Lipase-catalyzed glycerolysis of fish oil to obtain diacylglycerols

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SUMMARY

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Diaciglycerols (DAG) rich in n-3 residues were successfully produced by Novozym 435-catalysed glycerolysis of n-3 PUFA rich fish oil. Orbital and magnetic agitations were evaluated in order to minimize mass transfer limitations and thus assure the homogeneity of the reactant mixture. Different temperatures (65, 70, 75, 80, 85 and 90°C) and speeds (300, 500, 700 and 900 rpm) were tested. Optimal conditions to obtain the highest amount of DAG were: 65°C, with a substrate molar ratio of 3:1 (oil:glycerol), 500 rpm and 15% of enzyme load after 2.5 h, with a yield of 60%.

KEY-WORDS: Diacylglycerols – Fish oil – Glycerolysis – Novozym 435.

1. INTRODUCTION

Research has demonstrated that the ingestion of polyunsaturated fatty acids (PUFA) has specific physiological benefits for humans (Sardesai, 1992). The beneficial effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been well documented in the literature. EPA is useful in the prevention and treatment of certain medical conditions including coronary heart disease, aggregation of blood platelets, abnormal cholesterol levels, and several carcinomas (Kamali, 1996), while DHA reduces the risk of heart disease, the production of inflammatory cytokines (Simopoulos, 2002; Von Schacky, 2007) and inhibits the growth of tumour (Zhang et al., 2007). EPA and DHA can be consumed as acylglycerols for better absorption and less susceptibility to oxidation compared to its free fatty acid form. Recently, it has been reported that the DHA level in the erythrocytes and plasma in rats increased when they were fed DHA-high content monoacylglycerols (MAG), compared to those fed with DHA-ethyl esters (Valenzuela et al., 2005).

Fish oil is well known for its high content in n-3 fatty acids, with documented benefits for human health (Nettleton, 1994). Several reports on the enzymatic intereserification of fish oil are available in the literature (Jennings and Akoh, 1999; Garcia et al., 2000; Xu et al., 2000). Most approaches to intereserification reactions employ a large molar excess of fatty acids to maximize the level of incorporation of the associated acyl group via acidolysis reactions. Enzymatic glycerolysis has not been widely studied and there is still work to do.

A recent review on the production and applications of MAG and DAG depicts all the relevant aspects of these acylglycerols (Camino-Feltes et al., 2012). On the other hand, diacylglycerols (DAG) are natural components of various edible oils and recent studies have indicated that DAG beneficially affect lipid metabolism by increasing fat oxidation and decreasing the storage of TAG in adipose tissue (Nagao et al., 2000; Kamphuis et al., 2003), weight loss, lower liver fat content and lower abdominal fat content after the intake of DAG oil as compared to ordinary TAG oil (Murase et al., 2002; Yuan et al., 2010). Moreover, in different degrees of purity, DAGs are used as additives or carriers in the food, medicinal and cosmetic industries (Fureby et al., 1997). The versatility of DAG oil is evident in numerous applications, either enzymatically or chemically manufactured. Lipase-catalyzed glycerolysis is a potential alternative to the high temperature chemical process usually applied at an industrial scale for MAG and DAG production.
In a previous report we have employed response surface methodology to explore the influence of enzyme load, reaction temperature and substrate molar ratio and their interactions (Miranda et al., 2012) and thus, in the present study we focus on the evaluation of the type of agitation (orbital and magnetic), agitation speeds (300, 500, 700 and 900 rpm) and again temperatures (65, 70, 75, 80, 85 and 90°C) in order to increase the production of structured DAG and thus reduce mass transfer limitations because in solvent-free glycerolysis reactions, the glycerol and the oil phases are immiscible.

2. MATERIALS AND METHODS

2.1. Materials

The fish oil used was a gift from Ocean Nutrition (Nova Scotia, Canada). Glycerol (≥99.0%) was purchased from Sigma-Aldrich (Madrid, Spain). The biocatalyst employed was Novozym 435 (a lipase from Candida antarctica fraction B), kindly provided by Novozymes A/S ( Bagsvaerd, Denmark). The standards used were trilinolein (≥99%), 1,2-dipalmitin (≥99%), 1,3-diolein (≥99%), 1-monolein (≥99%) and arachidic acid (≥99%) and were purchased from Sigma-Aldrich, as well as 0.5 M sodium methoxide in methanol. Supelco 37 FAME Mix GC standard was purchased from Supelco (Bellefonte, PA). All the reagents for HPLC analysis were HPLC-grade from Scharlab (Barcelona, Spain).

2.2. Methods

2.2.1. Effect of the type of agitation

For the enzymatic glycerolysis, two types of agitation, orbital and magnetic, were evaluated. One set of reactors was agitated in an orbital incubator (SI 50, Stuart Scientific, UK) operating at 200 rpm and 60°C. Another set of reactors was agitated in a magnetic stirrer (Ikamag RCT, IKA®-Werke GmbH & Co. KG) operating at 200 rpm and 60°C. For the results obtained in our previous report, 3 g of the mixture of substrates were combined with the enzyme loading of 15% (with respect to the total weight of substrates) and allowed to react in 25 mL glass bottles (Miranda et al., 2012). Samples, 100 µL, were withdrawn periodically and dissolved in 1.5 mL CHCl₃:CH₃OH (2:1) to monitor the production of DAG in both systems for the same conditions indicated in section 2.2.1.

2.2.2. Effect of the agitation speed

After the first trials were conducted, we proceeded to evaluate different speeds (300, 500, 700 and 900 rpm) in 25 mL glass bottles under the same conditions indicated in section 2.2.1. Samples were withdrawn periodically to monitor the production of structured DAG as described in section 2.2.1.

2.2.3. Effect of the temperature

Due to the type of agitation and obviously the speed, both these parameters affect the homogeneity of the substrate reaction mixture and thus, we decided to evaluate a range of temperatures (65, 70, 75, 80, 85 and 90°C) for these new trials. Three grams of the mixture of substrates were combined with the enzyme (15%, with respect to the total weight of substrates) and allowed to react in 25 mL glass bottles. The conditions for these trials were 300 rpm, a substrate molar ratio of 3:1 (oil:glycerol) for 24 h. Samples were withdrawn periodically to monitor the production of DAG as described in section 2.2.1.

2.2.4. Analysis of glycerides profile

HPLC-ELSD analysis was carried out using a LaChrome L-7100 pump (Merck-Hitachi). The analytical column (250 mm × 4.6 mm ID × 5 µm) was a normal-phase silica Kromasil (Análisis Vínicos; Ciudad Real, Spain). An auto-injector LaChrom L-7200 (Merck-Hitachi) was employed to inject 20 µL of samples into the column. The chromatographic separation was carried out at 40°C as reported previously (Hernández-Martin and Otero, 2008). A Sedex (S.E.D.E.R.E., Alfortville, France) model 55 ELSD was used; the pressure of nebulizer gas (air) was set at 2.1 bar and the drift tube temperature was set at 90°C.

2.2.5. Fatty acids analysis

The samples withdrawn were analyzed by GC. Methyl esters of esterified fatty acids were prepared via the selective derivatization of 200 µL of reaction mixture with the addition of 0.5 mL 0.5 M sodium methoxide in methanol. After incubation for 5 min, 0.2 mL of water and 2 mL hexane were added. After vortexing, the methyl esters extracted in the hexane layer were collected and then dried with sodium sulphate. One µL of the extract was injected into an Agilent 6890N GC fitted with a Phenomenex ZB-WAX plus (30 m × 0.32 mm × 0.25 µm) capillary column. Injector and FID temperatures were set at 250 and 300°C, respectively. The oven temperature was kept at 50°C for 2 min, then was taken to 220°C at a rate of 30°C min⁻¹ and held at this temperature for 25 min. After that, the temperature was increased to 255°C and it was held at this temperature for 7 min. FA were identified by comparing their retention times with those of the Supelco 37 FAME Mix standard.

3. RESULTS AND DISCUSSION

As previously reported, the production of glycerides is a complex reaction scheme where
many variables affect product formation and overall reaction yield. In this study we evaluated the effect of agitation, temperature and speed in order to prepare high amounts of DAG.

3.1. Effect of the type of agitation

Due to the viscosity and the immiscibility of the reactants, it is important to ensure that the agitation system lead to homogeneity of the mixture. On the other hand, agitation minimizes the mass transfer limitations and favors complete interactions of the enzyme with its substrate (Babicz et al., 2010). In order to evaluate this effect, we compared orbital and magnetic stirrings to reach elevated amounts of DAG by the enzyme-mediated glycerolysis. The data obtained for these entries are contained in Fig. 1. According to the results, magnetic agitation was more effective; allowed us to produce the highest DAG content in a short reaction time. This indicates that magnetic stirring favored the mixing of reactants and the enzyme-substrate interaction. Thus, we selected magnetic agitation for further trials.

3.2. Effect of temperature

Due to the nature of the reactants employed, temperature is a significant variable of study. Glycerol has a low miscibility with oils and fats. At high temperatures, viscosity could be reduced, and also the substrate diffusion or its solubility could be improved, which means higher amounts of DAG and MAG. The results obtained for these experiments are depicted in Figure 2. These experimental runs at 300 rpm (see below the effect of agitation speed) involved abundant experimental difficulties because of the high heterogeneity of the reaction mixtures and their high viscosities, which is why some experimental data points are out of the curve defined by the rest of the experiments and have little statistical significance. Nevertheless, according to this Figure, DAG were formed at a faster rate at the highest temperatures (80-90 °C), with a yield of 50-53% for the first hour, while at the lowest temperatures (65-75 °C), 32-40% of DAG were produced after the same reaction time. However, when reaction was allowed to proceed for longer times, the maximum DAG yield (60%) was produced at the lowest temperatures. This can be explained because at higher temperatures reaction proceeds faster but when the most elevated content of DAG is reached, such percentage remains equilibrated. However, after 10 h of reaction at 75 and 85 °C the amount of DAG is lower compared to the other temperatures evaluated. The use of high temperatures has a negative impact on the operational stability of the biocatalyst. Therefore, based on this fact and considering the results obtained, we selected 65 °C as the best temperature for DAG production.

Camino-Feltes et al. (2010) reported that Novozym 435-catalyzed glycerolysis of fish oil by the addition of t-butanol at 600 rpm and 70 °C, with 15% enzyme load and a substrates molar ratio of 1. These authors obtained 43% of DAG and according to their statistical analysis the highest significant effect corresponded to temperature. Similar results were reported for olive oil, employing a substrates molar ratio of 0.5:1.5 (glycerol:oil), 10% of enzyme, 55 °C and 600 rpm (Criado and Otero, 2010). Torres et al. (2002) described that the glycerolysis of an oil rich in EPA by lipase-B from Candida antarctica in a solvent-free system produced 45% of DAG at 60 °C for a substrates molar ratio of 1.5:1 (fatty acid:glycerol) after 10 h. Compared to our results, we achieved 59.73% of DAG after 2.5 h. Wang et al. (2011), attained 51.62% of DAG at 50-60 °C from soybean. Valério et al. (2010) produced 17% of DAG at 70 °C, 600 rpm, glycerol:olive oil molar
ratio of 6:1, 16 wt% of surfactant (Tween 65) and 9 wt% of Novozym 435. Krüger et al. (2010) reached 50% of DAG at 70 °C, 600 rpm, using a glycerol to oil molar ratio of 0.5:1.5, after 8 h of reaction and an enzyme concentration of 10 wt%.

The mechanisms of the reaction explored involved MAG formation. The results are shown in Figure 3. The content of MAG in the mixture of acylglycerols was increased as the temperature increased during the first 2.5 h. At that time we attained the largest amount of MAG, 24.48 and 24.76%, at 75 and 90 °C respectively. After that time, the content of MAG decreased for all the temperatures explored, except at 65 and 70 °C. For the latter there was always an increment during the whole run. It was noteworthy that 24 h of reaction we were able to achieve 23.26% MAG at 90 °C. However, for an economical and practical approach we defined 70 °C as the best temperature for MAG formation. Valério et al. (2010) reached 26% of DAG at 70 °C, 600 rpm, glycerol:olive oil molar ratio of 6:1, 16 wt% of surfactant (Tween 65) and 9 wt% of Novozym 435. Krüger et al. (2010) produced 53% of DAG at 70 °C, 600 rpm, using a glycerol to oil molar ratio of 3:1/6:1, after 8 h of reaction and an enzyme concentration of 10 wt%.

3.3. Effect of agitation speed

Due to the low miscibility of glycerol with oils, the agitation speed clearly affected the production of both DAG and MAG. According to Figure 4, the more important effects of agitation speed were observed during the first 2.5 h of reaction, a time at which the use of agitation speeds of 500 and 700 rpm permitted us to obtain the highest amounts of DAG. The use of 900 rpm resulted in slightly lower amounts of DAG than those obtained with 700 rpm. This might confirm the fact that with high speeds, part of the enzyme is held in the walls of the vessel, leading to a lower amount of available biocatalyst to interact with the substrates. In contrast, the smallest amounts of DAG were produced at 300 rpm. In this case the agitation speed was insufficient to guarantee a good homogeneity of the mixture. Thus, the best yields were obtained at 500 and 700 rpm because we did not observe the opposite effects of high and low speeds. However, the greatest content of DAG was 60.31% at 500 rpm for a substrates molar ratio of 3:1 (oil:glycerol), 15% enzyme load and 65 °C. Compared to our results, Babicz et al. (2010) explored different agitation speeds for the Lipozyme TL IM-catalyzed hydrolysis of soybean oil and they reported the highest yield for DAG at 700 rpm.

Agitation speeds affected MAG formation in a different way. At the very beginning of the reaction (30 min), MAG formation was favored by the highest speed employed (900 rpm), but after that time, the largest amount of MAG (18.57%) was produced after 7.5 h at 500 rpm. The most interesting behavior was observed at 300 rpm. For the first hour, the lowest amounts of MAG were prepared at 300 rpm. At 2.5 h of reaction, the same amount of MAG was obtained for 300 and 900 rpm but at 5 h, 300 rpm resulted in a high formation of MAG compared to the other speeds. In the end, 300 and 900 rpm yielded the same production of MAG, 700 rpm was slightly greater but the highest synthesis was reached with 500 rpm. Consequently, we defined 500 rpm as the best agitation speed for the production of both DAG and MAG. We have already mentioned that higher speeds are better for the production of both glycerides.

With the best conditions indicated before for our entries, the composition of the DAG formed consisted of 89.37% n-3 PUFA: EPA (11.32%), DPA (8.34%) and DHA (69.71%). On the other
hand, the composition of the MAG prepared consisted of 90.28% n-3 PUFA: EPA (10.46%), DPA (7.61%) and DHA (72.21%).

4. CONCLUSIONS

For the two types of agitation tested, magnetic agitation proved to be the most adequate in order to ensure the homogeneity of the mixtures and the reduction of mass transfer limitations. In our all experiments, it was observed that the DAG production had a higher initial reaction rate than that verified for MAG production. However, we found that the lowest temperatures evaluated positively favored the synthesis of DAG; 65°C was the optimal temperature, reaching a 59.73% content of DAG. In the case of MAG, 75°C was the optimal temperature, achieving a 25% content. When agitation speed was evaluated, the greatest amount of DAG attained was 60.31% at 500 rpm. In the case of MAG we noted different trends, and still the largest content (18.57%) was also obtained at 500 rpm.

Given the health benefits of MAG and DAG, we will continue to develop further reaction schemes to reach higher amounts of MAG. For this attempt, 500 rpm allowed us to form both acylglycerols and even when two had different optimal temperatures, 75°C would be good for either. There is still work to do on the purification aspect of the acylglycerols, in order to make them available for pharmaceutical and food applications.

5. ACKNOWLEDGMENTS

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REFERENCES


Yuan QG, Ramprasath VR, Harding SV, Rideout TC et al., 2010. Diacylglycerol oil reduces body fat but does not alter energy or lipid metabolism in overweight, hypertriglyceridemic women. J. Nutr. 140, 1122-1126.


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