculated in relation to the total sunflower oil (HPOD/oil). These yields approach the yields obtained with the pure TL as substrate.

Regioselectivity. HPLC analysis of HPOD produced in the two-enzyme system shows one major peak (94%), which corresponds to the 13*S*-HPOD as in an aqueous medium. Chemoenzymatic synthesis of HOD from TL or sunflower oil does not change the regioselectivity (Table 3).

DISCUSSION

TABLE 3
Regioselectivity (%) of HPOD and HOD in Aqueous Medium[a] pH 9[b] After Chemoenzymatic Synthesis from TL[c] or Sunflower Oil[d] as Substrates

	13(*S*)9*Z*,11*E*	13(*S*)9*E*,11*E*	9(*S*)10*E*,12*Z*	9(*S*)10*E*,12*E*
HPOD[b]	95	0.5	2.7	1.8
HPOD[c]	94	0.5	3.3	2.2
HOD[c]	96.2	0.4	2.9	0.5
HOD[d]	96	0.6	2.5	0.9

[a]Experimental conditions are those in Table 1, where abbreviations can be found.
[b]Yield in aqueous medium, pH 9.
[c]Substrate: TL.
[d]Substrate: sunflower oil.

We have studied the synthesis of (+)-coriolic acid by a chemoenzymatic method. Three reactions were necessary to convert TL to (+)-coriolic acid. In the first step, lipase-catalyzed hydrolysis of TL and lipoxygenation of liberated LA were coupled in a biphasic system. Presence of an organic phase (octane) in the medium allows high solubility of acylglycerols and fatty acids. High substrate concentrations were then used. In the biphasic medium, mass transfer across the liquid–liquid interface is controlled. The moderately alkaline pH (9) facilitates LA solubilization and results in a sodium salt being formed. Recently (19), we showed that the substrate (LA) and the product (HPOD) of the LOX reaction have surface-active properties. During lipoxygenation in the biphasic system, the consumption of LA and the production of HPOD favor the fatty acid transfer to the aqueous phase, which allows an increase in LOX reaction. In the two-enzyme system (LPS/LOX-1), the phenomenon described previously favors lipoxygenation of LA. The consumption of this intermediate allows an increase in the degree of LPS reaction at the liquid–liquid interface. These interactions between the two reactions and the mass transfer increase the yields obtained in the two-enzyme reactor.

The optimal temperature for the production of HPOD from TL in the biphasic medium was 28°C and allows high yields of HPOD/TL. Generally, lipase-catalyzed hydrolysis could be carried out at high temperatures (30–50°C) (23,24), and LOX-1 oxidation was carried out at room temperature (21,25). Nevertheless, the optimal temperature in the two-enzyme bioreactor corresponds to the best performance of both hydrolysis and lipoxygenation. The yield decreases significantly at higher temperatures.

LPS-catalyzed hydrolysis gives high yields in the two-enzyme reactor. These yields are more important than the yields obtained when using lipases from *Mucor javanicus* and *Candida antarctica* in the same system (18). LOX-1 used in this work has the advantage of high selectivity. It forms preferentially 13*S*-HPOD from LA. This regioselectivity does not change when producing HPOD from TL in the two-enzyme system (LPS/LOX-1). In this system, LOX-1 acts exclusively on the free LA and does not oxidize TL, dilinolein, and monolinolein (18). Most of the HPOD (98–99%) remains in the aqueous phase; therefore, its synthesis in the biphasic reactor is interesting because of the near total recovery of the product by simple centrifugation. Thus, the aqueous phase, containing HPOD, can be separated from the organic phase and solid protein. Chemical reduction of HPOD in the presence of alcohol was complete. HOD then was extracted and stored in ethanol. The regioselectivity obtained is similar to the regioselectivity of HPOD. The single major compound is (+)-coriolic acid (>95%), product of the reduction of 13*S*-HPOD (major isomer of HPOD obtained in our system). The structure of (+)-coriolic acid was confirmed by ^{1}H NMR, MS, and IR.

Evolution of HPOD in the two-enzyme system had an optimal rate between 150 and 200 min. At 300 min, production of HPOD became much slower because lipoxygenase was inactivated at the octane/borate buffer interface (19). In the reduction medium, HPOD was rapidly converted to HOD (95% in 20 min). After one hour, the reduction was complete.

Yields were also high when using crude sunflower oil (60% LA) as the initial substrate. The quality of the final product [(+)-coriolic acid] after synthesis and extraction was the same as that obtained with TL as substrate. In fact, after hydrolysis of triglycerides, only LA was oxygenated in the presence of LOX-1; the medium did not contain other polyunsaturated fatty acids. After production of HPOD in the two-enzyme system, the aqueous phase was separated from the organic phase and enzyme. HPOD was then separated from impurities existing in the sunflower oil. The final extraction after synthesis of (+)-coriolic acid offered a high degree of purity.

The approach used in this work could be easily extended to a large scale to produce (+)-coriolic acid chemoenzymatically from high concentrations of TL with facile extraction.

ACKNOWLEDGMENTS

This work was supported by a grant from "La Région Poitou-Charentes," France. The authors are grateful to Dr. Marie Claire Parker for help with correction of the text.

REFERENCES

1. Blondin, G.A., Isolation Properties and Structural Features of Divalent Cation Ionophores Derived from Beef Heart Mitochondria, *Ann. NY Acad. Sci. 264*:98–111 (1975).
2. Kato, T., Y. Yamaguchi, T. Hirano, T. Yokoyama, T. Uyehara, T Namai, S. Yamanaka, and N. Harada, Unsaturated Hydroxy Fatty Acids, the Self Defensive Substances in Rice Plant Against Rice Blast Disease, *Chem. Lett.*:409–412 (1984).
3. Moncada, S., and J. Vane, Arachidonic Acid Metabolites and the Interactions Between Platelets and Blood-Vessel Walls, *New Engl. J. Med. 300*:1142–1147 (1979).
4. Buchanan, M.R., T.A. Haas, M. Lagarde, and M. Guichardant, 13-Hydroxyoctadecadienoic Acid Is the Vessel Wall Chemorepellant Factor, LOX, *J. Biol. Chem. 260*:16056–16059 (1985).
5. Buchanan, M.R., R.W. Butt, J. Hirsh, B.A. Markham, and D.J. Nazir, Role of Lipoxygenase Metabolism in Platelet Function: Effect of Aspirin and Salicylate, *Prostaglandins, Leukotrienes Med. 21*:157–168 (1986).
6. Grossi, I.M., L.A. Fitzgerald, L.A. Umberger, K. Nelson, C.A. Diglio, J.D. Taylor, and K.V. Honn, Bidirectional Control of the Membrane Expression and/or Activation of the Tumor Cell IRGpIIb/IIIa Receptor and Tumor Cell Adhesion by Lipoxyge-

nase Products of Arachidonic Acid and Linoleic Acid, *Cancer Res. 49*:1029–1037 (1989).
7. Rao, A.V.R., E.R. Reddy, G.V.M. Sharma, P. Yadagiri, and J.S. Yadav, A Stereoselective Synthesis of Coriolic Acid and Dimorphecolic Acid, *Tetrahedron Lett. 26*:465–468 (1985).
8. Bloch, R., and M.T. Perfetti, An Efficient Synthesis of 13(*S*)-Hydroxy-9*Z*,11*E*-octadecadienoic (coriolic) Acid, *Ibid. 31*: 2577–2580 (1990).
9. Chan, C., P.B. Cox, and S.M. Roberts, Chemo-enzymatic Synthesis of 13-*S*-Hydroxyoctadecadienoic Acid (13-*S*-HODE), *Biocatalysis 3*:111–118 (1990).
10. Martini, D., G. Iacazio, D. Ferrand, G. Buono, and C. Triantaphylides, Optimization of Large-Scale Preparation of 13-(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic Acid Using Soybean Lipoxygenase. Application to the Chemoenzymatic Synthesis of (+)-Coriolic Acid, *Biocatalysis 11*:47–63 (1994).
11. Croft, K.P.C., F. Jüttner, and A.J. Slusarenko, Volatile Products of the Lipoxygenase Pathway Evolved from *Phaseolus vulgaris* (L.) Leaves Inoculated with *Pseudomonas syringae* pv *phaseolicola*, *Plant Physiol. 101*:13–24 (1993).
12. Gardner, H.W., D. Weisleder, and R.D. Plattner, Hydroperoxide Lyase and Other Hydroperoxide-Metabolizing Activity in Tissues of Soybean, *Ibid. 97*:1059–1072 (1991).
13. Vick, B.A., and D.C. Zimmerman, Oxidative Systems for Modification of Fatty Acids: The Lipoxygenase Pathway, in *The Biochemistry of Plants, a Comprehensive Treatise*, Vol. 9, edited by P.K. Stumpf and E.E. Conn, Academic Press, New York, 1988, pp. 53–90.
14. Goldman, R., and E. Katchalski, Kinetic Behavior of a Two-Enzyme Membrane Carrying Out a Consecutive Set of Reactions, *J. Theor. Biol. 32*:243–257 (1971).
15. Mattiasson, B., and K. Mosbach, Studies on a Matrix-Bound Three-Enzyme System, *Biochim. Biophys. Acta 235*:253–257 (1971).
16. Mosbach, K., and B. Mattiasson, Matrix-Bound Enzymes. Part II: Studies on a Matrix-Bound Two-Enzyme System, *Acta. Chem. Scand. 24*:2093–2100 (1970).
17. Borzeix, F., F. Monot, and J.P. Vandecasteele, Bi-enzymatic Reaction for Alcohol Oxidation in Organic Media: from Purified Enzymes to Cellular Systems, *Enzyme Microb. Technol. 17*: 615–622 (1995).
18. Gargouri, M., and M.D. Legoy, Bi-enzymatic Reaction for Hydroperoxide Production in a Multiphasic System, *Ibid*., in press.
19. Gargouri, M., P. Drouet, J.F. Hervagault, and M.D. Legoy, Investigation of Behaviour of an Enzyme in a Biphasic System: Soybean Lipoxygenase-1, *Biotechnol. Bioeng. 51*:573–580 (1996).
20. Gardner, H.W., Recent Investigations into the Lipoxygenase Pathway of Plants, *Biochim. Biophys. Acta 1084*:221–239 (1991).
21. Drouet, P., D. Thomas, and M.D. Legoy, Production of 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic Acid Using Soybean Lipoxygenase 1 in a Biphasic Octane-Water System, *Tetrahedron Lett. 35*:3923–3926 (1994).
22. Gargouri, M., and M.D. Legoy, The Kinetic Behaviour of a Two-Enzyme System in Biphasic Media: Coupling Hydrolysis and Lipoxygenation, *Biochim. Biophys. Acta 1337*:227–232 (1997).
23. Bélafi-Bakó, K., A. Dombi, L. Szabó, and E. Nagy, Comparative Study on Hydrolysis of Triolein in Reversed Micellar System Catalysed by Different Types of Lipases, *Biotech. Tech. 9*: 59–64 (1995).
24. Kosugi, Y., H. Tanaka, and N. Tomizuka, Continuous Hydrolysis of Oil by Immobilized Lipase in a Countercurrent Reactor, *Biotechnol. Bioeng. 36*:617–622 (1990).
25. Piazza, G.J., Lipoxygenase Catalysed Hydroperoxide Formation in Microemulsions Containing Nonionic Surfactant, *Biotechnol. Lett. 14*:1153–1158 (1992).

[Received May 28, 1996; accepted February 19, 1997]