J Am Oil Chem Soc (2008) 85:635–639
DOI 10.1007/s11746-008-1248-4

ORIGINAL PAPER

Optimization of Selective Lipase-Catalyzed Feruloylated Monoacylglycerols by Response Surface Methodology

Yan Zheng · Jing Quan · Li-Min Zhu ·
Bo Jiang · Hua-Li Nie

Received: 5 March 2008 / Revised: 24 March 2008 / Accepted: 5 May 2008 / Published online: 22 May 2008

Abstract The use of solvent engineering to achieve selective enzymatic synthesis of feruloylated acylglycerols during the transesterification of ethyl ferulate with TAG was investigated. Novozym 435 catalyzed transesterification of ethyl ferulate and TAG resulted in a mixture of feruloylated monoacylglycerols (FMAG) and feruloylated diacylglycerols (FDAG). These feruloylated acylglycerols have recently received much attention because of their health benefits, antioxidant properties and UV absorption. However, FMAG in a pure form is more advantageous than the FMAG–FDAG mixture in exhibiting stabilizing, emulsifying and conditioning properties. Thus, it is significant to perform efficient selectivity in the synthetic process. In this present study, the effect of various solvent mixtures, including unitary, binary and ternary organic media selective enzymatic synthesis of feruloylated acylglycerols was investigated by response surface methodology. Selectivity towards FMAG substantially increased from 14.5% in the unitary solvent *n*-hexane to 94.2% in the binary mixtures of 2-methyl-2-butanol (2M2B) and toluene (1:1, v/v). The maximum conversion achieved was 75.4% in this binary mixture medium. Analysis of variance (ANOVA) showed that 99.6% of the observed variation was explained by the polynomial model. Lack of fit analysis indicated that the regress equation was adequate for predicting the degree of the selectivity.

Keywords Feruloylated monoacylglycerols · Lipase-catalyzed · Selectivity · Response surface methodology

Introduction

In recent years, the incorporation of ferulic acid into triacylglycerols (TAG) to produce novel feruloylated acylglycerols composed of feruloylated monoacylglycerols (FMAG) and feruloylated diacylglycerols (FDAG) has received much attention because of their health benefits [1–4]. They can reduce risk of coronary disease, prevent certain cancers, and improve immune functions. In addition, the antioxidant properties and UV absorption of the feruloylated acylglycerols have recently led to interest in them as natural ingredients for use in cosmetics and skin-care products [2]. However, each of the feruloylated acylglycerols molecular species is presumed to have special chemical and physical properties and FMAG in a pure form is more advantageous than the FMAG–FDAG mixture in achieving stabilizing, emulsifying and conditioning properties. Thus, it is significant to perform efficient selectivity in synthesis process.

Enzyme-catalyzed reactions are recognized as superior to conventional chemical methods in the selective modification of substrates due to the high selectivity of enzyme and mild operation conditions [4]. On the other hand, solvent mixtures as reaction media have been employed in the selective enzyme-catalyzed reactions, and the advantages of using solvent mixtures versus other strategies have been discussed [5]. However, few studies applied systematically statistical methods to investigate the effect of solvent mixtures on the selectivity in enzyme-catalyzed reactions [6, 7]. Response surface methodology (RSM) is a popular

Y. Zheng · J. Quan · L.-M. Zhu (✉) · B. Jiang · H.-L. Nie
College of Chemistry, Chemical Engineering and
Biotechnology, Donghua University,
Shanghai 201620, People's Republic of China
e-mail: lzhu@dhu.edu.cn

Y. Zheng
e-mail: zhengyan@mail.dhu.edu.cn

and effective statistical technique for the investigation of complex process. In addition to analyzing the effect of the independent variable, this method also generates a mathematical model that accurately describes the whole process.

In the present study, lipase catalyzed transesterification of ethyl ferulate and tributyrin in solvent mixtures to produce FMAG was investigated by RSM. Our objectives were to better understand the effect of solvent mixtures on the selectivity of the reaction and to determine the optimum solvent medium for the high purity of FMAG.

Materials and Methods

Materials

Novozym 435 (EC 3.1.1.3, an immobilized preparation of lipase from *C. antarctica* on macroporous acrylic resin, 10,000 U/g) was purchased from Sigma. Ethyl ferulate (purity $> 99\%$) was purchased from Suzhou Chang Tong Chemical Co., Ltd. (Suzhou, China). Tributyrin (purity $>$ 99%) was purchased from Tao He Chemical Co., Ltd. (Shanghai, China).

All solvents were of analytical grade and were dried by activated 4 Å molecular sieves before use. All other reagents used were of high purity commercially available unless otherwise noted.

Experimental Design

A three-variable simplex lattice design was performed to explore the effect of variable solvent on the response in the selective synthesis of FMAG. This design resulted in fourteen solvent mixtures (Table 1). The percentage of the composition of the solvent was presented at levels ranging from 0 to 100%.

Setting Initial a_w in a Closed System

The water activity (a_w) values of the solvent, enzyme, and substrate were adjusted before starting the reaction by the following method: solvent or solvent mixtures, ethyl ferulate, tributyrin and Novozym 435 were separately incubated in a chamber containing a desirable saturated salt solution (CH_3COOK $a_w = 0.23$), and the system was allowed to reach equilibrium for at least 7 days at 25 °C. The water content was measured by a Karl–Fisher titrator (Mettler-Toledo, DL31, USA).

Enzymatic Reaction

The enzymatic reaction was carried out in a closed, screw-capped tube containing 0.5 mmol ethyl ferulate, 1.5 mmol tributyrin, 120 mg Novozym 435 lipase at $a_w = 0.23$, in 3 ml solvent or solvent mixtures. The reaction was incubated in an orbital air shaking bath (210 rpm) at 50 °C for 120 h. All the experiments were done in duplicate and significantly different from tests at $P < 0.05$.

Analysis and Characterization

Analytical HPLC was performed using a Waters 510 with an Inertsil Ph-3 column (4.6 mm ID × 250 mm, 5 µm, GL Sciences, Japan),with detection at 325 nm. The mobile phase was solution A (water containing 0.1% acetic acid) and solution B (methanol containing 0.1% acetic acid) in

Table 1 Three-variable simplex lattice design, experimental value, and predicted value for response surface analysis[a]

Trial	Variable level[b]			Conversion (%)[c]	Selectivity (%)	
	n-hexane	Toluene	2M2B		Experimental value	Predicted value
1	3.00	0.00	0.00	52.8	13.9	14.5
2	1.50	1.50	0.00	63.9	49.9	48.8
3	1.50	0.00	1.50	64.5	52.6	51.0
4	0.00	3.00	0.00	70.6	57.9	58.8
5	0.00	1.50	1.50	75.4	94.2	92.3
6	0.00	0.00	3.00	67.4	69.9	68.6
7	2.00	0.50	0.50	61.3	45.0	43.7
8	0.50	2.00	0.50	73.7	72.5	72.2
9	0.50	0.50	2.00	71.5	73.1	76.2
10	1.00	1.00	1.00	67.8	67.9	69.6
11	3.00	0.00	0.00	53.5	14.4	14.5
12	0.00	0.00	3.00	68.3	67.9	68.6
13	0.00	3.00	0.00	71.4	59.2	58.8
14	1.50	1.50	0.00	61.7	48.2	48.8

[a] Mixture design results in fourteen formulations

[b] Components of solvent or solvent mixtures (ml)

[c] Conversion calculated under the experimental condition

all cases at 1 ml $\min^{-1}$ flow of A/B (30/70, v/v). The conversion of the reaction was quantified in terms of the mole percentage transesterification, i.e., the ratio of ethyl ferulate consumed to the total amount of ethyl ferulate before reaction.

The selectivity was defined as:

$$\text{selectivity} = M_1/M_2 \times 100\%$$

where M_1 was the mole percentage of FMAG formed and M_2 was mole percentage of ethyl ferulate consumed.

Statistical Analysis

The three-variable simplex lattice design was applied using Design-Expert 6.05 (State-Ease, United States). Experimental data based on the design was fitted to a quadratic canonical polynomial model described by Cornell (1983) as follows:

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1\beta_2 X_1 X_2 + \beta_1\beta_3 X_1 X_3 + \beta_2\beta_3 X_2 X_3$$

where Y is a predicted dependent variable (selectivity); β_1, β_2, β_3, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ are the corresponding parameter estimates for each prediction model; X_1 is the component of *n*-hexane; X_2 is the component of toluene; and X_3 is the component of 2-methyl-2-butanol (2M2B). The intercept was not included in the analysis because the mixture components should be equal to 100% of the mixture.

Results and Discussion

Systematically statistical methodology was employed to investigate the effect of solvent mixtures on the selectivity towards FMAG. The choice of the solvent was based on solvent polarity, which was the main factor influencing the selectivity of lipase-catalyzed reaction [8]. Several research groups pointed out that solvent with Log $P < 2$ could increase the specific production of monoacylglycerols [7]. On the other hand, solvent with Log $P > 2$ was favorable for relatively high activity of enzyme. Thus, we chose *n*-hexane, toluene and 2M2B as solvent components. Log P for *n*-hexane is 3.5, toluene 2, and 2M2B 1.5, respectively.

RSM Experiments and Fitting the Model

The experimental and predicted values of selectivity at points based on the experimental design were shown in Table 1. A quadratic regression model was obtained by using coded values from the estimation of data:

$$Y = 14.54\ X_1 + 58.78\ X_2 + 68.56\ X_3 + 48.67\ X_1X_2 + 37.93\ X_1X_3 + 114.54\ X_2X_3$$

Analysis of Variance (ANOVA) and Adequacy Test of the Model

ANOVA (Table 2) indicated that the quadratic regression model was highly significant ($P < 0.0001$) and the lack of fit was insignificant, which indicated that the model was prominent and adequate to explain most of the variability for selectivity. Nevertheless, a fitted response surface might give poor or misleading results if the model did not exhibit adequate fit [9]. Therefore, it is essential to check the adequacy of the model (Table 3). The low value of CV gave better reproducibility for the experimental point [10], and the CV for the selectivity was proved to be within the acceptable range as given in Table 3. The large value of "adj R-squared" indicated that the model could explain 99.33% of the total variation in the response. "Adeq precision" measured the signal-to-noise ratio, where a value greater than 4 was desirable. The plot of predicated values of selectivity versus the experimental values indicated a very good fit for the reaction with a correlation coefficient (Fig. 1). Consequently, this model could be used to navigate the design space.

Analysis of Responses Surfaces

The experimental and predicted values distribution at equilibrium of transesterification are presented in Table 1, meanwhile the conversion under the experimental condition is also given to investigate the catalytic ability of Novozym 435 in different organic media. Among the trials,

Table 2 Analysis of various (ANOVA) for the quadratic model for the regioselective synthesis of feruloylated monoacylglycerols

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value	Prob > F[a]
Model	6224.43	5	1244.89	388.21	<0.0001
Residual	25.65	8	3.21		
Lack of fit (model error)[b]	21.29	4	5.38	778.22	0.7701
Pure error (replicate error)	4.37	4	1.09		
Total	6250.08	13			

[a] Prob > F = level of significance

[b] Lack of fit = total – pure error

Table 3 Statistical analysis for the regioselective synthesis of feruloylated monoacylglycerols

Standard deviation	1.79
Mean	56.18
CV	3.19
PRESS	116.41
R-Squared	0.9959
Adjusted *R*-squared	0.9933
Predicted *R*-squared	0.9814
Adeq precision	66.341

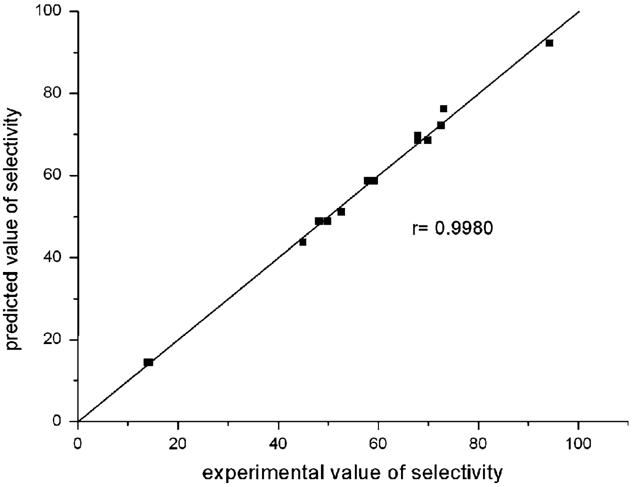

Fig. 1 Relationships between predicted value and experimental value

higher selectivity of the reaction was observed in 2M2B than in toluene (trial 4, 6), while the selectivity was the lowest in *n*-hexane for all reactions investigated (trial 11). When binary or ternary solvent mixtures were used, the selectivity was obviously changed. Overall, the product distribution was affected by the components in the solvent.

The triangular contour and three-dimensional surface plot are drawn to show the main first-order effect and interactive effect of the independent variables (solvent or solvent mixtures) on the dependent variables (selectivity). As shown in Fig. 2a, when binary solvent mixtures of *n*-hexane and toluene or the mixtures of *n*-hexane and 2M2B were used, the selectivity exhibited was almost on the same contour. When the solvent mixtures of toluene and 2M2B were used, the selectivity was greatly increased to 94.2% (Fig. 2b). This indicated that the selective synthesis of FMAG is highly induced by the binary solvent mixtures of toluene and 2M2B. Nevertheless, a similar contribution of toluene and 2M2B when mixed with hexane respectively could not be explained by polarity variation. According to the analysis of the effect of the ternary solvent mixtures on the selectivity (trial 7, 8, 9, 10), it was clear that as the proportion of *n*-hexane decreased, the selectivity towards FMAG increased rapidly (Fig. 2b), which indicates that a decreased polarity in the solvent mixture has a negative impact on the behavior of the reactant mixture, resulting in lower FMAG formation. It could be due to the low solubility of the ethyl ferulate in the less polar medium restrained the substrate access to the active site of the enzyme. Thus, it is concluded that the binary solvents system of 2M2B and toluene is suitable for selective synthesis of FMAG compared with unitary and ternary system. From the present investigation, it is notable that different organic solvent mixtures have a significant effect on the selectivity of the reaction, and that selectivity towards FMAG thus achieved in the optimum organic medium is higher than the method of Sun [11], which optimized the FMAG yield in a solvent free system.

In this study, RSM was used to investigate the effect of solvent mixtures on the selective synthesis of FMAG. The triangle contour and three-dimensional surfaces plots were successfully applied to analyze the optimum solvent mixtures for FMAG formation. A high selectivity of 94.2%

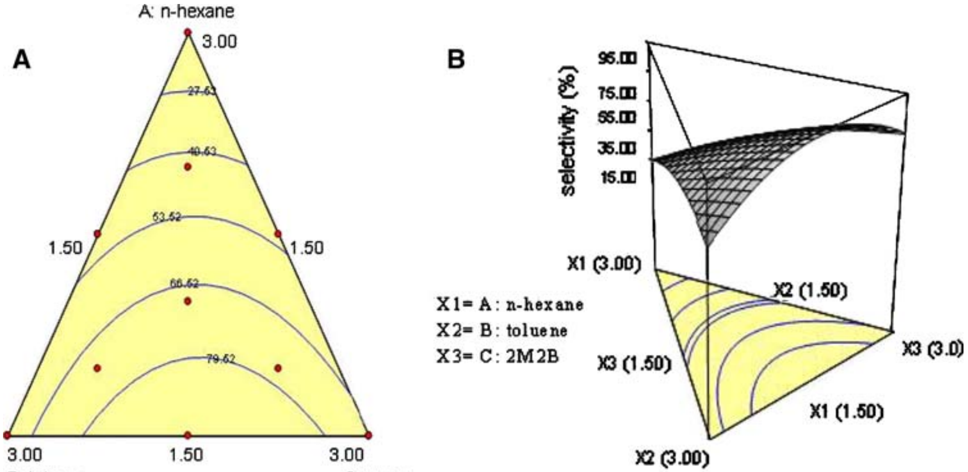

Fig. 2 **a** Contour plots using predicted model for lipase-catalyzed feruloylated monoacylglycerols. **b** Response surface plots using predicted model for lipase-catalyzed feruloylated monoacylglycerols

towards FMAG was achieved under the optimal conditions. Furthermore, the experimental values evaluated from the quadratic model showed a good agreement with the values predicted and the quadratic regression model describes the product distribution successfully.

Acknowledgments This work was financially supported by the National Nature Science Foundation of Chinese (No. 50773009), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0526).

References

1. Compton DL (2005) Sunscreens based on vegetable oil. Lipid Technol 17:276–279
2. Compton DL, Laszlo JA, Berhow MA (2006) Identification and quantification of feruloylated mono-, di-, and triacylglycerols from vegetable oils. J Am Oil Chem Soc 83:753–758
3. Warner K, Laszlo JA (2005) Addition of ferulic acid, ethyl ferulate, and feruloylated monoacyl- and diacyl-glycerols to salad oils and frying oils. J Am Oil Chem Soc 82:647–652
4. Sun SD, Shan L, Liu YF, Jin QZ, Wang XG, Wang ZM (2007) A novel, two consecutive enzyme synthesis of feruloylated monoacyl- and diacyl-glycerols in a solvent-free system. Biotechnol Lett 29:1947–1950
5. Damstrup ML, Abildskov J, Kiil S, Jensen AD, Sparso FV, Xu XB (2006) Evaluation of binary solvent mixtures for efficient monoacylglycerol production by continuous enzymatic glycerolysis. J Agric Food Chem 54:7113–7119
6. Liao HF, Tsai WC, Chang SW, Shieh CJ (2003) Application of solvent engineering to optimize lipase-catalyzed 1,3-diacylglycerols by mixtures response surface methodology. Biotechnol Lett 25:1857–1861
7. Kuo SJ, Parkin KL (1996) Solvent polarity Influences product selectivity of lipase-mediated esterification reactions in microaqueous media. J Am Oil Chem Soc 73:1427–1433
8. Rendon X, Lopez-Munguia A, Castillo E (2001) Solvent engineering applied to lipase-catalyzed glycerolysis of triolein. J Am Oil Chem Soc 78:1061–1066
9. Myers RH, Montgomery DC (2002) Response surface methodology. Wiley, United States
10. Daniel W (1991) Biostatistics: a foundation for analysis in the health sciences. Wiley, United States
11. Sun SD, Shan L, Liu YF, Jin QZ, Zhang LX, Wang XG (2008) Solvent–free enzymatic preparation of feruloylated monoacylglycerols optimized of feruloylated monoacylglycerols optimized by response surface methodology. J Agric Food Chem 56:442–447