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Enzymatic synthesis of fatty acid ascorbyl esters

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Abstract

Lipase B from the yeast *Candida antartica* was used to catalyze the enzymatic synthesis of fatty acid ascorbyl esters, in 2-methyl-2-butanol as a solvent. The influence of water activity (a_w) and reaction medium hydrophobicity on the synthesis of 6-*O*-palmitoyl L-ascorbic acid were investigated through a transesterification reaction involving L-ascorbic acid and palmitic acid methyl ester as substrates. The catalytic activity of the enzyme and the production at equilibrium were affected by the pre-fixed a_w, best results being obtained for the lowest a_w values. The activation of the lipase was promoted by an increase in the methyl palmitate/ascorbic acid molar ratio (R) up to 9, leading to 19 g/l of ascorbyl ester after a 5 h reaction time. That performance was not reached in a reaction medium of equal log P level, where methyl palmitate was partly substituted by a hydrophobic solvent. Results were applied to the syntheses of polyunsaturated fatty acid ascorbyl esters such as ascorbyl eicosapentaenoate, leading to a production range from 11 to 15 g/l, after a 3 h reaction time.

Keywords: *Candida antartica*; Lipase B; Transesterification; Water activity; Low-water media; log P; Ascorbyl palmitate; Polyunsaturated fatty acid ascorbyl esters

1. Introduction

Since a few years many studies have been reported, showing a growing interest in unsaturated lipids and more particularly, lipids of fish origin due to their beneficial effects on human health [1–3]. However, practical use of such fatty acids for a preventive purpose is limited because of their high susceptibility to autoxidation, which is responsible for the unfavorable off-flavour in rancid oils [4]. The simplest method for retarding lipid oxidation is the adjunction of antioxidant species. Synthetic permitted compounds such as BHA and BHT have been widely used to prevent the deterioration of foods containing fish oils [5]. However, since they were suspected to act as carcinogenesis promoters [6], their use has been falling off in favour of natural and safe antioxidants such as ascorbic acid, which is usually introduced through ascorbyl palmitate solubilization or through reversed micelles processes [7,8]. Unfortunately, these two protection methods may exhibit a loss of efficiency because of poor contact between highly oxidizable species and antioxidant species. A solution of great interest would be to bring closely polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5 $n-3$) or docosahexaenoic acid (DHA, C22:6

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$n-3$) and ascorbic acid, by forming an ester bond.

For that purpose, an enzymatic way involving lipases as biocatalyst is preferred to a chemical one due to high regioselectivity and mild reaction conditions, that avoid substrate alteration [9,10]. The aim of this work is to get more information about the catalytic activity of lipase B from *Candida antartica* for the enzymatic synthesis of fatty acid ascorbyl esters. In this scope, the influences of important biochemical and physico-chemical parameters such as the thermodynamic water activity or the hydrophobicity of the reaction medium were assessed through the enzymatic synthesis of the commercially available 6-*O*-palmitoyl L-ascorbic acid, as a reference reaction. This synthesis was previously shown to be effective by acylating L-ascorbic acid with methyl palmitate, in 2-methyl-2-butanol as a solvent, in the presence of the *C. antartica* lipase immobilized on an acrylic resin as a biocatalyst [11]. Results were applied to the synthesis of compounds of higher potential interest such as EPA and DHA ascorbyl esters.

2. Material and methods

2.1. Enzyme

The lipase B from *C. antartica*, immobilized on a macroporous acrylic resin (Novozym 435 ®, Novo Industri), was used as a biocatalyst.

2.2. Chemicals

The purity of L-ascorbic acid and methyl palmitate is over 99.5% and 97%, respectively (Fluka). EPA and DHA ethyl esters of purity over 85% were used as polyunsaturated acyl donors. The purity of solvents is over 99% for 2-methyl-2-butanol and over 98% for dodecane (Fluka). An ascorbyl palmitate reference range was prepared with 6-*O*-palmitoyl L-ascorbic acid of purity over 99% (Fluka).

2.3. Synthesis

The reaction is started by mixing enzyme preparation (330 mg) and organic phase composed of 2-methyl-2-butanol (20 ml), ascorbic acid (20 g/l) and methyl palmitate (276 g/l), in a stirring batch reactor, at 70°C and 400 rpm. Syntheses achieved with various acyl donor/ascorbic acid; molar ratio were conducted in reaction media containing methyl palmitate concentrations from 30 g/l to 340 g/l. Polyunsaturated fatty acid ascorbyl esters were produced at 55°C, in reaction media containing 2-methyl-2-butanol (20 ml), ascorbic acid (12 g/l) and EPA ethyl ester (112 g/l) or DHA ethyl ester (121 g/l). The course of the transesterification reaction was monitored through ascorbyl ester measurements by referring to an ascorbyl palmitate range, as previously described [11].

2.4. Initial rate measurement

Experimental data for the reaction course were fitted to a polynomial equation (a fifth grade giving generally the best fit). After first derivation, the initial rate was evaluated from the resulting equation.

3. Results and discussion

3.1. Influence of the thermodynamic water activity (a_w)

As first described by Goderis et al. [12], the influence of the thermodynamic water activity on the equilibrium and the rate of the transesterification reaction was assessed through experiments, where the reactant phase as well as the enzyme preparation were pre-equilibrated in separate containers with the water vapor of saturated salt solutions, at room temperature for 10 days (Table 1).

The pre-fixed water activity was shown to affect both catalytic activity of the lipase and

Table 1
Activities of selected H_2O-saturated salt solutions at 25°C [13]

Salt	a_w (25°C)
LiBr	0.07
LiCl	0.12
KAc	0.25
$MgCl_2$	0.33
K_2CO_3	0.45
$Mg(NO_3)_2$	0.55
NaCl	0.75
$K_2Cr_2O_7$	0.97

production of ascorbyl palmitate at equilibrium (Fig. 1). The observed decline in the initial reaction rate when increasing the initial a_w level may be firstly explained by a competition between hydrolysis and alcoholysis of the presumed acyl-enzyme intermediate due to water acting as a substrate. A second possible assumption would be the accumulation of water on the support surface of the Novozym leading to a physical aggregation of the individual catalyst particles. At last, there may be a limitation in the transfer of the substrates to the enzyme, due to accumulation of water around the catalyst particles; as the size of the resulting water layer increases the more difficult it is for the substrates and particularly for the hydrophobic methyl palmitate to accede to the enzyme molecules. A similar profile was obtained for the equilibrium conversion. The decline in ascorbyl palmitate production when growing the initial water activity is very likely to result from the direct participation of water as a substrate, leading to a shift of equilibria in favour of hydrolysis-side reactions.

3.2. Influence of reaction medium hydrophobicity

The influence of the reaction medium hydrophobicity was first assessed by increasing the methyl palmitate/ascorbic acid molar ratio (R), which results in increasing the log P level of the bulk organic phase (Fig. 2). A direct observation of Fig. 2 lays in the activation of the Novozym when increasing methyl palmitate concentration up to a R value of 9, for which 19 g/l of ascorbyl palmitate were produced after a 5 h reaction time. That result brings us to wonder whether the reaction medium hydrophobicity or the methyl palmitate concentration promotes the catalytic activity of the enzyme. Uppenberg et al. [14] supposed this *C. antartica* lipase may be an intermediate between an esterase which hydrolyzes water soluble substrates and a true interfacially activated lipase. According to these authors, its active site is accessible to the reactant phase through a narrow hydrophobic channel. α-helices of high mobility were identified at the entrance of this active site channel, as well as along its hydrophobic walls and were suggested to undergo conformational changes in the protein when contact occurs with a lipidic phase, leading to a transition from an

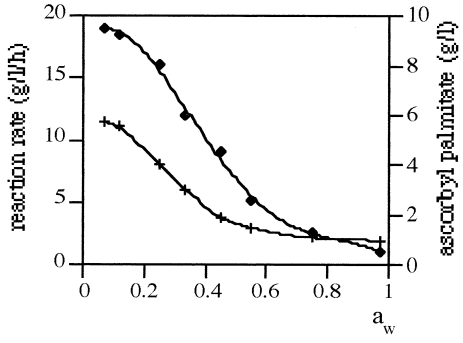

Fig. 1. Influence of the initial water activity on the reaction rate (◆) and the equilibrium conversion (+) of the transesterification reaction.

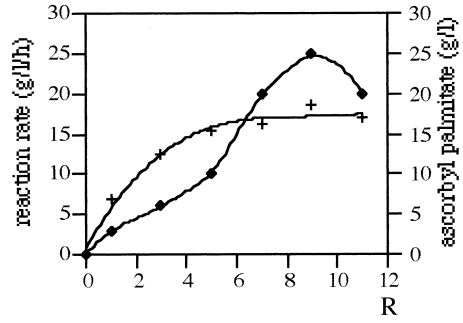

Fig. 2. Influence of the methyl palmitate/ascorbic acid molar ratio (R) on the reaction rate (◆) and the equilibrium conversion (+) of the transesterification reaction catalyzed by the Novozym.

inactive to an active form of the enzyme. As methyl palmitate concentration increases in the bulk organic phase, its concentration in the microenvironment of the lipase increases as well, probably inducing conformational changes that expose the active site of the enzyme to the substrates. A more surprising result, consisting in the apparent loss of activity for *R* values over 9, is supposed to result from high quantities of methyl palmitate interacting with the enzyme surface and the walls of the active site channel, which are thought to limit the access of the hydrophilic ascorbic acid to the catalytic site. The influence of the molar ratio of the substrates on the final production of ascorbyl palmitate may be explained by a thermodynamical shift of the equilibrium in favour of the synthesis of the ester due to methyl palmitate excess, the more as experiments were achieved in open reactors that allow the elimination of the by-product of the reaction, the methanol. However, for *R* values over 7, a stage in ascorbyl palmitate production was observed, suggesting a product inhibition.

To determine whether the reaction medium hydrophobicity or the methyl palmitate concentration is the major factor affecting the catalytic activity of the *C. antartica* lipase, syntheses were achieved at equal log *P* level, where methyl palmitate was partly substituted by a hydrophobic solvent such as dodecane. Fig. 3 presents results obtained in reaction media containing substrates molar ratios of 1 and 3 in the presence of added dodecane (4 ml and 5 ml, respectively), so as to reach the hydrophobicity level of a reaction medium containing substrates molar ratio of 9. The addition of dodecane was shown to exert no positive effect on the catalytic activity of the lipase. Even if the hydrophobicity property of the reaction medium is undoubtedly of importance for the creation and the stabilization of a catalytically active form of the enzyme [15], the establishment of appropriate bonds, which are specific to a substrate or a set of substrates, between the hydrophobic phase and the enzyme, is suggested to be a more determinant condition to undergo effective conformational changes in the protein.

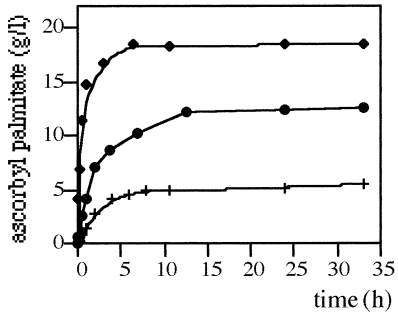

Fig. 3. Time course of ascorbyl palmitate synthesis catalyzed by the Novozym. Comparison was made between reactions achieved at equal log *P* level, and *R* values of 9 (♦) without any added hydrophobic solvent, 3 (·) and 1 (+) in the presence of 4 ml and 5 ml of added dodecane, respectively.

3.3. Synthesis of EPA and DHA ascorbyl esters

Syntheses were achieved at various temperature, showing the formation of undesirable secondary products at 70°C. A temperature of 55°C, that avoids substrate alteration, was then applied when working with the highly oxidizable EPA and DHA ethyl esters as acyl donor; ascorbic acid concentration was adjusted to 12 g/l so as to allow its total solubilization, at 55°C, in 2-methyl-2-butanol. Due to a relatively limited disponibility of the purified EPA and DHA ethyl esters, syntheses were performed in reaction media containing substrates molar ratios of 5. As previously shown for ascorbyl palmitate

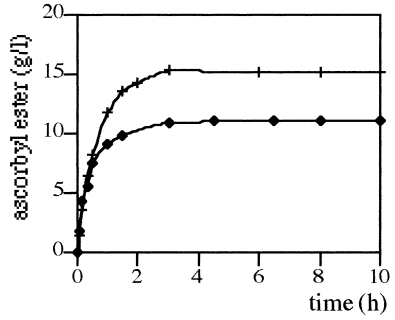

Fig. 4. Time course of the enzymatic syntheses of EPA (♦) and DHA (+) ascorbyl esters, catalyzed by the Novozym, at 55°C, with substrates molar ratios of 5.

synthesis, an increase in the substrates molar ratio up to 9 was shown to improve the production of these polyunsaturated fatty acid ascorbyl esters. Results obtained at 55°C, with substrates molar ratios of 5, are presented in Fig. 4.

Productions of 11 g/l and 15 g/l were obtained after a 3 h reaction time for the EPA and the DHA ascorbyl esters, respectively, suggesting a chain length influence. These promising results show that the enzymatic way can be applied to the synthesis of new substances of high potential interest such as stable polyunsaturated fatty acids. Similar syntheses are currently tested in an attempt to stabilize highly oxidizable colours involved in cosmetic or food preparations.

References

[1] J. Dyerberg, H.O. Bang, Lancet II 8140 (1979) 433.
[2] D. Kromhout, E.B. Bosschietor, C.D.L. Coulander, New Engl. J. Med. 312 (1985) 1205.
[3] G. Singh, R.K. Chandra, Prog. Food Nutr. Rev. 12 (1988) 371.
[4] P.J. Ke, R.G. Ackman, B.A. Linke, Prog. Food Nutr. Rev. 52 (1975) 349.
[5] J.K. Kaitaranta, JAOCS 69 (1992) 810.
[6] N. Ito, M. Hirose, S. Fukushima, H. Tsuda, T. Shirai, M. Tatematsu, Food Chem. Toxicol. 24 (1986) 1071.
[7] M. Jakobsson, B. Sivik, J. Dispersion Sci. Technol. 15 (1994) 611.
[8] D. Han, O.S. Yi, H.K. Shin, JAOCS 68 (1991) 740.
[9] K. Enomoto, T. Miyamori, A. Sakimae, R. Numazawa, Eur. Pat. Appl. EP 401704 (1990) .
[10] K. Sakashita, S. Myamoto, A. Sakimae, Eur. Pat. Appl. EP 514694 (1992) .
[11] C. Humeau, M. Girardin, D. Coulon, D. Miclo, Biotechnol. Lett. 17 (1995) 1091.
[12] H. Goderis, G. Ampe, M. Feyten, B. Fouwé, W. Guffens, S.V. Cauwenberg, P. Tobback, Biotechnol. Bioeng. 30 (1987) 258.
[13] G.W.C. Kaye, T.H. Laby, Tables of physical and chemical constants and some mathematical functions, 15th edn., Longman, London, 1986.
[14] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, Structure 2 (1994) 293.
[15] A.M. Brzozowski, U. Derewenda, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkenberg, F. Björkling, B. Huge-Jensen, S.A. Patkar, L. Thim, Nature 351 (1991) 491.