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SHORT COMMUNICATION

Hydrolytic Activity of Castor Bean Powder: Effect of Gum Arabic, Lipase and Oil Concentrations

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Abstract Lipase activity from castor bean seed powders was evaluated in hydrolysis reactions at 37 °C. The effects of different concentrations of lipase powder (LP), substrate (high oleic sunflower oil, *O*) and surfactant (gum arabic, *A*) on lipase activity (*R*) were assessed using experimental designs. Considered variable bounds were: 0.05–0.15 g_{LP}, 0.07–0.20 oil:aqueous phase (w/w) and 0–0.025 g gum arabic/mL. All variables had significant effects on the transformed response, $R^{1/2}$. The most important result was the negative effect of gum arabic in lipase activity, even when high oil concentrations were used. Experimental lipase activities involved in this work were within 0.32–16.90 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$. Using 0.05 g_{LP} and 0.20 oil:aqueous phase (w/w) without gum arabic, the activity of 20.47 ± 7.19 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$ was reached.

Keywords Castor bean lipase · Hydrolysis · Gum arabic · Response surface · Optimization

Introduction

Currently, plant lipases appear as an interesting alternative in oils and fats modification because of their wide availability, specificity and apparent low cost [1]. Several works using lipases extracted from castor bean seeds are available in the literature. Most of them investigate biological function, toxicity and structure of castor bean lipase; but only a few references have focused on its potential to be used as a commercial lipase in industrial processes such as modification of fats and oils [2–4]. Santos et al. [3] evaluated hydrolysis activity in lipase extract powders from castor bean and other plants in reactions where different substrates (oils) were emulsified using gum arabic. They determined optimal conditions with regard to reaction temperature, pH and buffer concentration. They also evaluated the reaction without using gum arabic, concluding that the measured decrease in activity could be attributed to the effects that the absence of an emulsifier could produce. Avelar et al. [4] studied the hydrolysis reaction catalyzed by the lipase extract powder from castor bean seeds, working at similar conditions that Santos et al. [3], without using an emulsifier. They optimized oil:buffer ratio, temperature and calcium chloride concentration. Although in both works the practical benefits of not using gum arabic in the reaction medium were stated, the effect of its concentration on the reaction performance was not studied. On the other hand, there are even less articles that use experimental designs and optimization to analyze castor bean lipase behavior under different conditions [3, 4]. These experiments are useful since they present advantages compared to changing one parameter at a time such as easier interaction identifications.

The aim of this work was to study castor bean seed lipase as a biocatalyst in hydrolysis reactions, in the form of lipase powder (LP), evaluating the presence of gum arabic as surfactant and its effect on reaction systems containing different substrate and LP concentrations. High oleic sunflower oil was used as substrate and gum arabic was chosen as surfactant since they had already been used with castor bean powders and other enzymes [2–6]. Actually, some authors [6] optimized emulsion stability before beginning lipase reaction experiments assuming that the best emulsion implies the best lipase performance.

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Surfactant concentration was included in the experimental design since it could affect lipase performance [3]. To the best of our knowledge, optimization of surfactant and LP concentrations, considering their interaction, was never studied in castor bean powders. On the other hand, previous works about plant lipases (including the extract from castor bean) used control reactions considering the incubation period of the reaction medium but not of the lipase powder. Taking into account that LP is not pure lipase, its presence in the reaction medium during all the reaction time could contribute to the final control fatty acid quantification particularly when using titration of the reaction medium. From unpublished previous results, we have detected that this aspect can be important, especially when low enzyme activities are involved and/or indirect methods (as reaction medium titration) are used to quantify fatty acid production. So, in this work, appropriate controls were made to take into account the powder contribution, and a chromatographic technique was used to assure that reported FA production effectively corresponds to lipolysis taking place under the conditions investigated.

Materials and Methods

Materials

Castor bean seeds (*Ricinus communis*) were obtained from a local ornamental plant (Bahía Blanca, Argentina). Refined high oleic sunflower oil was purchased from a local grocery store. FAME composition of this oil was determined by gas chromatography (GC) according to methods AOCS Ce 2-66 and AOCS Ce 1e-91 [7]: 85.25, 6.42, 3.93, and 2.65% of oleic, linoleic, palmitic and stearic acids, respectively. Gum arabic was extra pure grade (Anedra).

All reagents, gases and solvents used were of analytical or chromatographic grade.

Methods

Castor seeds were peeled and defatted according to Santos et al. [3]. Seeds (23 g) were ground with a hand blender for 5 min along with cold acetone (100 mL). For oil extraction, more acetone was added up to a ratio of 5:1 solvent:seed (mL/g). The mixture was placed overnight at 3 ± 1 °C with constant stirring and filtered on a Buchner funnel. After solvent evaporation, 7 g of powder (LP) were obtained and stored in the freezer until its use. Since this process only involves oil extraction, LP contained not only lipases, but also other vegetal components. Therefore, although LP remained as a solid throughout the reaction time, this does not imply that some compounds may not be partially solubilized (including the lipase).

Reaction medium contained 5 mL of acetate buffer (pH 4.4, 100 mM) and 5 mL of an emulsion of gum arabic:water solution and high oleic sunflower oil [3, 4]. Gum arabic:water solution (GAWS) is the aqueous phase in the substrate emulsion. Flasks with the reaction medium were introduced into a bath at 37 °C [2] and the reaction was started by the addition of LP. After 1.5 h under vigorous magnetic stirring (ensuring a homogeneous distribution of all components), ethanol (10 mL) was added to stop lipase activity.

Control reactions contained 5 mL of acetate buffer and LP. This was incubated at 37 °C. After 1.5 h, ethanol (10 mL) was added followed by 5 mL of the emulsion.

Box–Behnken design (BBD)

Parameters and their corresponding ranges were: *A*, gum arabic concentration in the GAWS (0–6% w/w); *O*, oil fraction in the emulsion (0.16–0.5 oil:GAWS w/w); and *L*, LP amount (0.05–0.15 g).

Two blocks of 18 runs (in random order) were performed, representing 12 replicates at the central point and two replicates in the remaining experimental runs. Data were statistically analyzed using Design-Expert 7.0.0 software.

Hydrolysis Products Extraction and Quantification

A solvent extraction using hexane and ethyl ether (1:1 v/v) was performed on hydrolysis samples in order to obtain a pure sample of the reaction products (lipid phase). The upper phase was collected and solvent was evaporated.

Recovered reaction products were dissolved in pyridine (JT Baker) and silylated using MSTFA (Sigma). Simultaneous quantification of free fatty acids (FFA), monoacylglycerols (MAG), and diacylglycerols (DAG) in derivatized samples was performed by GC according to Pacheco et al. [8]. A metallic capillary column (MXT-65TG, 30 m × 0.25 mm × 0.1 μm film thickness; Restek, Bellefonte, USA) and a flame ionization detector (FID) was used. The injector was used in split mode (ratio of 1:15) and held at 360 °C. The detector temperature was constant and equal to 380 °C. The oven temperature was programmed to be at 40 °C for 4 min, then increased from 40 to 350 °C at the rate of 15 °C/min and then to 360 °C at the rate of 0.2 °C/min. Hydrogen was used as carrier gas at a linear velocity of 33.6 cm/s. Data acquisition and peak integration were carried out using HP 3398A GLC Chemstation software. Glycerol and triacylglycerols (TAG) were quantified using mass balances.

Lipase activity (R) was calculated as $mmol_{FFA}/g_{oil}$ (using the information of FFA quantification by GC) normalized by the amount of LP used (g) and reaction time (h). For

hydrolysis percentages calculations (w/w), theoretical mass of FFA was considered to be equal to the initial oil mass.

Results and Discussion

Control reactions involved in this work were different from those used in other studies [2–4] and presented a significantly higher % FFA ($p \ll 0.01$) than experiments in which the reaction medium was incubated without LP (1.02 ± 0.09 and 0.55 ± 0.09% FFA, respectively). This showed that LP increases % FFA during incubation even when no oil was added to the medium, probably due to residual endogenous oil in the LP. Moreover, in a previous work (data not shown) where titration was used to quantify FFA according to Santos et al. [3], an activity overestimation was obtained indicating a contribution from other compounds. These aspects could generate wrong activity values especially when low production of FFA is obtained. That is why we chose the proposed control reaction and quantification method.

All BBD experiments in un-coded values are shown in Table 1 with their respective response. Activity values obtained in the whole experiment were within 0.32–16.90 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$ reaching hydrolysis percentages near 50% (run 11).

Table 2 shows the analysis of variance (ANOVA). A square root transformation of R was used in order to fulfill all ANOVA requirements. So, the model-dependent variable was $R^{1/2}$ instead of R. The obtained model was significant ($p < 0.0001$) and the lack of fit was not significant ($p = 0.4001$). Therefore, it can be used to study the behavior of the system in the considered independent variables' ranges. Variables L, A, and O, interactions $L{\cdot}A$ and $L{\cdot}O$, and the quadratic terms L^2 and A^2 of the final model were significant ($p < 0.0255$) while the quadratic term O^2 and interaction $A{\cdot}O$ could be considered statistically insignificant. Term O^2 was removed from the ANOVA since it increased lack of fit. On the other hand, removing $A{\cdot}O$ did not increase lack of fit so it was kept in the model ($p = 0.0968$). Coded parameters presented in Table 2 show that linear term of gum arabic concentration seems to have the most notable effect on $R^{1/2}$ followed by LP amount, both having negative coefficients. Since lipase activity (calculated as $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$) increased with higher oil concentrations, these results may be indicating that the lipase was not saturated by oil, the limiting reagent in the hydrolysis reaction, under the studied conditions. Equation (1) shows the final equation without O^2 term.

$$\boldsymbol{R}^{1/2} = 4.11 - 35.55\boldsymbol{L} - 0.82\boldsymbol{A} + 4.98\ O + 2.77\boldsymbol{L}\cdot\boldsymbol{A} - 15.67\boldsymbol{L}\cdot\boldsymbol{O} - 0.19\boldsymbol{A}\cdot\boldsymbol{O} + 89.71\boldsymbol{L}^2 + 0.05\boldsymbol{A}^2 \quad (1)$$

As can be easily seen in Fig. 1a and b, lipase activity also increased when using low enzyme and surfactant concentration. Surfaces obtained with $O = 0.16$ and 0.33, and $A = 3$

Table 1 Box–Behnken design experiments: parameters and response variable in un-coded values

Run	L: amount of LP (g)	A: gum arabic in GAWS (% gum arabic:water, w/w)	O: oil in emulsion (oil:GAWS, w/w)	R: lipase activity[a] ($mmol_{FFA}/(g_{oil}{\cdot}g_{LP}{\cdot}h)$)
1	0.05	0	0.33	16.90 ± 1.09
2	0.15	0	0.33	2.94 ± 0.41
3	0.05	6	0.33	2.76 ± 0.21
4	0.15	6	0.33	0.88 ± 0.40
5	0.05	3	0.16	2.48 ± 0.35
6	0.15	3	0.16	0.51 ± 0.09
7	0.05	3	0.50	7.23 ± 0.49
8	0.15	3	0.50	1.67 ± 0.09
9	0.10	0	0.16	4.10 ± 0.30
10	0.10	6	0.16	0.32 ± 0.10
11	0.10	0	0.50	10.96 ± 1.40
12	0.10	6	0.50	2.12 ± 0.02
13	0.10	3	0.33	1.89 ± 0.61
14	0.10	3	0.33	1.90 ± 0.17
15	0.10	3	0.33	1.47 ± 0.12
16	0.10	3	0.33	2.31 ± 0.61
17	0.10	3	0.33	1.95 ± 0.30
18	0.10	3	0.33	1.98 ± 0.44

LP: lipase powder; GAWS: gum arabic water solution

[a] Data reported as mean values of duplicate analyses ± standard deviation

Table 2 Analysis of variance for Box–Behnken design

Source	SS	*DF*	MS	*F* value	*p* value	Coded parameters
Model	26.1524	8	3.2690	129.4408	<0.0001	–
Model parameters						
L	7.2549	1	7.2549	287.2634	<0.0001	−0.6734
A	10.7393	1	10.7393	425.2310	<0.0001	−0.8193
O	3.7468	1	3.7468	148.3569	<0.0001	0.4839
L·A	1.3805	1	1.3805	54.6630	<0.0001	0.4154
L·O	0.1419	1	0.1419	5.6195	0.0255	−0.1332
A·O	0.0750	1	0.0750	2.9684	0.0968	−0.0968
L^2	0.4426	1	0.4426	17.5245	0.0003	0.2243
A^2	2.1483	1	2.1483	85.0633	<0.0001	0.4941
Residual	0.6566	26	0.0253			–
Lack of fit	0.4306	16	0.0269	1.1903	0.4001	–
Pure error	0.2261	10	0.0226			–

SS: sum of squares; *DF*: degrees of freedom; MS: mean square; *L*: amount of lipase powder; *A*: gum arabic concentration in the aqueous solution used to prepare substrate emulsion; *O*: oil fraction in the emulsion; *L·A*, *L·O* and *A·O*: the interactions between corresponding parameters; L^2 and A^2: quadratic terms for parameters *L* and *A*

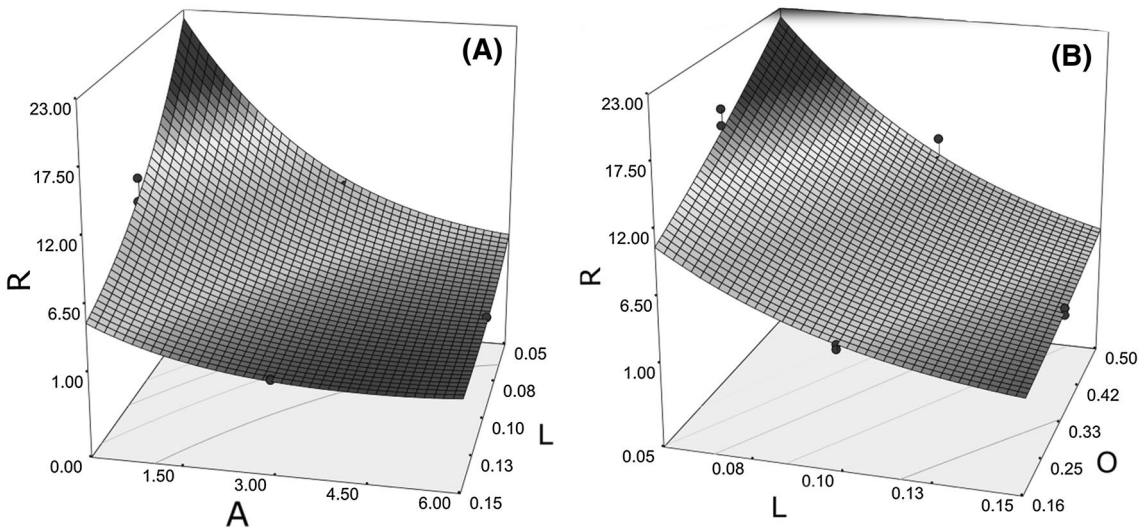

Fig. 1 Response surface plot: lipase activity (*R*, $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$) as a function of *A*: GAWS ratio (*A*, % w/w) and the amount of LP (*L*, g) when oil:GAWS ratio (*O*) is equal to 0.5 w/w; **b** *L* (g) and *O* (w/w) when *A* is equal to 0% w/w. *Black dots* represent experimental data

and 6 presented similar behavior to Fig. 1a and b, respectively, but lower *R* (data not shown). In this study, surfactant concentration showed an anti-intuitive effect since more stable emulsions and smaller oil droplets were not enough to improve, not even maintain, lipase activity. There are several hypotheses that could explain this behavior such as LP inactivation and/or gum arabic adsorption on the LP, hindering the effective substrate or products mass transfer. As Cavalcanti et al. [2] mention, an inhibition of LP because of any compound present in the gum arabic could also be possible. Regardless of the effect cause, it appears to overcome the increase in the oil–water interfacial surface since *A·O* was not significant. It was expected that at least at high oil concentrations this effect could be partially compensated for by higher substrate availability; but, obtained results do not support this. These results are complementary to other published works [3, 4]. We suggest that the absence of gum arabic would not only simplify industrial processes, as these authors have suggested, but it would also improve lipase performance under the studied conditions.

It would be possible to think that experiments with lower *L* would produce higher *R* only because L is smaller (since

L is used as denominator in the calculation of *R*, $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$). This is not true since those experiments using lower *L* without gum arabic in the medium yielded higher hydrolysis percentages (for example, 37 and 17% at L = 0.05 and 0.15 g, respectively, at $O = 0.33$). This fact could indicate that LP could be more efficiently dispersed in the reaction medium when lower amounts of powder are used. A homogeneous medium was observed by visual inspection suggesting an appropriate LP mixing in all samples. At the highest amount of gum arabic tested, this effect could not be detected, probably because of the influence of the aforementioned effects.

After optimization, *R* maximization resulted in an activity point of 22.13 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$ (predicted value) using $L = 0.05$ g, $A = 0\%$ and $O = 0.5$. As can be seen in Fig. 1a, this point is located at the edge of the response surface and, therefore, we cannot prove that it is a real maximum. More assays are needed in order to obtain the factor values that truly maximize *R*. This predicted maximum activity obtained within the BBD was then validated with experimental assays, which yielded a value of 20.47 ± 7.19 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$ for *R*, demonstrating a good prediction of experimental assays.

An additional experimental design using 0.19–0.32 oil:aqueous phase w/w and 0.05–0.005 g LP was performed without gum arabic but the model was not significant (data not shown). Despite this, 67% of the new experiments (runs) presented activities higher than 20.00 $mmol_{FFA}/g_{oil}{\cdot}g_{LP}{\cdot}h$, while in BBD, only 10% of the experiments exceeded that value.

In addition, glycerol was detected from early reaction times, increasing with hydrolysis degree. The FFA/glycerol molar ratios obtained were higher than 3.0 for all runs, suggesting castor bean lipase is not 1,3-positionally selective, in agreement with Noma and Borgström [9]. The cited paper mentioned that acyl migration did not take place between diacylglycerol isomers. However, the possible occurrence of acyl migration was not investigated herein.

Conclusion

This study provides an evaluation of lipase activity from castor bean seed powder in hydrolysis reactions using response surface modeling to take into account the simultaneous contribution of gum arabic, oil and LP concentration. The obtained model was highly significant as well as parameters associated to the linear term of the amount of LP, gum arabic concentration in the gum arabic:water solution and oil concentration in the emulsion, among others. Lipase activity was higher when using low LP concentration and high oil concentration, in the absence of gum arabic. It was concluded that gum arabic produced a negative contribution to the enzymatic reaction, and higher emulsion stability does not necessarily provide the best reaction medium. Furthermore, glycerol production points to the fact that this lipase may not show specificity for any of the FA positions in TAG, and/or a favored acyl migration reaction could be occurring in this reaction system.

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Compliance with Ethical Standards

Conflict of interest The authors have declared no conflicts of interest.

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