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Article

Enzymatic Hydrolysis of Salmon Oil by Native Lipases: Optimization of Process Parameters

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Três lipases microbianas nativas (*Aspergillus niger, Rhizopus javanicus* e *Penicillium solitum*) foram utilizadas na hidrólise do óleo de salmão (teor de AGPI *n*-3 de 30,1%) com o objetivo de concentrar o conteúdo de ácidos graxos poliinsaturados *n*-3 (AGPI *n*-3) nos acilgliceróis residuais. A metodologia de planejamento experimental e análise de superfície de resposta foi usada para se chegar às condições otimizadas de cada reação enzimática, utilizando as seguintes variáveis; temperatura (X_1), quantidade de lipase (X_2) e taxa de água/óleo (X_3). Com base nos resultados do planejamento, a lipase de *Aspergillus niger* foi a mais eficiente na concentração dos AGPI *n*-3, sendo que as condições ótimas de reação foram: concentração de enzima de 500 U g^{-1} óleo, temperatura 45 °C e taxa de água/óleo de 2:1 m/m após 24 h de reação. O grau de hidrólise (60%) conduziu a um aumento do conteúdo de ácido docosahexaenóico (DHA) de 14,4% para 34,0% (enriquecimento de 2,4 vezes) nos acilgliceróis residuais após a hidrólise do óleo de salmão.

In an attempt to concentrate the content of *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) in the residual acylglycerol, salmon oil (*n*-3 PUFA content of 30.1%) was hydrolyzed with three kinds of native microbial lipases (*Aspergillus niger*, *Rhizopus javanicus* and *Penicillium solitum*). For each lipase, a response surface methodology was used to obtain maximum PUFA content and to optimize the parameters of enzymatic reactions with respect to important reaction variables; temperature (X_1), amount of lipases (X_2) and water/oil ratio (X_3). Based on these results, optimal reaction conditions were established. *Aspergillus niger* lipase was the most effective in concentrating *n*-3 PUFA. The degree of hydrolysis (60%) led to an increase in the docosahexaenoic acid (DHA) content from 14.4% in the original oil to 34.0% (2.4-fold enrichment) in the residual acylglycerol under optimum conditions: enzyme concentration of 500 U g^{-1} oil, reaction temperature of 45 °C and water/oil mass rate of 2:1 (m/m) after 24 h reaction.

Keywords: polyunsaturated fatty acid, enzymatic hydrolysis, lipases, hydrolysis degree, experimental design, salmon oil

Introduction

The *n*-3 polyunsaturated fatty acids (PUFA) are commonly known to play an important role in human health. Eicosapentaenoic acid (C20:5 *n*-3, EPA) and docosahexaenoic acid (C22:6 *n*-3, DHA) have been shown to be of major importance in the prevention of a number of diseases, including coronary heart disease, inflammation, hypotriglyceridemic effect and diabetes.[1-5] During the last decade, several studies have shown positive effects of fish oils on cognitive development and vision enhancement in newborns, as well as in young children.[6] For medical or dietetic purposes, *n*-3 PUFA may be administered in different forms; as free fatty acids (FFA), as ethyl esters, or as acylglycerols. Some studies indicate that PUFA are most promptly absorbed from the intestines when FFA is given orally, are moderately absorbed as acylglycerols and are poorly absorbed as PUFA ethyl ester. However, free *n*-3 PUFA is oxidized most easily and, moreover, free *n*-3 PUFA is unacceptable as a food. Therefore, acylglycerols are considered to be the most desirable chemical form as food.[7] Lipases are known to catalyze hydrolysis reactions and have been shown as a good alternative for obtaining PUFA concentrates as acylglycerols.[8-11] Lipase-catalyzed enzymatic production of EPA and DHA concentrates from fish oil has shown potential in producing a high quality

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product, due to the mild conditions (e.g., neutral pH and low temperatures) of the process.[12]

Commercial lipases from *Candida rugosa* (*cylindracea*), *Geotrichum candidum, Humicola lanuginose, Chromobacterium viscosum*, and *Rhizomucor miehei* have been largely used for these purposes.[8-11,13,14] Few investigations have been carried out to exploit the kinetic properties and applications for native microbial lipases isolated from natural sources.[15-17]

In this paper, we evaluate the catalytic performance of three native lipases produced in our laboratory from *Aspergillus niger, Rhizopus javanicus* and *Penicillium solitum* for production of a salmon oil concentrate. In addition, a preliminary optimization to determine the optimal processing parameters for the hydrolysis degree of selected lipases was carried out. Three-factor response surface were employed to optimize the reactive conditions of lipase-catalyzed hydrolysis in order to obtain a maximum hydrolysis degree and acylglycerols rich in *n*-3 PUFA.

The method of process optimization by the response surface methodology is a faster and more economical method for gathering research results than the classical one-variable-at-a-time or full-factorial methods.[18,19]

Experimental

Chemicals and oil

Peptone and yeast extract were purchased from Difco Laboratories, (Detroit, MI, USA). The components for culture media, chemical reagents and other solvents were obtained from Merck (Darmstadt, Germany) and from Sigma-Aldrich Chemical Co. in the highest purity available. Low acidity olive oil was purchased at a local market. The salmon oil (saponification value, 185; acid value, 0.05) was purchased from Campestre Ltda (São Bernardo do Campo, São Paulo, Brazil).

Production and determination of lipases activities

This study was performed using *Aspergillus niger, Rhizopus javanicus* and *Penicillium solitum* lipases produced in our laboratory; these lipases achieved better kinetic properties in previous experiments.[15,17] The lipases were produced in a basal medium with an initial pH value of 6.0 and consisted of 2% (m/v) peptone, 0.5% (m/v) yeast extract, 0.1% (m/v) $NaNO_3$, 0.1% (m/v) KH_2PO_4, 0.05% (m/v), $MgSO_4.7H_2O$ and 2% (m/v) olive oil. Cultures were grown in Erlenmeyer flasks (500 mL) containing 120 mL of the growth medium. The cultures were inoculated with 1mL of spore suspension (10^5-10^6 spores mL^{-1}) and the flasks were stirred on a rotary shaker (130 rpm) at 35 °C for 72 h. After this period, the cultures were filtered and the supernatants were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed in water and lyophilized for use as extracellular crude lipase preparations in powder form. The residual water in the lyophilized lipases was 0.2-0.3% (m/m). Lipase activity were quantified by triolein using olive oil as substrate employing a standard oleic acid curve.[20] One unit (U) is defined as 1 µmoL of oleic acid released *per* minute of reaction. Lipases from *A. niger* with 7.5 U, *R. javanicus* with 6.8 U and *P. solitum* with 4.8 U were used. These lipases were used without further purification.

Hydrolysis reaction and experimental design

Three experimental designs, based on the 2^3 full factorial, were performed under the same experimental conditions for each lipase produced (*A. niger*, *R. javanicus* and *P. solitum*) in order to verify the power of hydrolysis of each native lipase using salmon oil. Reaction mixtures for enzymatic hydrolysis were carried out according to the experimental conditions in Table 1. Conical flasks (250 mL) were blanketed with nitrogen and kept sealed throughout the reaction for 48 h. The vessels were stirred in an incubator orbital shaker at 400 rpm at different temperatures. Different ratios of water (0.1 mol L^{-1} phosphate buffers) and salmon oil were mixed in the flasks and different amounts of lipase preparations (U of the enzyme g^{-1} of oil) were added. The initial water/oil amount was fixed at 30 g for all experiments. Statistical optimization experiments for all lipases were carried out according to the 2^3 full factorial designs with 4 center points. The independent variables or factors studied were temperature (°C; X_1), amount of lipases (U; X_2) and water/oil mass ratio (m/m; X_3). Response for the dependent variable studied was the degree of hydrolysis (DH) (%). The design matrix for *A. niger*, *R. javanicus* and *P. solitum* lipases shown in Table 2 was obtained by means of the Statistica 7.0 software. Significance of data was tested using the ANOVA statistical test. The experiments for all lipases were accomplished at four different times (6, 12, 24 and 48 h) in order to observe the kinetic behavior of each reaction using

Table 1. Experimental conditions

	Variables	Coded variable levels		
		-1	0	+1
X_1	T / °C	35	40	45
X_2	Enzyme (U/g)	100	300	500
X_3	Water/oil mass rate (m/m)	2:1	1:1	1:2

the different enzymes. Enzymes were deactivated by heating the reaction to approximately 90 ºC for 15 min in order to interrupt the reaction at a determined time.

Separation of reaction products

At the end of each enzymatic reaction, partially hydrolyzed oil containing residual acylglycerols (mono-, di- and triacylglycerols) and released fatty acids fractions were separated by the method described by Shimada *et al.*[8] After hydrolysis by *Aspergillus niger* lipase, the yield of fatty acids isolated (g) *per* oil (g) was 0.5-0.7.

Determination of degree of hydrolysis (DH)

Degree of hydrolysis (DH) was determined by measuring the acid value of both unhydrolyzed (original oil) and hydrolyzed oil at different treatment times (6, 12, 24 and 48 h) and the saponification value of unhydrolyzed oil, according to the American Oil Chemists' Society methods.[21] Blanks (no enzyme) were determined for each treatment. DH was calculated according to the following equation:

$$\text{DH (\%)} = \frac{\text{acid value (hydrolyzed oil} - \text{blank at each condition)}}{\text{saponification value (original oil)} - \text{acid value (original oil)}} \times 100$$

Acid values indicate relative amount of free fatty acids released by the hydrolysis reaction, as measured by the mg of potassium hydroxide necessary to neutralize fatty acids in 1 g of salmon oil.

Determination of fatty acid composition

Fatty acids in unhydrolyzed salmon oil, residual acylglycerols and liberated fatty acid fractions were converted after hydrolysis into fatty acid methyl esters (FAME), according to the method of Hartman *et al.*,[22] and their compositions were determined by gas chromatography (GC). A Chrompack gas chromatograph (CP-9001) equipped with a flame ionization detector and a CP-Sil 88 capillary column (Chrompak, WCOT Fused Silica 50 m × 0.25 mm i.d.; split ratio, 100:1) were used for analyzing FAME. The initial temperature of the column was 50 °C for 2 min and then the temperature was increased at a rate of 10 °C min^{-1} up to 210 °C; hydrogen was used as a carrier gas. The temperature of the injector was 220 °C and the detector's temperature was 250 °C. Fatty acids were identified by comparing the times of retention of the components present in each sample with the times of retention of the components present in the standard FAME Mix C4-C24 (Supelco).

Results

Degree of hydrolysis

A three-factor response surface methodology (RSM) with 4 center points was employed to study the degree of hydrolysis (DH) by lipase-catalyzed synthesis of salmon oil for each native lipase. Experimental results of DH for catalyzed-hydrolysis reactions with salmon oil for each RSM using *A. niger*, *R. javanicus* and *P. solitum* lipases are given in Table 2. All the reactions were accomplished at four different times (6, 12, 24 and 48 h), although only results for the 48 h reaction were analyzed statistically.

DH´s of salmon oil from different lipases, conditions of run and reaction times are shown in Figure 1. In general, salmon oil was hydrolyzed rapidly during the initial 12 h. Afterwards, hydrolysis was more gradual, and only

Table 2. Three-factor designs and responses for lipases from *Aspergillus niger, Rhizopus javanicus* and *Penicillium solitum*

Run	Coded value			Experimental value (DH / %)[a]		
	X_1	X_2	X_3	*A. niger*	*R. javanicus*	*P. solitum*
1	–1	–1	–1	19.2	12.9	2.8
2	+1	–1	–1	21.9	6.2	1.9
3	–1	+1	–1	44.1	29.7	5.0
4	+1	+1	–1	57.2	20.2	3.0
5	–1	–1	+1	4.6	7.6	3.4
6	+1	–1	+1	4.7	5.3	1.0
7	–1	+1	+1	6.6	9.6	2.7
8	+1	+1	+1	7.5	6.9	1.8
9	0	0	0	12.8	7.2	3.5
10	0	0	0	17.4	7.6	3.6
11	0	0	0	15.8	7.4	2.8
12	0	0	0	14.8	8.0	3.7

[a]DH = degree of hydrolysis after 48 hours of reaction.

minor increases were shown throughout the 24 and 48 h time periods. *A. niger* was the lipase that most effectively hydrolyzed salmon oil (*ca.* 60% of hydrolysis after 24 h under experimental conditions).

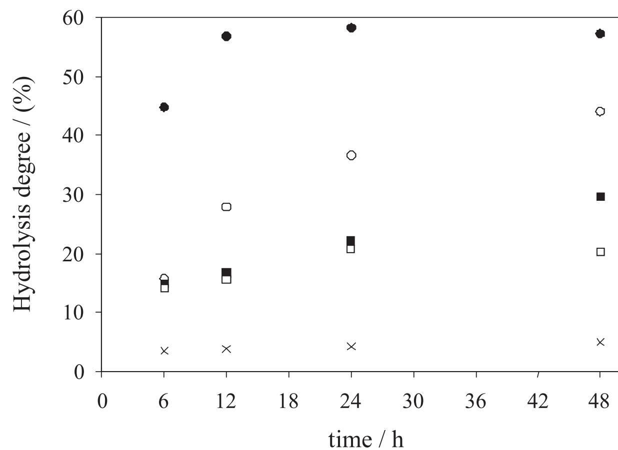


Figure 1. Hydrolysis degree as a function of time (h) for the lipases in different conditions: (●) *Aspergillus niger* - run 4; (○) *Aspergillus niger* - run 3; (■) *Rhizopus javanicus* - run 3; (□) *Rhizopus javanicus* - run 4; (×) *Penicillium solitum* - run 3.

Table 3 depicts the analyses of the reaction parameter effects for the DH when the *A. niger* lipase was used. The concentration of the *A. niger* lipase, the water/oil mass ratio and the interaction between these parameters significantly affected the DH. Moreover, temperature did not affect the degree of hydrolysis, nor the interactions that involved this parameter. Therefore, these results suggest a linear model whose parameters are mainly X_2, X_3 and X_2X_3. The coefficients of independent variables determined for the linear model for the DH, when the *A. niger* lipase was used, are given in equation 1.

$$DH_{(AN)} = 18.33 + 8.12 \times X_2 - 14.87 \times X_3 - 6.92 \times X_2X_3 \quad (1)$$

The analysis of variance for the DH model is given in Table 4. Based on the *F*-test, the model (equation 1) is predictive, since the percent of explained variance is high (93.24%) and the calculated *F*-value (regression/residual) is more than 4 times higher than the critical *F*-value at 95% of confidence ($F > F_{0.95,4.7}$ of 6.09). In addition, there is little evidence of a lack of fit for the fitted model, since the calculated *F*-values (lack of fit/pure error) are lower than the critical *F*-value ($F < F_{0.95,3.2}$ of 19.16) at 95% confidence.[23]

The same analysis of effects of parameters and analysis of variance for *R. javanicus* and *P. solitum* lipases were studied (Tables 5 and 6). Observing the percent of explained variance (R^2) and *F*-test, the results demonstrate that only

Table 3. Statistical analysis of the operating variables effects for the *Aspergillus niger* lipase on DH

Factor	Effect	SE[a]	*t*-Value	*p*-Value
X_1	4.20	3.2255	1.3021	0.24963
X_2	16.25	3.2255	5.0379	0.00397[b]
X_3	-29.75	3.2255	-9.2232	0.00025[b]
$X_1 X_2$	2.80	3.2255	0.8680	0.42505
X_1 X3	-3.70	3.2255	-1.1470	0.30325
$X_2 X_3$	-13.85	3.2255	-4.2938	0.00776[b]

[a]Standard Error; [b]significant factors (*p*-value < 0.05).

Table 4. Analysis of variance (ANOVA) to fit the model presented in equation 1

Source	Sum of squares	Degree of freedom	Mean squares	F-value
Regression	2681.89	4	670.47	25.73
Residues	182.38	7	26.05	
Lack of fit	171.26	2	34.25	9.24
Pure error	11.12	3	3.70	
Total	2864.27	11		

R^2 = 0.93 %; explained variance: 93.24%.

a first-order polynomial model does not represent the behavior of lipase-catalyzed reactions for these lipases, since the high lack of fit of the models and the regression of the parameters are not significant statistically. Whilst the results of the lipases from *R. javanicus* and *P. solitum* were not statistically significant enough to predict a model, they might be used to observe the behavior of the reactions in function of the parameters studied (temperature, amount of enzyme and water/oil mass ratio) when these native lipases were employed and compared with others in relation to the hydrolysis performance of each.

The next results show the hydrolysis performances of the lipases and their effects on the enzymatic reactions when submitted to different experimental conditions. Figure 2 illustrates the variation of the DH when, simultaneously, the concentration of lipase and the temperature are analyzed. For *A. niger* lipase (Figure 2 (a)), practically no influence of the temperature on the response was observed. On the other hand, the increase in temperature decreases the DH for the native lipases from *R. javanicus* and *P. solitum*, as can be seen in Figures 2 (b) and 2 (c).

Figure 3 shows that, although the three native lipases have differences in the DH (%), the same behavior was observed in relation to the concentration of lipases and ratio of water and oil in order to increase the DH. Higher DH is reached, increasing the concentration of lipases and increasing the amounts of water in the reaction, simultaneously.

Table 5. Statistical analysis of the operating variables effects for the *Rhizopus javanicus* and *Penicillium solitum* lipase on the DH

	R. javanicus			*P. solitum*		
	Effect	SE[a]	p-Value[b]	Effect	SE[a]	p-Value
X_1	10.71667	0.098601	0.000002	2.85000	0.117851	0.000155[b]
X_2	–5.30000	0.241523	0.000207	–1.80000	0.288675	0.008319[b]
X_3	8.60000	0.241523	0.000049	1.10000	0.288675	0.031777[b]
$X_1 X_2$	–9.90000	0.241523	0.000032	–1.20000	0.288675	0.025313
X_1 X3	–0.80000	0.241523	0.045314	0.35000	0.288675	0.312117
$X_2 X_3$	2.80000	0.241523	0.001378	–0.35000	0.288675	0.312117

[a]Standard Error; [b]significant factors (*p*-value < 0.05).

Table 6. ANOVA of regression parameters for the response surface model

	Sum of squares	Pure error	R^2	F-value ($F > F_{0.95;4;7}$)[a]	F-value ($F < F_{0.95;2;3}$)[b]
R. javanicus	570.80	0.35	0.89	14.56	19.16
P. solitum	16.81	0.50	0.76	5.72	10.30

$F_{0.95;4;7} = 6.09$; $F_{0.95;2;3} = 19.16$; [a]indicative of statistically significant regression; [b]evidence of lack of fit for the model.

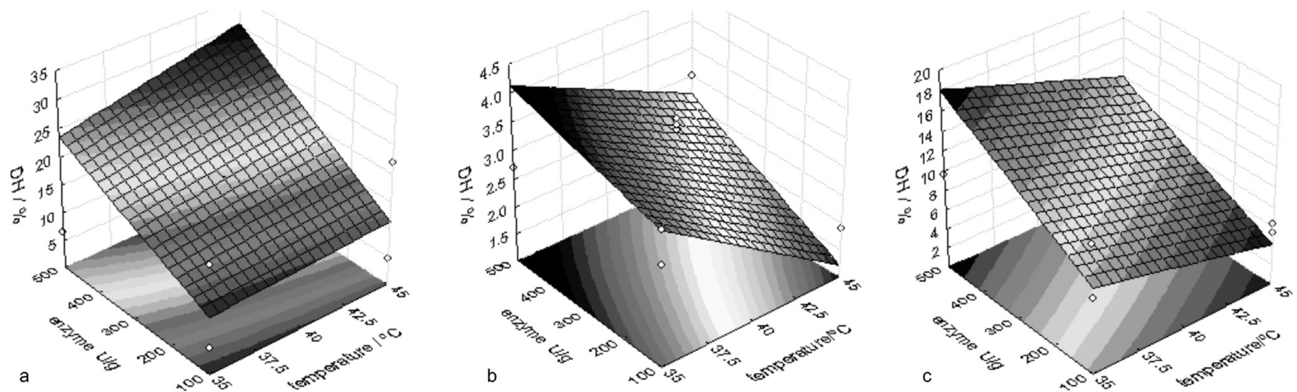


Figure 2. Varying concentrations of lipases and temperature as a function of DH for lipases: a) *Aspergillus niger*; b) *Penicillium solitum*; c) *Rhizopus javanicus*.

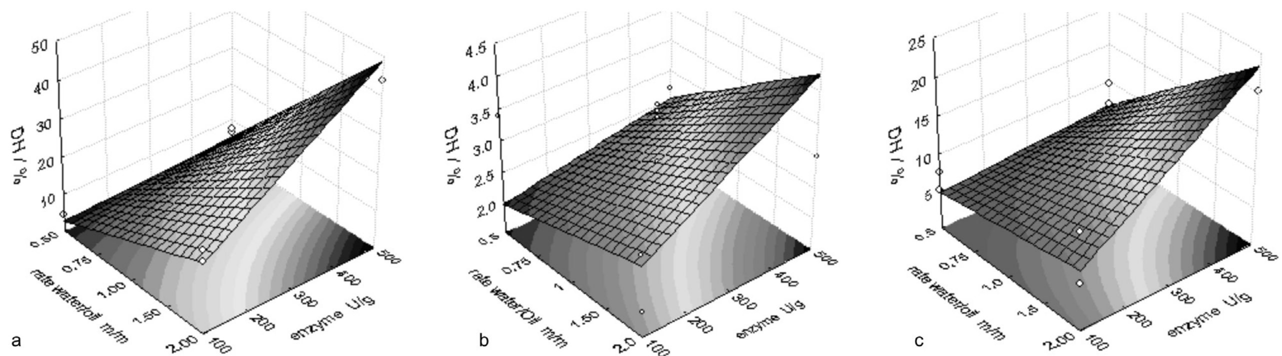


Figure 3. Varying water/oil mass ratio and concentrations of lipases as a function of DH: a) *Aspergillus niger*; b) *Penicillium solitum*; c) *Rhizopus javanicus*.

Table 7. Composition of the main fatty acids of the reaction product after 45 and 60% hydrolysis of salmon oil by *Aspergillus niger* lipase

		45% of hydrolysis[a]		60%[b] of hydrolysis[a]	
Fatty acids	Original oil	Hydrolyzed acylglycerol	Liberated fatty acids	Hydrolyzed acylglycerol	Liberated fatty acids
C16:0	9.4 ± 0.4	5.3 ± 0.8	15.8 ± 1.6	4.6 ± 0.5	14.4 ± 1.9
C16:1	4.3 ± 0.3	2.7 ± 0.4	7.9 ± 1.4	2.2 ± 0.3	6.8 ± 1.3
C18:0	3.5 ± 0.2	1.9 ± 0.8	4.3 ± 0.6	2.5 ± 0.3	4.3 ± 1.0
C18:1	15.3 ± 0.4	8.5 ± 0.4	18.8 ± 1.5	7.9 ± 2.1	16.8 ± 1.7
C20:5	15.7 ± 0.4	16.6 ± 1.4	10.8 ± 2.6	11.0 ± 1.7	17.3 ± 2.6
C22:6	14.4 ± 0.4	28.7 ± 2.5	5.1 ± 0.4	34.0 ± 2.6	3.9 ± 0.4
n-3 PUFA	30.1	45.3	15.9	45.0	21.2
DHA/EPA	0.9	1.7	0.5	3.0	0.2

[a] Corresponding to *ca.* 45% hydrolysis degree after 6 hours of reaction (run 4). [b] Corresponding to *ca.* 60% hydrolysis degree after 24 hours of reaction (run 4).

Fatty acid composition of the reaction products

The composition of the main fatty acids in the residual acylglycerol and liberated fatty acid fractions after hydrolysis of salmon oil with *A. niger* lipase are shown in Table 7.

After 45% hydrolysis (6 h of reaction), the total *n*-3 PUFA content of the residual acylglycerol fraction increased by approximately 1.5-fold, compared to the original oil, giving a final concentration of approximately 45%. Most of the enrichment was due to DHA, which increased two-fold, whereas the EPA content remained relatively constant with no significant differences, compared to the feed oil. This difference in selectivity is reflected in the increased DHA/EPA ratio.

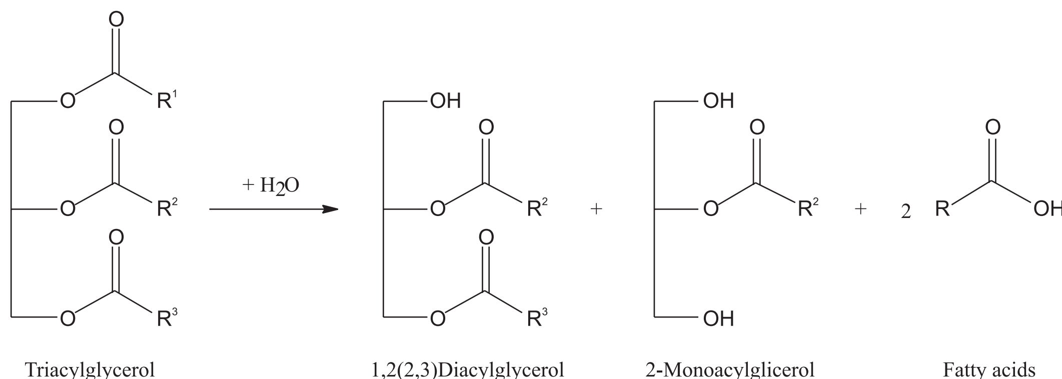
The enrichment of *n*-3 PUFA was compensated by a reduction in the content of C16-C18 saturated and monounsaturated fatty acids. The liberated fatty acid fractions were correspondingly increased in the C16-C18 saturated and monounsaturated fatty acids, with a great depletion in DHA, compared to the feed oil.

When the reaction reached 60% hydrolysis (24 h of reaction), the residual acylglycerol fraction were further enriched in DHA, however the EPA content was reduced. Total *n*-3 PUFA was similar to that found after 45% hydrolysis, although the DHA/EPA ratio reached approximately three at this stage.

Discussion

Salmon oil was submitted to hydrolysis by three 1,3-specific lipases from *A. niger*, *R. javanicus* and *P. solitum* to preserve the long chain *n*-3 PUFA, which are preferentially located at the 2 position on the glycerol backbone.[24] Due to the several *cis-cis* unsaturations of PUFA, these molecules are strongly bent, thus enhancing the steric hindrance effect. Therefore, the ester moiety of PUFA-containing acylglycerols is hardly split by lipase, in contrast to saturated and monounsaturated fatty acids (Scheme 1).

From the experimental design using native lipase from *A. niger*, it was possible to obtain a first order model to



Scheme 1. Hydrolysis of triacylglycerol catalysed by 1,3-specific lipases.

optimize the parameters of reaction in function of the DH of salmon oil. When lipase from *A. niger* was used, about 60% of hydrolysis is required after 24 hours of reaction to obtain a high content of DHA and a high DHA/EPA ratio in the unhydrolyzed fraction. Under these optimized conditions, the total *n*-3 PUFA content of the residual acylglycerol fraction was 45% (34% DHA + 11% EPA), approximately 2.4-fold greater than the initial values obtained with the non-optimized system.

In contrast, salmon oil was not hydrolyzed as much with the other lipases; the *R. javanicus* and *P. solitum* lipases achieved just 20% and 3% hydrolysis under the same conditions. The temperature significantly influenced the process, causing a 30-40% decrease in DH for both the *R. javanicus* and *P. solitum* lipases. A higher reaction temperature (45 ^{o}C) caused the denaturation of two lipases. In a previous report, we showed that lipase from *A. niger* was more thermostable than other native lipases, demonstrating stability at the range 30 and 50 ^{o}C and maintaining at least 60% of its hydrolytic activity at 60 ^{o}C after 1 h.[16] A probable explanation for the low lipases activities of *R. javanicus* and *P. solitum* is their enzyme structures, which do not accommodate the esters of PUFA from salmon oil in their active sites.

To our knowledge, this is the first finding of an enzymatic hydrolysis of salmon oil by native lipases. The commercial lipases from *Candida rugosa* (*cylindracea*) and *Geotrichum candidum* are useful for the production of oil containing high concentrations of PUFA. *Geotrichum candidum* lipase can enrich DHA and EPA and *Candida rugosa* can enrich DHA, but not EPA.[8] In general, the DH is higher in oil hydrolyzed by non-specific lipases (*Candida sp.*), compared with 1,3-specific lipases (*Mucor javanicus, Rhizopus delemar* and *Aspergillus niger*).[10,11,25] Tanaka *et al.*[25] reported that *Candida cylindracea* lipase shows low reactivity for DHA esters, and is successful in concentrating DHA in the residual acylglycerol fraction during the hydrolysis (*ca.* 70% rate) of fish oil. DHA was concentrated from 8.9% to 30.5% and EPA was changed from 13.3% to 9.8%, resulting in 40.3% of total *n*-3 PUFA in the residual acylglycerol fraction of the fish oil.

Some authors have reported that hydrolysis exceeding 50 hours might increase hydrolysis to around 90%.[26] In this study, a DH of 60% and an increase of 50% in *n*-3 PUFA were reached after 24 h and no further increase was noted following longer reaction times (data not shown).

Okada and Morrisey (2007)[11] investigated the production of *n*-3 PUFA concentrate from Pacific sardines using several commercial lipases. Oil hydrolyzed by *Aspergillus niger* lipase exhibited only a minor increase in DHA content (13.6% to 18.5% with 250 U after 1.5 h and to 18.7% with 500 U after 3 h) and no significant changes in EPA content.[11]

Our results clearly demonstrate the superiority of the lipase from *Aspergillus niger* in the enrichment of DHA by selective hydrolysis of salmon oil, which demonstrated to be a feasible method for the development of salmon oil health products. The experimental design procedure provided a powerful tool to optimize the hydrolysis conditions that permit an important improvement in the degree of hydrolysis of this lipase. Under optimum conditions, a good enrichment of DHA (2.4-fold) was achieved, which is considerably higher than those results previously reported using this lipase.

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