Pergamon

Tetrahedron Letters, Vol. 35, No. 23, pp. 3923-3926, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)E0704-2

Production of 13(S)-Hydroperoxy-9(Z),11(E)-Octadecadienoic Acid using Soybean Lipoxygenase 1 in a Biphasic octane-water system.

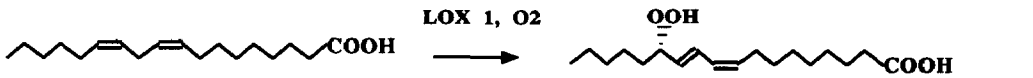
Philippe Drouet*, Daniel Thomas and Marie Dominique Legoy.

Laboratoire de Technologie Enzymatique. Université de Technologie de Compiègne.
BP 649. 60206 Compiègne. France.

Abstract: The synthetic potential of soybean lipoxygenase 1 (LOX 1) for the synthesis of 13(S) hydroperoxy-9(Z),11(E)-octadecadienoic acid is investigated in a biphasic medium (octane; borate buffer, pH 9.6) Improvement of the reaction yield (compared to an aqueous system) is observed at very high concentrations (20-40 g/L). The regioselectivity of the reaction is not affected by the presence of the organic phase.

Lipoxygenase (E.C. 1.13.11.12.) is a non heme iron dioxygenase which catalyses the incorporation of a dioxygen into polyinsaturated fatty acids possessing a (1Z, 4Z) pentadienyl insaturation [1]. The product obtained is a E, Z conjugated diene hydroperoxide. 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (HPOD) is formed from linoleic acid (Scheme 1).

Scheme 1:



linoleic acid (9Z,12Z) **13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid**

Hydroperoxides after reduction lead to hydroxy-fatty-acids which could serve as chiral synthons in chemical industry [2].

Soybean lipoxygenase (LOX 1) has been extensively studied; but the mechanism of the reaction is still not clear [3,4]. Nevertheless, all the authors agree that this enzyme shows a strong substrate inhibition [5]. Therefore, some studies have recently been reported with the use of the soybean lipoxygenase in an unconventional medium: oxidation under high pressure of oxygen [6], action of the lipoxygenase in a microemulsion system containing a surfactant [7,8].

We report here the use of LOX 1 in a biphasic system (octane, borate buffer 0.1M, pH 9.6) without any surfactant [9].

The influence of the ratio of the two phases and the influence of the substrate concentration on the reaction yield are investigated. The effect of the solvent on the regioselectivity of the reaction is studied.

LOX 1 has its pH optimum in the basic range (9-10). This can be an advantage since fatty acids are more soluble as salts in aqueous solution. However at high substrate concentration (higher than 10 g/L), they form aggregates which are not easily dispersible in the basic buffer.

The presence of a non-polar solvent such as octane can help the solubilization of these aggregates. No surfactant has to be added to the medium because the substrate has by itself tensioactive properties [10].

In a preliminary experiment, it is found that the whole of the product is in the aqueous phase (98-99%) ; therefore investigation of the behaviour of the LOX 1 in a biphasic system is interesting because of the total recovery of the product by a simple centrifugation.

The interface between the non-polar solvent and the aqueous media can dramatically decrease the half life of the enzymes in general. Nevertheless, the reaction time for this enzymatic oxidation is short (45 min to 60 min). Thus, the denaturing effect of the interface is minimal in this case.

The ratio (buffer : octane, 8:1) is the most appropriate for an improvement of the reaction yield; a more important quantity of octane decreases the HPOD production (Table 1). The initial concentration of linoleic acid in a biphasic system is defined as the initial quantity of substrate divided by the volume of the aqueous phase (10 ml).

As shown in Table 2, the presence of an organic phase does not change the yield of the reaction for an initial substrate concentration of 10 g/L. On the other hand, significative improvement of the yield is observed (23%-45%) for higher concentration (20-40 g/L). In this range of concentration, more and more fatty acids start to aggregate in pure buffer and the accessibility of the substrate to the enzymatic site decreases. In a biphasic medium, all the linoleic acid is solubilized and there is no mass transfer constraint.

Table 1 : Evolution of the Reaction Yield with different volume of organic phase (octane).

Ratio borate buffer: octane (v:v)	Pure buffer	16:1	8:1	4:1	2:1
Yield (%)	49.8	48.6	60.2	44.3	34.2

Aqueous phase is kept constant (10 ml). Linoleic acid: 200 mg is diluted in octane and the organic phase is mixed with the aqueous phase which contains the lipoxygenase (4 mg).

HPLC analyses of the product [11] show one major hydroperoxide peak (see Table 3) as in an aqueous medium. Thus the selectivity of LOX 1 is not affected by the presence of the organic solvent. The single major peak is identified as 13S HPOD according to the literature.

The structure assignement is confirmed by ^{1}H NMR [12]. ^{13}C NMR spectroscopy exhibits the same chemical shifts described by Van-Os and al [13].

Table 2 : Evolution of the Reaction Yield versus the initial substrate concentration.

Substrate Concentration (g/L) / Reaction yield (%)	10	20	30	40
Aqueous medium	74.5	49.8	32.8	20.3
Biphasic medium (8:1,v:v)	77.9	60.2	40.3	30.1

Aqueous medium pH 9.6 (10 ml). Biphasic medium 8:1. Linoleic acid: 100 mg; LOX 1: 2 mg.

Table 3 : Comparison of the Regioselectivity of the Soybean lipoxygenase 1 in a biphasic medium and in an aqueous system.

Product	13S OOH, 9Z, 11E	13S OOH, 9E, 11E	9S OOH, 10E, 12Z	9S OOH, 10E, 12E
Biphasic medium	92.1	0.8	4.6	2.5
Aqueous medium	92.5	0.5	5.5	1.5

Aqueous medium pH 9.6 (10 ml). Biphasic medium 8:1. Linoleic acid: 100 mg; LOX 1: 2 mg.

Conclusion

Soybean lipoxygenase-1 catalyses the formation of HPOD in a biphasic medium formed with borate buffer and octane. The high solubility of the substrate in this system helps to improve the reaction yield. This method can be easily extended to a preparative scale in order to produce large quantity of HPOD.

Acknowledgement

This work was supported by a grant from Limagrain company, France.

References

1. Veldink, G.A.; Vliegenthart, J.F.G. *Advances in Organic Biochemistry.* Elsevier Scientific Publishing Co.; Amsterdam, **1984**, *6*, 139.
2. Vignolo, R.; Naughton, F. **1991**, *Inform. 2*, 692-699.
3. Wang, Z.X.; Killilea, S.D.; Sristava D.K. *Biochemistry.* **1993**, *32*, 1500- 1509.
4. Schilstra, M. J.; Veldink, G. ; Verhagen, J.; Vliegenthart, J.F. *Biochemistry.* **1992**, *31*, 7692-7699.
5. Egmond, M.R.; Brunori, M.; Fasella, P.M. **1976**. *Eur.J.Biochem.*, *61*, 93-100.
6. Iacazio, G.; Langrand, G.; Baratti, J.; Buono, G.; Triantaphylidès, C. *J. Org. Chem.* **1990**, *55*, 1690-1691.
7. Piazza, G. J. *Biotechnol. Lett.* **1992**, *14*, 1153-1158.
8. Hiruta, O.; Nakahara, T.; Yokochi, T.; Kamasaka,Y.; Susuki, O. *J. Am. Oil. Chem. Soc.* **1988**, *65*, 1911-1914.
9. Substrate and enzyme were purchased from Sigma Chemical Co. Octane (99%+) was obtained from Aldrich. Linoleic acid is diluted in octane (2.5 ml for a 4:1 ratio). The organic phase is mixed with a 0.1M pH 9.6 borate buffer solution containing the soybean l lipoxygenase 1 (10 ml, enzyme/substrate= 0.02, 20°C). The biphasic medium and the aqueous medium are bubbled with pure oxygen (30 ml/min) and agitated with a magnetic stirrer (800 rpm). High speed stirring is very important in order to prevent artefacts due to a lower diffusion of oxygen.The reaction progress can be followed by UV analysis. The octane phase is removed by centrifugation (10000 rpm, 1min) and an aliquot of the aqueous phase is diluted in ethanol (10µL/12mL). Measurements were carried out at 234 nm assuming a molar extinction coefficient for the hydroperoxide of 25000 $M^{-1}.cm^{-1}$[14]. Care was taken that absorption at 270nm was inferior to 0.02 OD. The reaction yield was calculated after 1h.
10. Verhagen, J.; Vliegenthart, J.F.G.; Boldingh, J. *Chem. Phys. Lip.* **1978**, *22*, 255-259.

11. HPLC analysis was based on the protocol described in the reference 6, except that the silica column is a Waters μPorasil (300 mm x 3.9 mm). Retention times and isomer distribution for HPOD : 15,2 min; 17,1 min; 18,2 min; 19,6 min.
12. Reduction of the hydroperoxide to hydroxy-acid with $NaBH_4$ and purification with a Waters μPorasil (300 mm x 7.8 mm), flow rate: 2.5 ml/min. Elution time : 26.5 min. 13(S)-Hydroxy-9(Z),11(E)-octadecadienoic acid: ^1H NMR (300 MHz, $CDCl_3$) : δ 6.53 (dd, J_{11-12} = 15Hz, J_{10-11}= 11Hz, H-11), 6.0 (t, J_{10-11}= J_{9-10}= 11Hz, H-10), 5.65 (dd, J_{11-12}= 15Hz, J_{12-13}= 7Hz, H-12), 5.45 (t, J_{9-10}= 11Hz, J_{10-11}= 7Hz, H-9), 4.2 (q, J_{12-13}= J_{13-14}= 7Hz,H-13), 2.43 (t, J= 8Hz, H_2-2), 2.2 (q, J= 8Hz, H_2-8), 1.6 (m, H_2-14), 1.5 (m, H_2-3), 1.4 (br m, H_{14}-4,5,6,7,15,16,17), 0.85 (t, J= 8Hz,H_3-18).
13. Van Os. C.P.A.; Rijke-Schilder, G. P. M.; Van Halbeck, H.; Verhagen, J.; Vliegenthart, J.F.G. *Biochim. Biophys. Acta.* **1981**, *663*, 177.
14. Grossman, S.; Zakut, R. *Meth. Biochem. Anal.* **1979**, *25*, 303-329.

(*Received in France* 18 *February* 1994; *accepted* 8 *April* 1994)