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Enzymatic synthesis of monoglycerides by esterification reaction using *Penicillium camembertii* lipase immobilized on epoxy SiO_2-PVA composite

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ABSTRACT

Glycerol-fatty acid esterification has been conducted with lipase from *Penicillium camembertii* lipase immobilized on epoxy SiO_2-PVA in solvent-free media, with the major product being 1-monoglyceride, a useful food emulsifier. For a given set of initial conditions, the influence of reaction was measured in terms of product formation and selectivity using different fatty acids as acyl donors. Results were found to be relatively dependent of the chain length of the fatty acids, showing high specificity for both myristic and palmytic acids attaining final mixture that fulfills the requirements established by the World Health Organization to be used as food emulsifiers.

1. Introduction

The use of mono- and diacylglycerols as non-ionic emulsifiers in the food and pharmaceutical industries and as synthetic chemical intermediates has been a growing research area in recent years [1,2]. Furthermore, they have a generally recognized as safe status, which contributes to their larger application. Mono- and diglycerides are consumed at an annual level of 85,000,000 kg in the United States, corresponding roughly to 70% of the total emulsifiers used in the food industry [3]. Besides bulk applications in the food and dairy industries, some other applications for special monoacylglycerols have been described [4,1,5]. Recently, the antimicrobial activities of particular types of monoglycerides such as monolaurin, monomyristin, monolinolein, and monolinolenin have been reported [6]. It has also been proposed that fatty acids and monoglycerides (lauric acid, monolaurin) can be used by the humam or animal to destroy lipid-coated viruses, such as HIV, herpes, cytomegalovirus, influenza, various pathogenic bacteria, including *Listeria monocytogenes* and *Helicobacter pylori*, and protozoa such as *Giardia lamblia* [7].

The conventional chemical method to produce monoglycerides (MG) involves the glycerolysis of fats and oils at higher temperatures (220–260 °C) and elevated pressure under nitrogen atmosphere while employing inorganic alkaline catalysts. The major drawbacks of this process include high-energy consumption, low yield, and poor product quality [8]. Molecular distillation is usually needed for the production of high purity MG [1,4]. Moreover, this process produces environmentally undesirable by-products [8]. The product is a mixture containing 35–60% monoacylglycerols, 35–50% diacylglycerols, 1–20% triacylglycerols, 1–10% free fatty acids, and their alkali metal salts [3].

Synthesis of MG by means of an enzymatic process is an environmentally friendly approach. Highly stable lipases in organic solvent offers the possibility of employing various approaches to the enzyme-catalyzed synthesis of MG, such as selective hydrolysis or alcoholysis using 1,3-regiospecific lipases [9], esterification of glycerol with fatty acids [10] and glycerolysis of fats or oils [11]. The advantages of enzymatic synthesis are higher yields and mild reaction conditions, resulting in products of higher quality and lower energy consumption [4,12].

When the production of a high degree pure monoglyceride is desired, an interesting route is the direct lipase-catalyzed esterification. Thus, the objective of the present work was to study the synthesis of monoacylglycerols in a medium solely composed of substrates by direct lipase-catalyzed esterification of glycerol with fatty acids, without any solvent or surfactant. Such a system avoids the problems of separation, toxicity, and flammability of organic solvents, thereby lowering the cost of the final product and allowing product recovered without further complex purification or evaporation steps. Moreover, a solvent-free system was chosen because the target products have a potential use in foods [13].

Lipase-catalyzed synthesis of these partial acylglycerols by the direct esterification of glycerol with fatty acids in different reaction media have been studied and the media investigated include the use of aqueous-organic two-phase systems [14], microemulsions [14,15] and anhydrous organic solvents [15] as well as the use of solvent-free systems [3,16]. The selection of a lipase is another factor affecting the success of this method.

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Previous work demonstrated that lipases from *Candida antarctica* and *Penicillium camembertii* (Lipase G) immobilized on SiO_2-PVA composite were found to be potential catalysts to produce monolaurin by direct esterification of glycerol with lauric acid [17]. Reaction conditions were optimized by factorial design and results indicated that *P. camembertii* gave the best performance, in terms of lauric acid incorporation into glycerol giving about twice the concentration of diglyceride and low amount of trilaurin [17].

In this paper, we report further studies dealing with the direct acylation of glycerol with different fatty acids using *P. camembertii* lipase immobilized on SiO_2-PVA composite. This lipase has been shown consistent high production of monoglyceride in the direct acylation of glycerol with fatty acids having different chain lengths. Although several reports have been found using this lipase preparation [18,19,20], data relating its performance on an immobilized state is scarce in the literature. However, the use of immobilized lipases is often recommended for lipase-catalyzed reactions, since it is provided non-aqueous conditions necessary for synthetic reactions [21]. In addition, immobilized lipase can be reused many times without significant loss of activity and may also exhibit substantial thermo stability, which is another advantage for industrial application. A promising approach to lipase immobilization makes use of epoxy SiO_2-PVA composite [22,23]. This type of support can be easily recognized by the lipases, at molecular level, as solid surfaces. Thus, lipases can be immobilized on such supports leading to open immobilized structures. In accordance with this methodology, microbial lipase from *P. camembertii* was successfully immobilized on epoxy SiO_2-PVA rendering active and stable samples [24]. In this work, the ability of this immobilized preparation to catalyze the direct esterification of glycerol with short, medium and long fatty acids was investigated.

2. Experimental

2.1. Materials

Lipase G (*P. camembertii*) from Amano Pharmaceutical (Japan) was used. Tetraethoxysilane was acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). Epichlorohydrin, hydrochloric acid (minimum 36%), ethanol (minimum 99%), polyvinyl alcohol (MW 72,000) and polyethylene glycol (molecular weight-1500) were supplied by Reagen (Brazil). Substrates for esterification reactions (glycerol and fatty acids: lauric, myristic, palmytic, stearic and oleic) were purchased from Merck and were dehydrated with 0.32 cm molecular sieves (aluminum sodium silicate, type 13 X-BHD Chemicals, Toronto, Canada), previously activated in an oven at 350 °C for 6 h. Other used reagents were hexane (Quimex; Brazil), ethyl acetate (Reagen; Brazil), tetradecane (minimum 99%, Fluka, Germany).

2.2. Support synthesis and lipase immobilization

A silica-polyvinyl alcohol (SiO_2-PVA) hybrid composite was prepared by the hydrolysis and polycondensation of tetraethoxysilane according to methodology previously described [22] rending particles having the following properties: average pore diameter (22.91 Å); surface area BET (461.00 m^2/g) and porous volume (0.275 cm^3/g) [23]. The activation of SiO_2-PVA particles was carried out with epichlorohydrin 2.5% (w/v) at pH 7.0 for 1 h at room temperature, followed by exhaustive washings with distilled water. Afterwards, the support was dried at 60 °C for 24 h. Epoxy SiO_2-PVA particles were soaked into hexane under stirring (100 rpm) for 1 h at 25 °C. Then, excess of hexane was removed and lipase was added at a ratio of 1:4 g of enzyme/g of support. PEG-1500 (5 mg/g) was added together with the enzyme solution at a fixed amount (100 μl/g of support). Lipase-support system was maintained in contact for 16 h at 4 °C under static conditions. The immobilized lipase derivative was filtered (nylon membrane 62HD from Scheiz Seidengazefabrik AG, Thal Schweiz, Switzerland) and thoroughly rinsed with hexane. The esterification activity of resulting immobilized derivative was in range from 35 to 40 U/g following methodology previously described [24].

2.3. Monoglycerides synthesis

Esterification reactions between glycerol and fatty acids (lauric, myristic, palmytic, steriac and oleic) at a fixed molar ratio (molar ratio 8:1 between glycerol:fatty acid) was performed in solvent-free system. These reactions were carried out in a closed spherical glass reactor containing 40 g of medium and 5% (w/w) of lipase G immobilized on SiO_2-PVA corresponding to 5 U/g (activity units) of substrate. The reaction mixture was incubated in a thermo stated bath at temperature range from 45 to 65 °C, according to the melting point of each fatty acid. The reaction medium was magnetically stirred at 200 rpm and samples taken at regular intervals were treated for extraction of the water and glycerol following methodology previous described [16].

2.4. Gas chromatography analysis

Mono-, di- and triacylglycerols were analyzed by gas chromatograph using a Varian 3800 model equipped with flame-ionization detector and with a (10 m × 0.25 mm × 0.12 μm CP Sil 5CB) capillary column (Varian Inc., Corporate Headquarters, Palo Alto, CA, USA). The chromatograms were processed using a Varian data integrator version 4.51 computational program. Nitrogen was used as the carrier gas with a flow rate of 2 ml min^{-1}. The detector and injector temperatures were 300 °C. The column temperature was set to 80 °C for 1 min and was then programmed at 20 °C min^{-1} to 320 °C which was maintained constant for 2 min. Other conditions were split ratio of 1:20 and attenuation equal to 1 [16]. Reaction medium was previously treated for extraction of the water and glycerol and organic phase was dissolved in hexane/ethyl acetate (proportion of 1:1) which contained tetradecane as internal standard, and a direct injection was carried out into the gas chromatograph. For the determination of calibration curves, solutions of pure glyceride (Sigma–Aldrich) were used. Concentrations were expressed as molar fractions calculated from the peak area using calibration curves. The selectivity values obtained in the reaction systems were determined by Eq. (1) [3].

$$\%\text{selectivity} = \frac{\%\text{MG}}{\%\text{MG} + \%\text{DG} + \%\text{TG}} \quad (1)$$

where %MG = is the amount of monoglycerides formed in the reaction, %DG = is the amount of diglycerides formed in the reaction and %TG is the amount of triglycerides formed in the reaction.

3. Results and discussion

Lipases display varying degrees of selectivity towards the substrates with which they interact. Steric hindrance (branching, unsaturation and chain length) and electronic effects of the substrates are the two major factors that determine selectivity. Since it is difficult to generalize the effect of chain length on esterification as this depends on individual lipase preparation and its specificity, in this work the influence of fatty acids in terms of carbon chain size and unsaturated degree on the performance of the esterification reactions catalyzed by lipase from *P. camembertii* were investigated using lauric, myristic, palmytic, steriac and oleic acids as acyl donors. These acids are the major components in the oil extracted

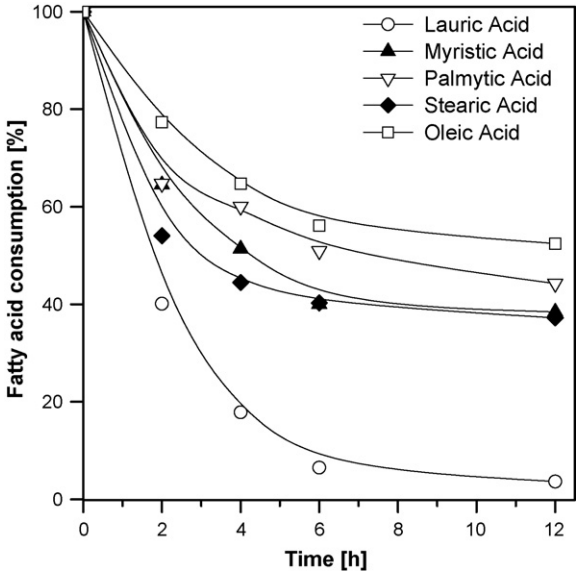

Fig. 1. Profile curve for fatty acid consumption in the esterification reaction of glycerol with fatty acids catalyzed by lipase G immobilized on SiO_2-PVA.

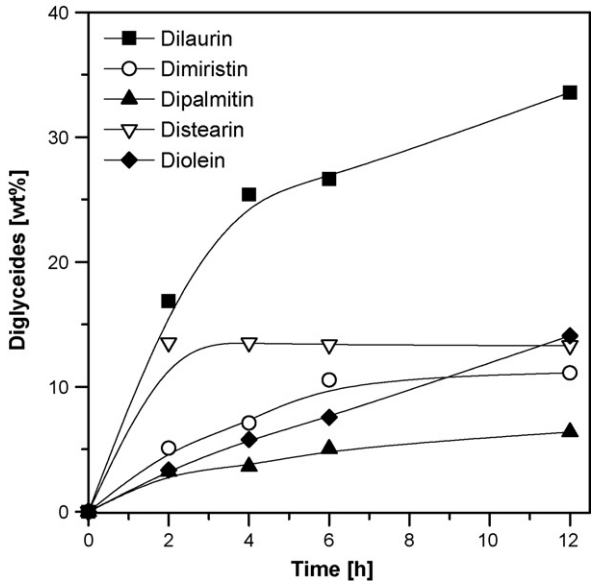

Fig. 3. Influence of fatty acid chain length on the diglycerides formation in the esterification reaction of glycerol using lipase G immobilized on SiO_2-PVA.

from babassu palm trees (*Orbinya martiana*) a potentially raw material source for fatty acids found in abundance in tropical countries, such as Brazil [25].

In all reaction systems, lipase G was immobilized on epoxy SiO_2-PVA and used as biocatalyst. These experiments were carried out using glycerol in excess (molar ratio glycerol to fatty acid 8:1) and 5% (w/w) immobilized derivative.

Fig. 1 displays the curve profile for consumption for each tested fatty acid as a function of time and the formation of the corresponding monoglycerides and diglycerides are shown in Figs. 2 and 3.

Different profiles were obtained depending on the fatty acid studied (Fig. 1). The rate at which the fatty acid was converted decreased in the order of lauric C12, myristic C14, palmitic C16, steric C18 and oleic C18:1, attaining in 6 h conversions of 93%, 60%, 50%, 60% and 46% of the corresponding acids. Extending the reaction for an additional 6 h, did not favor the acid consumption. This rank order can be attributed in part to differences in the solubilities of these fatty acids at the reaction temperature used. However, the

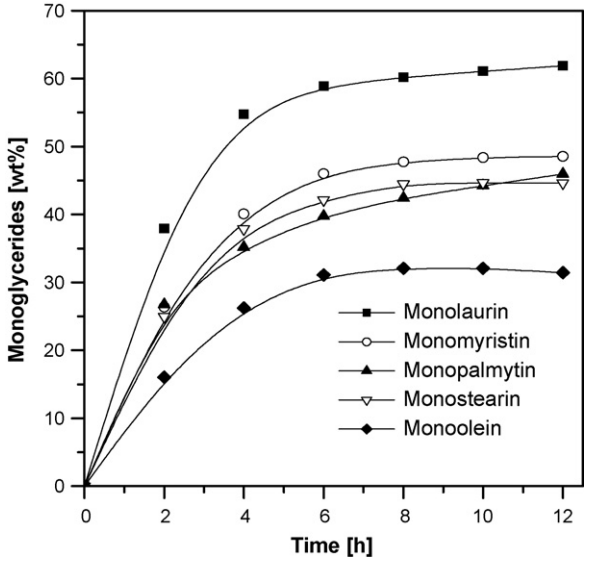

Fig. 2. Influence of fatty acid chain length on the monoglycerides formation in the esterification reaction of glycerol using lipase G immobilized on SiO_2-PVA.

rank order is also a consequence on the selectivity of this enzyme for medium chain fatty acids rather than for long chain fatty acids. In particular, this enzyme prefers saturated chain fatty acids lower than C18.

In terms of monoglycerides formation the curve profile using different fatty acids (Fig. 2) showed that the highest concentration were obtained for the shortest fatty acid (C12), attaining almost 60 wt%, this performance decreased with increasing chain length. Therefore, for myristic acid there was a decreased on formation of monomyristin to 48 wt%. Further decreased on the monoglycerides formation were also observed when palmytic and steriac acids were used as acyl donors, attained, respectively, 46 and 44 wt% and even lower monoglyceride formation was found using oleic acid (C18:1) achieving only 32 wt% monoolein. Note also that the monoester accumulation in the medium was as consequence of disproportionation and glycerolysis reactions of the diester formed earlier as shown in Fig. 3. Nearly quantitative conversions to the corresponding monoesters were found for myristic and palmytic acids. However, both saturated (C18) and unsaturated C18:1 fatty acids produce lower amounts of the corresponding diesters.

This could be related with the active site of the lipase G having difficulty in interacting with longer chain fatty acids (steric hindrance, unsaturation and chain length), decreasing in this way its catalytic activity. This suggests that the acylation migration in lipase G catalyzed synthesis is the rate-determining step and the extent of the deacylation step will affect the overall reaction rate. If one supposes that the acylation step is dependent on the affinity between the fatty acids and enzyme and that deacylation of the acyl-enzyme intermediate by alcohols is merely thermodynamically controlled nucleophilic reaction, then it is expected that for an enzymatic reaction for which the acylation is the rate-determining step, the relative rate of synthesis of esters using fatty acids of different carbon chain length will be more dependent on the carbon chain length of the fatty acids. Interpretation of these findings, with respect to the relationship between the chain lengths of fatty acids, seems to be consistent with the fact that the active site of lipase G is composed of a cavity large enough to accommodate substrates whose total carbon chain length occupies a space equivalent to 15 – CH – moieties. A similar spatial arrangement of the active site has been also suggested for bile-salt stimulated human milk lipase [26]. Thus, even though the longer chain fatty acids may intrude

Table 1
Selectivity for monoglyceride production in the esterification reactions of glycerol with different fatty acids.

Fatty acid	MG (%)	DG (%)	TG (%)	Selectivity[a] (%)
Lauric acid (C_{12})	59.45	26.67	8.38	62.91
Myristic acid (C_{14})	47.92	10.56	1.48	79.92
Palmytic acid (C_{16})	45.86	6.39	3.51	83.80
Stearic acid (C_{18})	42.16	13.37	4.20	70.58
Oleic acid ($C_{18:1}$)	32.92	7.56	3.40	75.02

[a] Values calculated for 6 h reaction.

into this active site, they will also block the pathway for approach by the alcohols to form the products from the acyl-enzyme intermediate, thereby causing a decrease in the net rate of the monoesters production.

Regarding the selectivity of these reactions as displayed in Table 1, the highest values were obtained when both myristic and palmytic acids were used as acyl donors (about 80%). Langone et al. [3], found similar values to 70% of selectivity for the monomyristin formation in the esterification reaction catalyzed by lipozyme. The results presented in Table 1 confirmed that although the lipase from *P. camembertii* possesses a high affinity for lauric acid, higher selectivity was shown for C14 and C16 fatty acids.

Selectivity values can be also related with the enzyme affinity and reaction rates, as already previously discussed. Increasing the fatty acid chain length led to a decrease on the reaction rate which in this turn also decreased the diglycerides formation. However, for C18:0 and C18:1 fatty acids which had much lower global reaction rate, the monoglycerides formation from the glycerolysis of the diglycerides earlier produced occurred at lesser extend, resulting in a decrease in the selectivity, compared with those attained using myristic and palmytic acids as acyl donors.

According to the directives of the World Health Organization, the requirements for these mixtures for utilization as food emulsifiers are: (1) to have at least 70% of mono + diglycerides, (2) to have a minimum of 30% of monoglyceride, and (3) to present contents of both glycerol and triglyceride below 10% [27]. The enzymatic synthesis of both monomyristin and monopalmytin as carried out in this work, resulted in a final mixture that fulfills these requirements.

4. Conclusions

The nature and structure of the fatty acid lead to different interactions with the enzyme. Hence, variations in the values of solubilities of both the precursor fatty acids and the product glycerides are observed as the structure of the precursor acid is changed. The results indicated the feasibility to produce monoglycerides in a solvent-free medium at high yields and selectivities. Conversions (defined as the percentage of consumed fatty acid) reached 80%, with monoglyceride being the major product. Monoglyceride was produced at about twice the concentration of diglyceride, and only trace amounts of triglyceride were detected. The final composition observed after 6 h reaction in the monoglyceride synthesis fulfills the requirements for emulsifier utilization in food, cosmetic, and pharmaceutical industries. The results obtained by enzymatic synthesis under mild experimental conditions were similar to those achieved by chemical glycerolysis of fat and oils, without the drawback of producing some secondary products such as acrolein, polyethers, or polyesters of glycerol.

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