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The pronounced effect of water activity on the positional selectivity of Novozym 435 during 1,3-diolein synthesis by esterification

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ABSTRACT

We have investigated the effect of water activity on the positional selectivity of the immobilized lipase Novozym 435 during the esterification of oleic acid with glycerol for 1,3-diolein preparation. The highest preferential selectivity of Novozym 435 to 1-position over 2-position of the glycerol molecular was achieved when the water activity was 0.53. The mechanism by which the enzyme favors or disfavors the selectivity at various water activities may be related to the changes in the internal flexibility of the enzyme which depends on the effective hydration.

1. Introduction

The activity and selectivity of enzymes in organic media are usually greatly affected by the level of water present in the medium. As reported by many authors, water availability in organic media cannot be quantified using its concentration because the capacity of organic phase to dissolve water varies considerably with the polarity of solvent. It is best analyzed in terms of water activity since the hydration of the enzyme in different reaction media is the same when the same water activity were controlled [1]. And it has been widely accepted that each enzyme has the specific water activity requirements [2].

There are several reports about the effect of water activity on the selectivity of enzymes [3–8], but some groups find that water activity does not influence the selectivity of an enzyme [9,10]. So it will be interesting to study the effect of water activity on the positional selectivity of the immobilized lipase Novozym 435 during 1,3-diolein synthesis by esterification of oleic acid with glycerol. In view of this, we present here a systematic investigation into the water activity effect on the positional selectivity of the enzyme from the points of view of kinetics, based on an earlier literature which described the effect of water activity on the equilibrium and rate constants during triolein preparation catalyzed by immobilized *Mucor miehei* lipase [11]. *t*-Butanol was chosen for this study because it was an excellent solvent for this reaction proved by our preliminary experiments.

2. Experimental

2.1. Biological and chemical materials

Novozym 435 (immobilized lipase from *Candida antarctica*, type B) was a generous gift from Novozymes (Denmark). 1-Monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein and triolein were purchased from Sigma and were chromatographically pure. All other chemicals and reagents were obtained commercially and were of analytical grade.

2.2. General procedure for 1,3-diolein synthesis at constant water activity (a_w)

The method of maintaining a constant water activity throughout the lipase-catalyzed esterification proposed recently [8] was adopted here. The reaction solution of oleic acid (0.15 M) and glycerol (0.06 M) in *t*-butanol was prepared and used in all reactions. The reaction solution and Novozym 435 were separately equilibrated with aqueous saturated salt solution of known a_w for 16 h in two air tight desiccators (fitted with a rubber septum on the lid). The following salts were used: LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.53$), NaCl ($a_w = 0.75$) and K_2SO_4 ($a_w = 0.97$). The reaction was started by transferring 10 mL of the equilibrated reaction solution with a syringe into the beaker

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containing Novozym 435 via the rubber septum without opening the lid. The reaction mixture was magnetically stirred.

2.3. Analysis of the samples

The acylglycerol in the reaction mixture was analyzed by a Shimadzu 20A HPLC system (Shimadzu, Kyoto, Japan) with an ELSD-LT II low temperature-evaporative light scattering detector. Two microliters sample and 1 mL acetone were mixed thoroughly. Twenty microliters of the aforementioned mixture was injected. The stationary and mobile phases were an C_{18} column (5 μm, 250 mm × 4.6 mm) (Dikma Technology, PLATISIL ODS, China) and a gradient elution program (Table 1) by acetonitrile and dichloromethane at 1.5 mL min^{-1}, respectively. The column temperature was 40 °C. The drift pipe temperature was 70 °C, and the nitrogen pressure was 320 kPa. In addition, the oleic acid and glycerol concentration were detected using the methods described by Du [12].

2.4. Rate constants identified

For modeling the kinetic behaviors of 1,3-diolein preparation catalyzed by Novozym 435 in *t*-butanol systems, we adopted the synthetic scheme shown in Scheme 1 on the basis of the model proposed previously [13,14]. The esterification reaction followed second-order reversible reactions, while the acyl migration reactions between 1-MO and 2-MO, 1,2-DO and 1,3-DO, respectively, belonged to one-order reversible reactions; the mass transfer limitation in the reaction system could be neglected. Based on the above assumptions, the differential equations characterizing the whole reaction process were listed as follows (OA, 1-MO, 2-MO, 1,3-DO, 1,2-DO, TO and Gly referred to oleic acid, l-monoolein, 2-monoolein, 1,3-diolein, 1,2-diolein, triolein and glycerol, respectively):

$$\frac{d[\text{OA}]}{dt} = k_2[\text{1-MO}] + k_{10}[\text{2-MO}] + k_4[1,\text{3-DO}] + (k_6 + k_{12})[1,\text{2-DO}] + (k_8 + k_{14})[\text{TO}] - ((k_1 + k_9)[\text{Gly}] + (k_3 + k_{11})[\text{1-MO}] + k_5[\text{2-MO}] + k_7[1,\text{2-DO}] + k_{13}[1,\text{3-DO}])[\text{OA}]$$

$$\frac{d[\text{1-MO}]}{dt} = k_1[\text{OA}][\text{Gly}] + k_4[1,\text{3-DO}] + k_{12}[1,\text{2-DO}] + k_{16}[\text{2-MO}] - (k_2 + k_5 + (k_3 + k_{11})[\text{OA}])[\text{1-MO}]$$

$$\frac{d[\text{2-MO}]}{dt} = k_6[1,\text{2-DO}] + k_9[\text{OA}][\text{Gly}] + k_{15}[\text{1-MO}] - (k_5[\text{OA}] + k_{10} + k_{16})[\text{2-MO}]$$

$$\frac{d[1,\text{3-DO}]}{dt} = k_3[\text{1-MO}][\text{OA}] + k_{14}[\text{TO}] + k_{18}[1,\text{2-DO}] - (k_4 + k_{13}[\text{OA}] + k_{17})[1,\text{3-DO}]$$

$$\frac{d[1,\text{2-DO}]}{dt} = (k_5[\text{2-MO}] + k_{11}[\text{1-MO}])[\text{OA}] + k_8[\text{TO}] + k_{17}[1,\text{3-DO}] - (k_6 + k_7[\text{OA}] + k_{12} + k_{18})[1,\text{2-DO}]$$

$$\frac{d[\text{TO}]}{dt} = (k_7[1,\text{2-DO}] + k_{13}[1,\text{3-DO}])[\text{OA}] - (k_8 + k_{14})[\text{TO}]$$

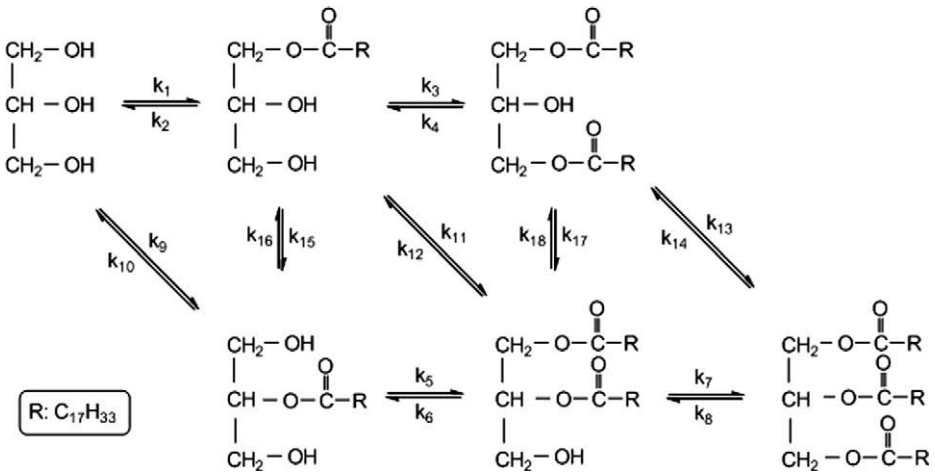
$$\frac{d[\text{Gly}]}{dt} = k_2[\text{1-MO}] + k_{10}[2-\text{MO}] - (k_1 + k_9)[\text{OA}][\text{Gly}]$$

Table 1
Gradient elution program.

Time (min)	Flow rate (mL min^{-1})	Acetonitrile–acetic acid (99.85:0.15) (V/V, %)	Dichloromethane (V/V, %)
0	1.50	100	0
4	1.50	100	0
12	1.50	90	10
25	1.50	90	10
30	1.50	70	30
35	1.50	70	30
45	1.50	20	80
55	1.50	20	80
60	1.50	100	0
65	1.50	100	0

3. Results and discussion

A range of water activities (from 0.11 to 0.97) were selected to investigate their effect on the positional selectivity of the immobilized lipase Novozym 435 during the esterification of oleic acid with glycerol carried out in *t*-butanol system. A detailed comparison on enzymatic 1,3-diolein synthesis with different water activity has been made in Table 2. It can be clearly seen that, the diolein yield (entry 2) and the ratio of 1,3-diolein to 1,2-diolein (entry 3) were distinctly dependent on the water activity. The diolein yield was increased with the increasing water activity. This corresponded to the activation of enzyme by hydration, involving a general role of water in making the enzyme structure more flexible, increasing the enzymatic rate [15,16]. But high water levels have also been shown to reduce the rate of the lipase-catalyzed esterification owing to increasing the hydrolysis rate. So, the trend was reversed when water activity reached a particular value (0.53), further rise led to a drop in diolein yield. It was also worth noting



Scheme 1.

Table 2
Influence of water activity on the enzymatic 1,3-diolein synthesis.

Water activity (a_w)	0.11	0.33	0.53	0.75	0.97
Diolein yield (%)	66	78	84	80	75
1,3-Diolein/1,2-diolein	13.2	20.5	24.1	22.6	18.7
k_1	0.535	0.901	1.231	1.112	0.082
k_3	0.846	1.148	1.347	1.267	1.056
k_9	0.083	0.097	0.126	0.115	0.091
k_{11}	0.0025	0.0027	0.0030	0.0028	0.0025
k_1/k_9	6.42	9.30	9.73	9.54	8.03
k_3/k_{11}	344	426	480	450	380

that, the ratio of 1,3-diolein to 1,2-diolein increased to 24.1 and then decreased along with the increase of the water activity. As we all known, a bigger ratio of 1,3-diolein to 1,2-diolein could be attributed to a higher preferential selectivity of Novozym 435 towards 1-position over 2-position of the glycerol molecular. So, to get a better understanding of the phenomena involved in the changes in the ratio of 1,3-diolein to 1,2-diolein, an insight into the influence of water activity on the positional selectivity of Novozym 435 from the points of view of kinetics could be of great interest.

The rate constants were identified by solving the differential equations described previously with an adaptive step-size Runge–Kutta method within a nonlinear regression procedure, using the Levenberg–Marquardt algorithm, so as to obtain the best fit between the experimental data and the results calculated. And some rate constants which were relevant to the positional selectivity of Novozym 435 were listed in Table 2.

As shown from Table 2 (entries 4–7), when the water activity was increased from 0.11 to 0.53, k_1, k_3, k_9 and k_{11} increased. It could be interpreted that an increase in the internal flexibility of the enzyme caused by increasing water activity in general, resulted in the increase of enzymatic activity [15,16]. Among the increase of these four rate constants, k_1 and k_3 increased noticeably; k_9, and k_{11} increased mildly. As a result, k_1/k_9 and k_3/k_{11} increased with the increasing water activity. It was demonstrated that the preferential selectivity of Novozym 435 to 1-position over 2-position of the glycerol molecular was raised with the increase of water activity. The reason may be that the increase of the water activity resulted in more water molecular binding near the active site of the enzyme and made it more flexible. Accordingly, the selectivity of the enzyme increased with the increasing water activity [9,17–19].

When the water activity was increased from 0.53 to 0.97, the opposite effect was obtained, i.e. the rate constants decreased. Among the decrease of the four rate constants, k_1 and k_3 decreased obviously; k_9, and k_{11} decreased slightly. As a consequence, k_1/k_9 and k_3/k_{11} decreased with the increasing water activity, indicating that the preferential selectivity of Novozym 435 to 1-position over 2-position of the glycerol molecular was dropped with increasing water activity. The reason involved in this case was supposed to be that, after the optimum hydration level of the enzyme was reached, the further increase of water activity would allow excess water molecular to bind close to the active site of the enzyme, made it too flexible, leading to the decrease of the difference in binding of the different hydroxyl groups of the glycerol molecular; on the other hand, the excess water would form a partial obstruction near the active site [15], thereby inhibiting the access of the glycerol molecular to the enzyme. So, the positional selectivity decreased.

Interestingly, it was also observed that k_3/k_{11} was much bigger than k_1/k_9 for each water activity. A speculative explanation could be that after one 1-position of the glycerol molecular was acylated, the preferential selectivity to the other 1-position over 2-position would be enhanced strongly.

Based on the above results, it was concluded that the water activity has a pronounced influence on the preferential selectivity of Novozym 435 to 1-position over 2-position of the glycerol molecular during the 1,3-diolein synthesis by esterification. The mechanism by which the enzyme favors or disfavors the selectivity at various water activities may be related to the changes in the internal flexibility of the enzyme which depends on the effective hydration. Recently, the molecular modeling revealed that there was a possibility for the water molecular to bind in the stereospecificity pocket of *C. antarctica* lipase B specifically and reduced its size, enhancing the difference in binding of the substituents on the secondary alcohols molecular, and then increased the enantioselectivity [4,5]. So, the molecular modeling may be a helpful tool for exploring the molecular mechanism of the positional selectivity of Novozym 435 during 1,3-diolein synthesis by esterification of oleic acid with glycerol.

4. Conclusion

In summary, the influence of water activity on enzymatic selectivity is complex, but essential for the biocatalysis. The results of this work allowed a better understanding of the pronounced effect of water activity on the positional selectivity of the immobilized lipase Novozym 435 during 1,3-diolein synthesis by esterification of oleic acid with glycerol. The molecular modeling may be a promising tool for elucidating how the enzyme serves the task of distinguishing between the isomers behind water activity effect.

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References

[1] R.H. Valivety, P.J. Hallmg, A.R. Macrae, Biochem. Biophys. Acta 1118 (1992) 218–222.
[2] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178–206.
[3] A. Jonsson, W. Van Breukelen, E. Wehtje, P. Adlercreutz, B. Mattiasson, J. Mol. Catal. B: Enzym. 5 (1998) 273–276.
[4] V. Leonard, L. Fransson, S. Lamare, K. Hult, M. Graber, ChemBioChem 8 (2007) 662–667.
[5] V. Leonard, Z. Marton, S. Lamare, K. Hult, M. Graber, J. Mol. Catal. B: Enzym. 59 (2009) 90–95.
[6] L. Ma, M. Persson, P. Adlercreutz, Enzyme Microb. Technol. 31 (2002) 1024–1029.
[7] P. Pepin, R. Lortie, Biotechnol. Bioeng. 63 (1999) 502–505.
[8] S. Sabbani, E. Hedenstrom, J. Mol. Catal. B: Enzym. 58 (2009) 6–9.
[9] M. Persson, D. Costes, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 30 (2002) 916–923.
[10] E. Wehtje, D. Costes, P. Adlercreutz, J. Mol. Catal. B: Enzym. 3 (1997) 221–230.
[11] Y. Dudal, R. Lortie, Biotechnol. Bioeng. 45 (1995) 129–134.
[12] W. Du, D.H. Liu, L.L. Li, L.M. Dai, Biotechnol. Prog. 23 (2007) 1087–1090.
[13] R. Lortie, M. Trani, F. Ergan, Biotechnol. Bioeng. 41 (1993) 1021–1026.
[14] T. Watanabe, M. Shimizu, M. Sugiura, M. Sato, J. Kohori, N. Yamada, K. Nakanishi, J. Am. Oil. Chem. Soc. 80 (2003) 1201–1207.
[15] J.L. Schmitke, C.R. Wescott, A.M. Klibanov, J. Am. Chem. Soc. 118 (1996) 3360–3365.
[16] V. Kurkal, R.M. Daniel, J.L. Finney, M. Tehei, R.V. Dunn, J.C. Smith, Biophys. J. 89 (2005) 1282–1287.
[17] J. Broos, A.J.W.G. Visser, J.F.J. Engbersen, A. van Hoek, D.N. Reinhoudt, J. Am. Chem. Soc. 117 (1995) 12657–12663.
[18] A. Jonsson, E. Wehtje, P. Adlercreutz, B. Mattiasson, Biochim. Biophys. Acta 1430 (1999) 313–322.
[19] J. Broos, Biocatal. Biotransfor. 20 (2002) 291–295.