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Optimization of Enzymatic Hydrolysis of Chicken Fat in Emulsion by Response Surface Methodology

Dike Teng · Rensi Le · Fang Yuan · Jia Yang · Li He · Yanxiang Gao

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Abstract Chicken fat in an emulsion prepared with mechanical shearing and high pressure homogenization (HPH) was hydrolyzed using *Candida cylindracea* lipase. A homogenization pressure of 50 MPa, which can generate smaller droplets and higher hydrolysis efficiency than mechanical shearing, was fixed to prepare the emulsion for hydrolysis optimization. Response surface methodology (RSM) was applied to study the effect of temperature, enzyme loading, shaking rate and reaction time on the hydrolysis process. The results showed that all three-second-order polynomial models adequately fitted the experimental data. Additionally, hydrolysis parameters for the optimal yields of free oleic and linoleic acids were also obtained using the desirability function: a temperature of 38 °C, an enzyme loading of 0.48% (g/g fat basis), a shaking rate of 100 rpm and a reaction time of 80 min. Under the optimal conditions, the yields of free oleic and linoleic acids were predicted as being 0.470 and 0.118 g/g fat with recoveries of 94.6 and 93.7%, respectively. During the hydrolysis process, the particle size increased with concomitant boosting of the degree of hydrolysis and the stability of the emulsion system was gradually undermined by this reaction process.

Keywords Chicken fat · High pressure homogenization · Particle size · Lipase · Oleic acid · Linoleic acid

D. Teng · R. Le · F. Yuan · J. Yang · L. He · Y. Gao (✉)
College of Food Science and Nutritional Engineering,
China Agricultural University,
100083 Beijing, China
e-mail: gyxcau@126.com

Introduction

In the prosperous chicken processing industries, large amounts of chicken fat are produced annually as a co-product. Chicken fat has a higher degree of unsaturation than tallow because of its high content of unsaturated fatty acids (about 60%). Most chicken fat has a substantial nutrient value due to its high unsaturated fatty acid content, which mainly contains oleic and linoleic acids [1]. It has the potential to be further processed to upgrade the nutritive properties for human consumption. However, chicken fat is restricted to the field of food additives such as meat flavor, and the development of this biomass resource is still at a preliminary stage. In the previous decade, much attention was paid to lipase-catalyzed reaction carried out under milder conditions (temperature lower than 70 °C, atmospheric pressure) with higher selectivity than its chemical counterparts. Free fatty acids (FFA), the raw material of oleochemistry, are produced predominantly from the hydrolyzed natural triacylglycerols of vegetable oils or animal fats. For the enzymatic reaction, substrate concentration, temperature, enzyme loading, reaction time, shaking rate and pH value could affect the enzymatic hydrolysis efficiency significantly [2–6]. Furthermore, the possible interaction of multiple variables may also influence the output [7]. Consequently, a considerable number of studies have been carried out on lipase-catalyzed hydrolysis aiming at studying hydrolysis process of lipids rich in functional and nutritional fatty acids [8, 9]. However, publications aiming at the investigation of the optimization of chicken fat enzymatic hydrolysis, which focus on enriching free oleic and linoleic acids, are not yet available.

Since free lipase catalyzes the fat hydrolysis reaction at the water–oil interface, increasing the interface could accelerate the hydrolysis rate. Nevertheless, due to the

difficulty of interface analysis, the particle size was used as the parameter to estimate the interface behavior according to the relevant kinetics of hydrolysis in the emulsion [10]. In order to decrease the droplet size, reaction substrates were frequently agitated or sheared to prepare an emulsion in the presence of surfactant [11, 12]. High pressure homogenization (HPH) is an effective alternative to lower and stabilize the droplet size in the emulsion and the hydrolysis process in the emulsion prepared with the aforementioned method had not been covered in relevant publications.

In the present work, the comparative results of the hydrolysis in the emulsion prepared with HPH and mechanical shearing revealed the advantage of HPH in terms of droplet size and the degree of hydrolysis. The effects of temperature, enzyme loading, shaking rate and reaction time on the hydrolysis in the HPH prepared system were investigated. The optimal parameters for the yields of free oleic and linoleic acids were also determined. Under the optimal hydrolysis conditions, the gradually increase in chicken fat droplet diameter was observed with an escalating degree of hydrolysis during the reaction process.

Materials and Methods

Materials

Chicken fat was purchased from Tanggu Muyang Oils & Fats Co., Ltd. (Tianjin, China). The chicken fat mainly consists of triglycerides and its saponification value (SV) is 195. *Candida cylindracea* lipase (Lipomod-34P), which was selected among 13 different resource lipases according to their activities in a previous study by our research group [13], was kindly donated by the Biocatalysts (Cardiff, UK). Standards of fatty acid methyl esters (FAME), oleic acid, linoleic acid, *p*-bromophenacyl bromide (PBPB) and 1, 4, 7, 10, 13, 16-hexaoxacyclooctadecane (18-crown-6) were purchased from Sigma Aldrich (Shanghai, China). All the solvents (HPLC grade) were provided by Merck (Shanghai, China) and other chemicals (analytical grade) were from the Beijing Chemical Co. (Beijing, China).

Preparation of Chicken Fat Emulsion

Chicken fat and an aqueous solution of phosphate buffer (pH = 7) at a ratio of 1:1 (w/w) in the presence of 1% Tween-80 were homogenized using a high speed blender (Model HD-1, Huayuanhang Experimental Equipment Factory, Beijing, China) at a speed of 5000 rpm at 40 °C for 5 min to form a mechanically sheared coarse emulsion. The fine emulsions prepared by HPH were formed with an identical formula that was treated by a two-stage high pressure homogenizer (Model NS 10011L 2K, Niro-Soavi, Parma, Italy) at a given pressures at 40 °C. The particle size of the droplets in the emulsions was determined by a dynamic light scattering technique using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). The measurement was carried out at a fixed angle of 90°, and the samples were diluted approximately 1000 times with Milli-Q water at 0 °C, which quenched the hydrolysis reaction in the emulsion. The particle size of the droplets in the emulsions was described by the cumulants mean (z-average) diameter.

Chemical Hydrolysis of Chicken Fat

The chemical hydrolysis of chicken fat was based on the method of Shen et al. [14]. Chicken fat was saponified with 40 mL 0.5 M potassium hydroxide 95% ethanol solution for 24–48 h at room temperature, until all the oil drops had disappeared. Subsequently, 100 mL distilled water was added to the saponified mixture, followed by an extraction with three aliquots of anhydrous diethyl ether. The pH value of the aqueous phase was adjusted to pH 2 by adding 6 M HCl, and the FFA was extracted with anhydrous diethyl ether. After the removal of the organic solvent under a vacuum, the FFA sample was stored at −18 °C until the analysis of total amount of oleic and linoleic acids in the chicken fat was carried out.

Enzymatic Hydrolysis of Emulsified Chicken Fat

The enzymatic hydrolysis of chicken fat was performed by a shake flake method, using a stoppered, flat-bottomed conical flask (150 mL). About 75 g emulsion was heated up to the desired temperature in a water bath. The reaction was triggered by the addition of a given amount of enzyme (w/w fat basis). Reactions were performed in duplicate at the designed shaking rate. A sample of 0.1 g was withdrawn and dissolved in a neutral mixture of ethanol/acetone (1:1, v/v) with a volume of 15 mL, in order to quench the enzymatic reaction when the reaction time had elapsed. Subsequently, the aforementioned mixture was titrated with 0.1 mol/L potassium hydroxide ethanol solution to determine the acid value (AV) for estimating the amount of FFA according to Yan et al. [15]. The degree of hydrolysis of the chicken fat was calculated by using the following equation.

$$\text{Hydrolysis degree } (\%) = \frac{AV \text{ of hydrolyzed emulsion} - AV \text{ of feed emulsion}}{SV \text{ of feed emulsion}}. \quad (1)$$

Analysis of Fatty Acids

Samples of 0.5 g from the reaction mixture were drawn off, and a methanolic NaOH (0.5 M, 25 mL) solution was added to the mixture to neutralize the FFA from the hydrolysis and the triglycerides were extracted three times with *n*-hexane (3 × 100 mL). The lower layer containing the saponified FFA released during the hydrolysis was collected to prepare the FAME. The chicken fat (0.25 g) was saponified in the above-mentioned methanolic NaOH (25 mL) at 80 °C until the oil had disappeared. The saponified chicken fat and FFA were converted into FAME for fatty acids composition according to the method described by Xu et al. [16]. Fatty acids analysis was performed on an Agilent GC (Model 6890 Series) fitted with a flame ion detector. The FAME in 1 μL *n*-heptane was injected into the GC in a split mode (split ratio 1:50). The separation was carried out in a fused silica capillary column (HP-Innowax, 30 m × 0.25 mm i.d., 0.32 μm film thickness) with an initial temperature of 170 °C for 14 min, then the temperature was increased to 240 °C at a rate of 10 °C/min and was kept at 240 °C for 8 min. The detector temperature was set at 300 °C. Nitrogen was used as the carrier gas with a flow rate of 1.0 mL/min. Identification of FAME was achieved by comparing their retention times with those of authentic compounds under the same conditions. Each sample was analyzed in triplicate. The percentage composition of the fatty acids was calculated from their peak areas shown in the detector signals.

The quantitative determination of free oleic and linoleic acids in the chemical or enzymatic hydrolysate was carried out according to the HPLC method previously reported by Brindisi et al. [17] with slight modifications. A hydrolysate sample of 0.15 g at a given reaction time was transferred to 10 mL of a mixture of 1:1 (v:v) chloroform: methanol. And then 1 g anhydrous sodium sulfate was added to the mixture in order to keep it dry. The mixture was centrifuged at 1000 rpm for 15 min to facilitate emulsion destabilization. The supernatant was passed through a 0.45-μm nylon membrane filter. Then, a 0.25-mL portion of the filtrate was combined with 1.25 mL PBPB and 0.05 mL 18-crown-6. PBPB and 18-crown-6 stock solutions were prepared at a concentration of 1 mg/mL with acetonitrile as the solvent. An aliquot of 0.2 g potassium carbonate was added for its buffering capacity. The mixture was heated at 80 °C for 30 min. 60 mL of formic acid (4%, v/v) was added and the mixture was heated for an additional 5 min at 80 °C. Then the mixture was cooled for 1 h at 4 °C and the final mixture was then passed through a 0.45 mm nylon membrane filter. An aliquot of 20 μL final filtrate was injected into the HPLC column. A Zorbax SB-C18 reverse column (250 mm × 4.6 mm i.d., 5 μm) was used to separate the FFA. The analysis was performed on an Agilent 1100 HPLC system equipped with a diode array detector. A 60/40 (v/v) acetonitrile/water gradient profile was used for the elution with a 4% increase in acetonitrile over the first 10 min, followed by 100% acetonitrile for 25 min. The flow rate was constant at 1.6 mL/min, and the column temperature was maintained at 10 ± 0.5 °C. The FFA were detected at 254 nm with an ultraviolet detector. Oleic and linoleic acids standards were prepared as stated for the aforementioned hydrolyzed chicken fat samples. External standard curves were generated for free oleic and linoleic acids. The recoveries of fatty acids were determined in term of the percentage of oleic and linoleic acids released during enzymatic hydrolysis of chicken fat.

Experiment Design

RSM was applied to evaluate the effect of the factors that might have an impact on the degree of hydrolysis of chicken fat, and on the yields free of oleic and linoleic acids. The parameters and their ranges were chosen on the basis of the preliminary experimental results. The experiments were designed according to the central composite design (CCD) using a 2^n factorial and star design with three central points (Table 2). Runs at the central point of design were performed to estimate the possible pure error. Three-second-order polynomial equations were used to express the degree of hydrolysis of the chicken fat (Y_1), yields of oleic acid (Y_2) and linoleic acid (Y_3). Functions of the independent variables are as follows:

$$Y_k = a_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ii} X_{ii} + \sum_{i \neq j=1}^{4} a_{ij} X_i X_j \quad (2)$$

where Y_k represents the response variables, a_0 is a constant, a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients, respectively. X_i and X_j are the levels of the independent variables. Star points were carried out using an α of 1.547.

Three-dimensional surface response plots were generated by changing two of all the variables within the experimental range while holding the other variables at constant values at the central point. The coefficients of the response surface equation were estimated by using Statgrphics Centurion XV (Statpoint, Inc., 2005). The statistical significance test was based on the total error criteria with confidence level of 95.0%.

The desirability function was performed in Design-Expert 7.1.3, and each response was assigned an important value (1–5). The objective function is given as follows:

$$D = (d_1 r_1 \times d_2 r_2 \times \cdots \times d_n r_n)^{1/\sum r_i} \quad (3)$$

where D reflects the overall desirability of the function, d_i is the partial desirability function of each response obtained from the transformation of the individual response of each experiment, n is the number of responses in the measure and r_i represents the importance of each response.

Results and Discussion

Fatty Acid Analysis

The fatty acid composition of chicken fat is listed in Table 1. Saturated fatty acids accounted for 31.5% of the total fatty acids. The percentage of unsaturated fatty acids was about 68.4%, in which monounsaturated and polyunsaturated fatty acids were 52.9 and 15.6%, respectively. Among all the unsaturated fatty acids in chicken fat, oleic acid was the most abundant fatty acid, which was over 46%; while linoleic acid was approximately 15%. According to the quantitative determinations by HPLC, the absolute content of oleic and linoleic acids in chicken fat were 0.497 and 0.126 g/g fat, respectively.

Droplet Sizes and the Degrees of Hydrolysis of Chicken Fat in Emulsions Prepared by Different Methods

The particle size distributions in the emulsions prepared by HPH are plotted in Fig. 1a and a significant effect was not observed with regard to the average particle size ($P > 0.05$) with the rise of pressure from 50 to 110 MPa. Their particle sizes were 532, 509 and 516 nm for 50, 80 and 110 MPa, respectively. The particle size analysis (not shown in Fig. 1) showed that the diameter of emulsified chicken fat prepared with mechanical shearing at 40 °C, 5000 rpm for 5 min was 41.2 μm which was roughly 100 times larger than that prepared with HPH. The hydrolysis process of emulsified chicken fat at a temperature of 40 °C, enzyme loading of 0.6% and a shaking rate of 100 rpm are shown in Fig. 1b. Although the hydrolysis curve of chicken fat in the coarse emulsion prepared with shearing resembles the pattern of nanoemulsion prepared with HPH under

Table 1 Fatty acids composition for chicken fat and FFA in the hydrolysate

Fatty acids methyl ester (area %)			
		FFA	Chicken fat
C 8:0	Octanoic acid methyl ester	0.03 ± 0.00	0.01 ± 0.01
C 10:0	Decanoic acid methyl ester	0.02 ± 0.00	0.02 ± 0.01
C 12:0	Dodecanoic acid methyl ester	0.17 ± 0.01	0.03 ± 0.00
C 13:0	Tridecanoic acid methyl ester	0.02 ± 0.01	0.02 ± 0.00
C 14:0	Tetradecanoic acid methyl ester	23.70 ± 0.05	24.1 ± 0.01
C 14:1	9-Tetradecenoic acid methyl ester	0.69 ± 0.11	0.13 ± 0.04
C 15:1	Pentadecanoic acid methyl ester	0.07 ± 0.03	0.06 ± 0.00
C 16:0	Hexadecanoic acid methyl ester	0.23 ± 0.02	0.63 ± 0.25
C 16:1	9-Hexadecenoic acid methyl ester	7.49 ± 0.21	5.22 ± 0.17
C 17:0	Heptadecanoic acid methyl ester	0.09 ± 0.04	0.11 ± 0.07
C 17:1	(*Z*)-10-Heptadecenoic acid methyl ester	0.11 ± 0.00	0.09 ± 0.00
C 18:0	Octadecanoic acid methyl ester	3.72 ± 0.08	6.25 ± 0.24
C 18:1	Oleic acid methyl ester	47.08 ± 0.89	46.98 ± 0.56
C 18:2	Linoleic acid methyl ester	15.49 ± 0.64	14.71 ± 0.36
C 18:3	γ-Linolenic acid methyl ester	0.22 ± 0.07	0.15 ± 0.00
C 18:3	Linolenic acid methyl ester	0.42 ± 0.2	0.53 ± 0.08
C 20:0	Eicosanoic acid methyl ester	–	0.08 ± 0.01
C 20:1	(*Z*)-11-Eicosenoic acid methyl ester	–	0.55 ± 0.00
C 22:2	(*Z*)-11,14-Eicosadienoic acid methyl ester	–	0.08 ± 0.01
C 23:3	(*Z*)-8,11,14-Eicosatrienoic acid methyl ester	–	0.12 ± 0.05
C 24:2	(*Z*)-13,16-Eicosadienoic acid methyl ester		0.05 ± 0.01
∑SFA		28.0	31.5
∑MUFA		55.4	52.9
∑PUFA		16.7	15.6

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

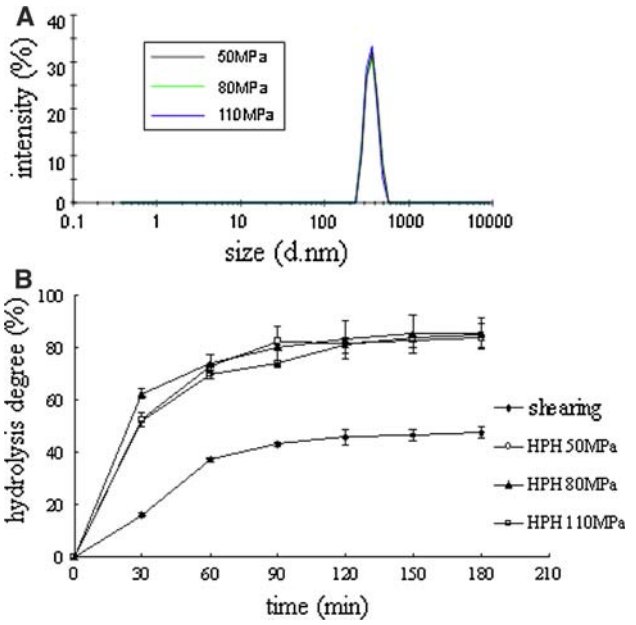

Fig. 1 Time course of hydrolysis of emulsified chicken fat prepared with different methods (**a**) and the particle size distributions of chicken fat emulsion prepared with HPH under different pressures (**b**)

different pressures, their hydrolysis efficiencies were obviously different as expected. However, the hydrolysis processes within 180 min did not vary significantly with the increase in pressure, which were consistent with the effect of pressure on the droplet diameters. Even though HPH enhanced the degree of hydrolysis compared with the strong shearing process, it did not exhibit any significant influence beyond 50 MPa, and therefore the HPH pressure was fixed at 50 MPa in the following optimization trials.

Fitting the Model

The degree of hydrolysis of chicken fat, the yields of free linoleic and oleic acids produced from all the hydrolysis trials are listed in Table 2. The experimental data were used to calculate the coefficients of the second-order polynomial equations, and the regression coefficients obtained are summarized in Table 3. The results from analysis of variance (ANOVA) showed that the resultant second-order polynomial models adequately represented the experimental data, of which the coefficient of multiple determinations (R^2) for the responses of the degree of hydrolysis, yields of free linoleic and oleic acids were 0.94, 0.93 and 0.88, respectively.

ANOVA was used to evaluate the significance of the model (Table 3). For each parameter in the model, a small P value indicates a significant effect on response variables. Thus all the linear terms except the shaking rate showed significant effects on the degree of hydrolysis, yields of free oleic and linoleic acids ($P < 0.05$). The linear variable with the most significant effect on all of the three response values was temperature ($P < 0.01$), followed by the reaction time and the enzyme loading. For the quadratic terms, besides the significant effect of quadratic terms of all parameters on the degree of hydrolysis, the significant effect of temperature, reaction time as well as enzyme loading on free linoleic acid yield was also observed in the model. However, only the quadratic terms of temperature revealed a significant effect on the yield of free oleic acid. Furthermore, two interactive terms presented significant effects on the free linoleic acid yield, namely, the interactions between enzyme loading and reaction time ($P < 0.05$) as well as enzyme loading and shaking rate ($P < 0.05$).

Analysis of Response Surfaces

The effect of independent variables was visualized by varying two variables within the experimental range while holding the other two at constant values at the central point. Figure 2a is a response surface plot indicating the effect of reaction temperature and time on the degree of hydrolysis of chicken fat at a fixed enzyme loading of 0.4% and with a shaking rate of 100 rpm. Reaction time had a positive linear effect on the degree of hydrolysis when the temperature increased from 35 to 40 °C. For the temperature higher than 44 °C, the negative quadratic effect of temperature on the degree of hydrolysis became significant ($P < 0.05$). This is possibly due to the decrease of the free lipase activity during the reaction period. At low temperature, the rate of enzyme heat-inactivation was slower in comparison with the rate of the enzyme catalyzed reaction. At high temperature, the increased heat-inactivation rate led to a faster decrease in the number of active catalyst molecules [18]. Reaction time had a positive linear effect ($P < 0.05$) on the degree of hydrolysis for the designed temperature range. However, further an increase in reaction time resulted in little increase in the degree of hydrolysis (P value of negative quadric effect of reaction time <0.05). This phenomenon could be probably explained as: during hydrolysis of chicken fat, the existed fatty acids might form a film covering the outer surface of the free lipase and such film acted as a barrier for chicken fat to enter the active site of the enzyme [19].

Figure 2b illustrates the effect of the enzyme loading and the shaking rate on the degree of hydrolysis while maintaining the temperature at 42.5 °C and the reaction time at 60 min. It can be seen that the enzyme loading had a significant positive linear effect ($P < 0.05$) on the degree of hydrolysis when the enzyme loading was lower than 0.5%. For a loading over this critical value, the negative

Table 2 Experimental data for the degree of hydrolysis of chicken fat, yields of free oleic and linoleic acids obtained from the central composite experimental design

Experimental number[a]	Temperature-X_1 (°C)	Enzyme-X_2 loading (%)	Shaking rate-X_3 (rpm)	Time-X_4 (min)	The degree of hydrolysis-Y_1 (%)	Linoleic acid-Y_2 (g/g fat)	Oleic acid-Y_3 (g/g fat)
1	50.0	0.60	30	50	16.8	0.017	0.077
2	42.5	0.40	60	23	61.3	0.085	0.298
3	50.0	0.60	30	150	13.5	0.026	0.102
4	50.0	0.20	30	150	4.7	0.009	0.026
5	50.0	0.20	90	50	12.5	0.017	0.060
6	35.0	0.60	30	50	61.1	0.102	0.255
7	30.9	0.40	60	100	75.5	0.085	0.332
8	50.0	0.60	90	50	37.4	0.034	0.145
9	42.5	0.09	60	100	31.1	0.051	0.145
10	42.5	0.40	14	100	36.4	0.038	0.136
11	42.5	0.40	106	100	94.9	0.117	0.510
12	42.5	0.40	60	100	84.5	0.111	0.425
13	35.0	0.20	30	50	20.2	0.026	0.068
14	50.0	0.20	30	50	6.35	0.009	0.043
15	50.0	0.60	90	150	23.8	0.026	0.077
16	35.0	0.60	90	150	78.8	0.085	0.315
17	35.0	0.20	30	150	40.1	0.068	0.153
18	35.0	0.20	90	150	73.2	0.094	0.332
19	42.5	0.40	60	100	82.5	0.102	0.391
20	54.2	0.40	60	100	8	0.009	0.034
21	50.0	0.20	90	150	8.5	0.068	0.255
22	42.5	0.40	60	178	81.2	0.102	0.383
23	35.0	0.20	90	50	65.4	0.094	0.298
24	42.5	0.40	60	100	81.1	0.094	0.374
25	42.5	0.71	60	100	97.1	0.106	0.493
26	35.0	0.60	30	150	70.5	0.085	0.289
27	35.0	0.60	90	50	77.5	0.111	0.391

[a] Experiments were performed in random order

quadratic effect became important ($P < 0.05$). Little enhancement in the degree of hydrolysis attributable to the enzyme increase was observed, which could be explained by the fact that the enzyme had saturated the interface between the oil and water [12].

As illustrated in Fig. 2b, an increase in the shaking rate which was slower than 100 rpm led to a rise in the degree of hydrolysis although the positive linear effect was not significant. It could be due to the fact that the enzyme located at the interface produced a faster rate of reaction than that taken from the bulk [11]. The faster shaking rate promoted the dispersion of enzyme toward the interface. Conversely, the significant negative quadratic effect ($P < 0.05$) of the shaking rate on the degree of hydrolysis was observed. Further an increase in the shaking rate led to an evident decrease in the degree of hydrolysis. A similar result was also obtained for the hydrolysis of grease catalyzed by free lipase [5].

As the most two valuable fatty acids in the chicken fat, the yields of free oleic acid and linoleic acids (g/g fat basis) during the enzymatic hydrolysis were investigated.

Figure 3 shows the effect of the reaction temperature and time on the yields of free oleic and linoleic acids produced during the hydrolysis with an enzyme loading of 0.4% and a shaking rate of 100 rpm. Linear terms of temperature showed a significant ($P < 0.05$) influence on the yields of free oleic and linoleic acids. Along with the increase of temperature from 35 to 38 °C, the yields of free oleic and linoleic acids went up to 0.465 and 0.116 g/g fat, respectively. For the temperature range over 38 °C, the negative effect of the quadratic term of temperature on these two responses was observed, as a further increase in

Table 3 Analysis of variance and the regression coefficient of the quadratic equation for the degree of hydrolysis of chicken, yields of free oleic and linoleic acids

Variable[a]	The degree of hydrolysis (%)		Linoleic acid (g/g fat)		Oleic acid (g/g fat)	
	Regression coefficient	*P* value	Regression coefficient	*P* value	Regression coefficient	*P* value
a_0	−707.4283		−0.769		−4.49814	
Linear						
a_1	24.1647	<0.0001	0.03295	<0.0001	0.19265	<0.0001
a_2	355.6896	0.0003	0.59929	0.0185	2.81891	0.0078
a_3	1.2109	0.3758	0.00014	0.2836	0.00466	0.2793
a_4	2.6653	0.0006	0.00274	0.0007	0.01579	0.0006
Quadratic						
a_{11}	−0.3485	<0.0001	−0.0004	0.0002	−0.00234	0.0002
a_{22}	−257.1481	0.0087	−0.254	0.0417	−1.36250	0.0524
a_{33}	−0.0029	0.0467	−0.000002	0.3924	−0.00002	0.1519
a_{44}	−0.0106	0.0127	−1.170	0.0361	−0.00006	0.0562
Interaction						
a_{12}	−1.2205	0.5226	−0.00425	0.1171	−0.01583	0.2893
a_{13}	−0.0101	0.1966	0.0000085	0.4151	0.000006	0.9134
a_{14}	−0.0172	0.1871	−0.000005	0.7832	−0.00006	0.5219
a_{23}	−0.1757	0.5392	−0.00085	0.0439	−0.00323	0.1571
a_{24}	−0.3371	0.4810	−0.00142	0.0439	−0.07226	0.0658
a_{34}	−0.0014	0.4748	−0.0000007	0.7832	−0.000002	0.8996
R^2	0.94		0.93		0.88	
R^2 (adj)	0.88		0.84		0.75	

[a] a_0 is a constant, a_i, a_{ii}, and a_{ij} are the linear, quadratic and interactive coefficients of quadratic polynomial equations, respectively

the temperature lowered the yields of FFA. For both free oleic and linoleic acids, the yields increased linearly with the extension of the reaction time, which was reflected by the *P* value (<0.05) of linear terms of reaction time. However, while the reaction time was extended, the increase in the yields became slow due to the negative effect of the quadratic of the reaction time. The effect of enzyme loading on the free oleic and linoleic acids yields closely resembled the effect of reaction time. The plot in Fig. 4 shows the effect of the enzyme loading and the shaking rate on two responses when holding the temperature at 42.5 °C and the reaction time at 60 min. Linearly increasing yields of free oleic and linoleic acids were observed along with the higher enzyme loading. More free oleic and linoleic acids were not detected in the model when the enzyme loading was higher than 0.55%. Moreover, the shaking rate had the least important effect on free oleic and linoleic acids yields (*P* value of linear and quadratic terms of shaking rate >0.05, Table 3).

In general, it can be seen that the effect of linear and quadratic terms of the factors on the yields of oleic and linoleic acids were very similar to that on the degree of hydrolysis. This phenomenon was probably due to the fact that the Lipomod-34P adopted in the experiment, *Candida cylindracea* lipase, did not possess hydrolysis selectivity toward different fatty acids in the chicken fat [2].

However, another two interactions (enzyme loading and shaking rate, as well as enzyme loading and reaction time) on the linoleic acid were found. The interaction effect between the enzyme loading and the shaking rate on the yield of linoleic acid can be clearly seen in Fig. 4b. At low enzyme loading levels, the yield increased with the rise of the shaking rate mostly for the same reason as that of the shaking rate on the degree of hydrolysis. On the other hand, when the enzyme loading was higher than 0.55%, an opposite effect of the shaking rate on the linoleic acid yield was found, where a slight reduction in yield was revealed with a rising shaking rate.

Optimization of the Hydrolysis Conditions of Chicken Fat

The hydrolysis parameters could be considered as optimum if the yield of free oleic and linoleic acids simultaneously reached their highest values by using the desirability function approach according to Liao et al. [20]. The yields

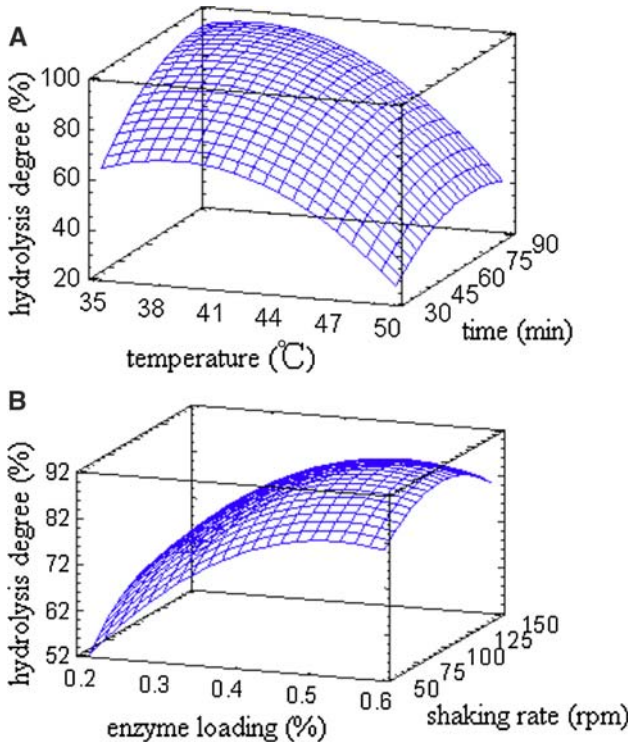

Fig. 2 Effect of temperature and time on the degree of hydrolysis at a fixed enzyme loading of 0.6% and a shaking rate of 100 rpm **a** and effect of enzyme loading and shaking rate on the degree of hydrolysis at the fixed temperature of 42.5 °C and time of 60 min **b**

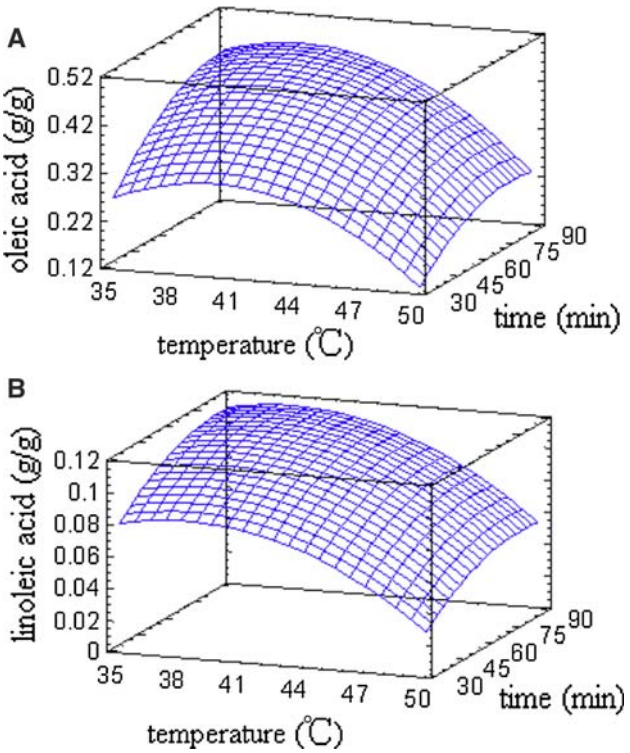

Fig. 3 Effect of temperature and time on the yields of free oleic acid (**a**) and linoleic acid (**b**) at a fixed enzyme loading of 0.4% and a shaking rate of 100 rpm

of oleic and linoleic acids were converted into an individual desirability and given the equal important values of 5. The desirability values scale ranged from 0 to 1, and the yield was expected as being as high as possible. The two individual desirability functions from the oleic and linoleic acids yields were combined to obtain the overall desirability, defined as the geometric average of individual desirability.

The highest overall desirability of 0.96 was obtained at a temperature of 38 °C and a reaction time of 82 min along with a shaking rate of 100 rpm and an enzyme loading of 0.48%. Under the optimum condition, the predicted yields of free oleic acid and linoleic acid were 0.470 and 0.118 g/g hydrolysate with recoveries of 94.6 and 93.7%, respectively. Compared with chicken fat, a similar composition of released FFA at optimal condition was observed (Table 1), however, for the long chain fatty acids (>C_{18}), the content in the sample was under the analysis limit. This might be due to the selectivity of lipase toward comparative short chain fatty acids (according to the instructions of Lipomod-34P) and because of the low content of long chain fatty acids in the chicken fat, the composition of other fatty acids in the released FFA did not vary remarkably after the hydrolysis.

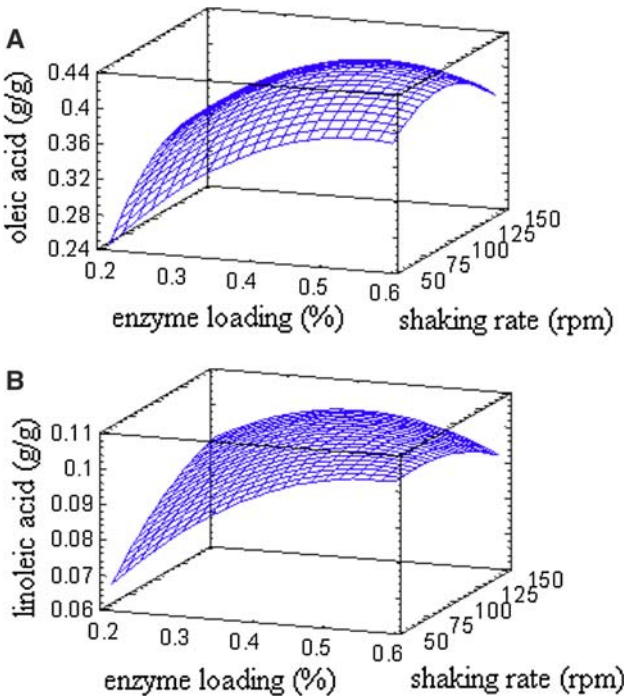

Fig. 4 Effect of enzyme loading and shaking rate on the yields of free oleic acid (**a**) and linoleic acid (**b**) at a fixed temperature of 42.5 °C and a time of 60 min

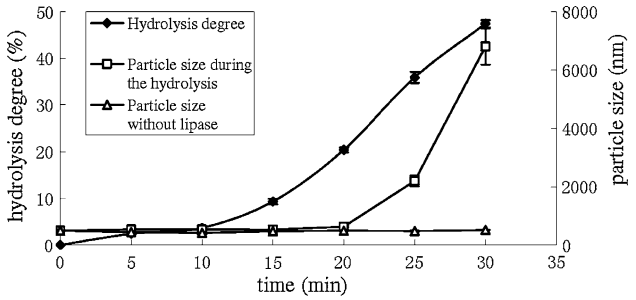

Fig. 5 The degree of hydrolysis and particle size change during the enzymatic reaction at temperature of 38 °C, enzyme loading of 0.48% and shaking rate of 100 rpm

Aggregation of Droplets of Chicken Fat During the Hydrolysis

The change in the degree of hydrolysis and the particle size during the hydrolysis process under the optimal condition was indicated in the Fig. 5. Only a marginally increase in the degree of hydrolysis in the first 15 min can be seen. However, a sharp rise was observed thereafter from 10 to nearly 50% in the next 15 min. For the particle size change of droplet during the hydrolysis progress, although the similar overall phenomenon is shown in the same graphic, the change did not accurately followed the degree of hydrolysis increase.

The lipase catalyzed the reaction at the interface of chicken fat droplets and water, and the products including FFA mono- and diglycerides are surface-active compounds which tend to accumulate at the interface. This could explain the reason no significant change was detected in the droplet diameter within the first 15 min in spite of the degree of hydrolysis of 10%. The droplet diameter became larger by about 100 nm with an increase of 10% in the degree of hydrolysis between 15 and 20 min. During this period, more products could saturate the interface and those surface-active compounds began to dissolve in the water phase [10]. Due to the high concentration of chicken fat (50%) in the system, those products in water began to aggregate once the hydrolysis above critical degree. With the increase of the hydrolysis, the particle size of chicken fat in the emulsion escalated sharply, and the emulsion was separated into oil and water layers at the end of the hydrolysis under the optimal condition. Although those products could act as emulsifiers, the stable emulsion was not able to be formed without HPH and the particle size began to increase with the aggregation of more products in the water phase. More products released from the interface aggregated and formed bigger droplet of oil phase (achieving 6800 nm) when the degree of hydrolysis reached almost 50%. The particle distribution index of such an emulsion was 1 after 30 min, which means that the system was not stable and was able to separate into two different layers without shaking. Therefore, the particle size was not analyzed any longer for such a hydrolyzed emulsion. A control trial was carried out under exactly the same condition without lipase in order to verify that the change of the particle size was caused by the enzymatic hydrolysis rather than factors such as temperature or shaking (Fig. 5).

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