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ORIGINAL PAPER

Synthesis of trimethylolpropane esters of oleic acid by Lipoprime 50T

Vita Kiriliauskaitė · Vida Bendikienė · Benediktas Juodka

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Abstract The ability of the commercial lipolytic enzyme Lipoprime 50T to catalyze the biotechnologically important synthesis of the biodegradable and environmentally acceptable trimethylolpropane (2-ethyl-2-(hydroxymethyl)-1,3-propanediol) ester of oleic acid was investigated. Simple and accurate thin-layer chromatography and computer analysis methods were used that enable one to follow changes of all reaction mixture components simultaneously. The processes of transesterification and esterification were compared. The effects of the molar ratio of the substrates, reaction temperature, time, and medium on the composition of the reaction mixture were analyzed. Esterification was determined to be more preferable than transesterification in both studied solvents. Under the optimal conditions identified (15% w/w water, temperature 60°C, trimethylolpropane to oleic acid molar ratio 1:3.5, and reaction time 72 h), the highest trimethylolpropane trioleate yield of around 62% and trimethylolpropane mono-, di-, and trioleate overall yield of about 83% were obtained. Although the yields are not high enough for industrial application, the process shows the potential to be optimized for higher yields in the near future as the conversions were obtained at ambient pressure, whereas many other processes described in the literature are conducted under vacuum at a specific pressure.

Keywords Trimethylolpropane · Lipase · Transesterification · Esterification · Thin layer chromatography

V. Kiriliauskaitė (✉) · V. Bendikienė · B. Juodka
Department of Biochemistry and Biophysics, Faculty of Natural Sciences, Vilnius University, M.K. Čiurlionio 21, 03101 Vilnius, Lithuania
e-mail: vita.kiriliauskaite@chf.stud.vu.lt

Introduction

In recent years, an increasing environmental awareness in society has given rise to intensified efforts to bring biolubricants derived from renewable resources into the market due to their biodegradability, low toxicity, and environmentally benign nature [4]. Moreover, such lubricants exhibit better lubricity, an excellent viscosity index, and lower volatility than petroleum-based lubricants. These characteristics should result in a lower engine friction, increased fuel economy, and prolonged engine life. Despite these advantages, vegetable oil-based lubricants have been slow in gaining wide acceptance as lubricants [13]. One of the reasons is a poor ageing stability of vegetable oils as they are composed of some polyunsaturated fatty acids which are responsible for the poor oxidation stability. However, the existence of unsaturated compounds is important for the low-temperature, tribological and biodegradable properties of the product. Numerous chemical and biological modifications have been applied to reduce the content of polyunsaturated fatty acids and increase the content of monounsaturated fatty acids. However, preference is being given to the use of synthetic esters composed of oleic acid and polyhydric alcohol as the base fluids for industrial applications [15]. Common fat-based synthetic lubricants are of the following types: branched polyol esters, mono- and dibasic esters, and glycol esters. The most important group is the polyol esters derived from branched polyol. The absence of a hydrogen atom on the β-carbon of its structure ensures a high thermal stability of these esters, a feature rare in vegetable oils. A combination of selected fatty acids or fatty acid esters with alcohol can produce synthetic esters with an appropriate structure for various applications [14]. Preference is being given to a lipase-catalyzed synthesis of

such esters as an environmentally acceptable alternative to chemical synthesis.

The commonly accessible trimethylolpropane (TMP) was used for the synthesis of the esters. The synthesis of TMP esters was performed by transesterification of methyl oleate (MetO) and esterification of oleic acid (OA), using the commercial Lipoprime 50T enzyme as a biocatalyst. The products of the reactions are TMP esters which can be used as raw materials for a biodegradable hydraulic fluid and other lubricants. The conversions were obtained at ambient pressure, whereas many other processes described in the literature are conducted under vacuum at different pressures [7, 11, 14]. The main focus of this paper is the development of an easy, simple, and rapid method for product analysis during large-scale experiments, highlighting the significance of such simple methods as thin-layer chromatography during various optimization processes.

Materials and methods

Materials

LipoPrime® 50T (hereafter Lipoprime) was kindly provided by Biopolis Ltd, the distributor of Novozymes A/S in Lithuania. Lipoprime is classified in the Chemical Abstracts Service Registry as "Lipase, triacylglycerol, CAS no. 9001-62-1." The corresponding Enzyme Classification number (International Union of Biochemistry) is EC 3.1.1.3. According to the Novozymes' product data sheet, Lipoprime is a protein-engineered lipase produced by submerged fermentation of a genetically modified *Aspergillus* microorganism. Lipoprime hydrolyzes fat by cleaving the ester bonds in the 1 and 3 position in triglyceride molecules. The enzyme has a broad substrate specificity promoting the hydrolysis of a wide range of different fats and oils. The product is produced by a non-pathogenic microorganism and is classified as non-toxic. The product preparations are biodegradable. The components of Lipoprime are listed in the relevant inventories, e.g., in the European Inventory of Existing Commercial Chemical Substances (EINECS) and Toxic Substances Control Act (TSCA).

All chemicals used in the study were of analytical grade. Oleic acid, diolein (DO, comprising 85% 1,3-DO and 15% 1,2-DO), monoolein (MO), methyl oleate, and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Fluka and Sigma; ethanol, 2-propanol, *tert*-butanol (2-methyl-2-propanol), petroleum and diethyl ethers, *n*-hexane, *n*-heptane, sodium hydroxide, ethyl acetate, acetic, *ortho*-boric, *ortho*-phosphoric, and hydrochloric acids were purchased from Lachema and Roth; trimethylolpropane and trimethylolpropane trioleate (TMP-TO) were purchased from Aldrich; silica gel G-25 plates for thin-layer chromatography (TLC) were purchased from Merck.

Standard spectrophotometric assay of hydrolytic activity

The hydrolytic effect of lipase on *p*-NPB solution in 2-propanol [5, 9] was investigated by measuring the change of optical density at 400–410 nm within 3–6 min at 30°C and pH 7.0–10.0, 100 mM universal buffer (Britton–Robinson buffer, composed of acetic, *ortho*-boric and *ortho*-phosphoric acids at a ratio of 1:1:1 providing buffering capacity over a wide range of pH) [2, 10].

Enzymatic synthesis of TMP esters

Transesterification and esterification for the synthesis of TMP esters were carried out in closed 20-ml batch reactors at a constant stirring speed, coupled to condensers in order to avoid the loss of reaction mixture components by volatilization, typically as follows: trimethylolpropane (0.60 g, 4.5 mmol) was dissolved in distilled water or *tert*-butanol (15% w/w of the total mass of the substrates), and then oleic acid methyl ester (4.67–6.00 g, 15.75–20.25 mmol) or oleic acid (4.45–5.72 g, 15.75–20.25 mmol) and a lipase preparation (40% w/w) were added [11]. According to the Novozymes' product data sheet, Lipoprime 50T has an activity of 50 KLU/g. The activity is determined relative to an enzyme standard under the assay conditions (hydrolysis of tributyrin, 30°C, pH 7.0). Novozymes uses a pH-stat method for measuring the standardized activity of Lipoprime 50T.

The reactions were conducted at a temperature of 37, 47 or 60°C. The reaction progress was followed by extracting 50-μl aliquots of reaction mixture at defined time intervals and analyzing by TLC. For analysis by the TLC method, samples were diluted with diethyl ether (350 μl), mixed vigorously, and kept at −20°C until chromatographic analysis [3].

Chromatographic analysis

The products of transesterification and esterification reactions were analyzed by TLC on TLC plates (5 × 10 and 10 × 10 cm) precoated with 0.25 mm Silica Gel 60 (Merck). TLC plates were run in triplicate. The samples were applied to the marked start edge of a TLC plate (1.0 cm above the lower edge of the plate) using a Hamilton syringe. The sample volume for all experiments was 2 μl. The plate was then air-dried for 10–15 min before transferring it to the TLC tank. The chromatograms were developed by employing eluents of light petroleum

(b.p. 40–60°C)/diethyl ether/acetic acid (85:15:2, v/v) [3] or *n*-heptane/ethyl acetate (96:4, v/v) [11] for the analysis of both reaction products. The tank containing the eluent was covered with a lid and thereby pre-saturated with vapor for at least 20–30 min at room temperature before use. A sample-loaded TLC plate was transferred to the TLC tank and developed for no less than a 4 cm (for 5 × 5 cm TLC plate) and 8 cm (for 5 × 10 cm TLC plate) migration distance of the solvent from the start line. The developed TLC plates were air-dried for about 10–15 min. Spots were visualized using a saturated iodine chamber and identified with reference to standards. Pure MetO, OA, TMP, TMP-TO, diacylglycerol (DAG), and monoacylglycerol (MAG) solutions in diethyl ether were used as standards.

Quantitative analysis (%) of reaction products separated by TLC (average of 3 assays) was performed with a Uvitec Cambrige Fire-reader imaging system and Uvitec Fire-reader software by photodensitometry assessing spot area and color intensity. Verification of the products yield was performed also using the Micro image 4.0 program which had been successfully applied in our previous experiments. The accuracy of this method was tested by determination of the residual (esterification reaction) or released (transesterification reaction) oleic acid content by titration against sodium hydroxide using phenolphthalein as indicator. The yields determined by TLC and titrimetry were in good agreement. The validation of the analysis was described elsewhere [1].

Statistics

Each experiment was carried out in triplicate. The results are presented as means ± standard error of the mean (SEM).

Results and discussion

The products of transesterification and esterification reactions were analyzed by TLC. The solvent system of *n*-heptane and ethyl acetate (96:4, v/v) was chosen according to the method described by Uosukainen and co-authors [11] and compared with our commonly used system comprising light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (85:15:2, v/v). An image of a TLC plate developed with each system is shown in Fig. 1.

As shown in Fig. 1, significant differences could be observed using different solvent systems. Our commonly used system (Fig. 1, lane 2) was found to be more informative for the separation and identification of reaction mixture components as compared with the system used by Uosukainen and co-workers (Fig. 1, lane 1).

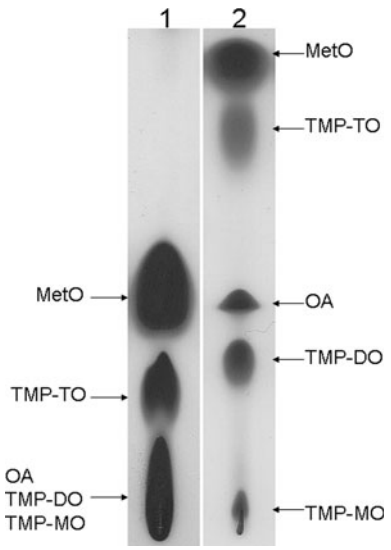

Fig. 1 Thin-layer chromatogram of reaction mixture components. Solvent systems: *1*, *n*-heptane/ethyl acetate (96:4, v/v); *2*, light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (85:15:2, v/v). *MetO*, methyl oleate; *TMP-TO*, trimethylolpropane trioleate; *TMP-DO*, trimethylolpropane dioleate; *TMP-MO*, trimethylolpropane monooleate; *OA*, oleic acid

The effects of the molar ratio of substrates, reaction temperature, and time on the efficacy of the MetO transesterification reaction with TMP in the environmentally friendly medium of water containing no organic solvents (hereafter aqueous solution) were investigated. No vacuum was used, and all experiments were carried out at ambient pressure. Figure 2 shows the effects of reaction temperature, time, and the molar ratio of TMP and MetO on the relative amount of reaction mixture components. A maximum total conversion to TMP-TO of around 30% was obtained with both studied molar ratios of the substrates at 47°C in 48 h, with only an insignificant incremental increase within 72 h. Although it is generally accepted that a large excess of free fatty acid or fatty acid alkyl ester is necessary to drive the reaction toward completion, from a practical point of view, a small amount of di- or monoesters is a much smaller problem than a large excess of unreacted free fatty acid or fatty acid alkyl ester. In compliance with the results shown in Fig. 2 and for the reasons described above, a molar ratio of TMP to MetO of 1:3.5 was chosen for the further experiments. Preliminary experiments clearly suggested that the conversion of MetO to the desired TMP esters increased with increasing temperature. For this reason, a temperature of 60°C was used for the further experiments in order to obtain higher yields of the product.

Considering the fact that reaction medium is a very important parameter for the effectiveness of lipase-catalyzed processes, the reactions in two different media were

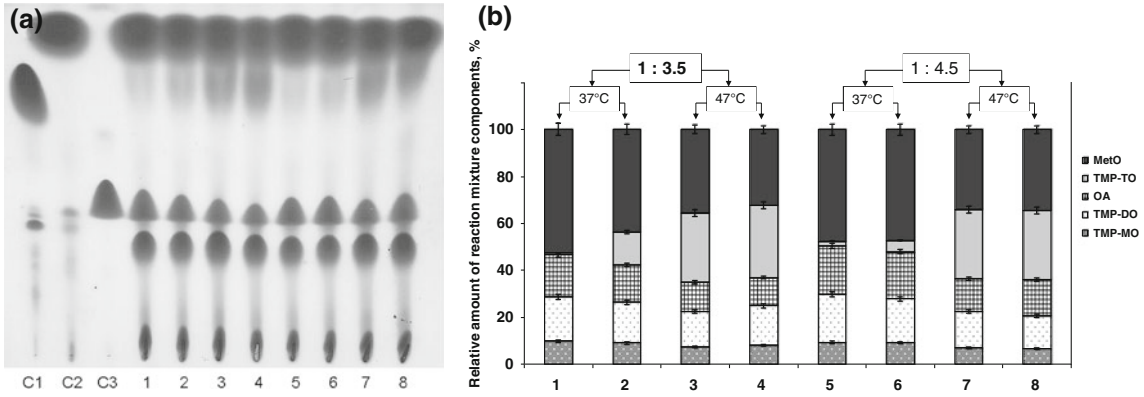

Fig. 2 Effects of temperature, time, and the molar ratio of substrates on Lipoprime 50T-catalyzed conversion of trimethylolpropane to trimethylolpropane esters in aqueous solution. **a** Thin-layer chromatograms. Control samples: C1, trimethylolpropane trioleate; C2, methyl oleate; C3, oleic acid. **b** Quantitative analysis (%). *1*, TMP/MetO = 1:3.5 (37°C, 48 h); *2*, TMP/MetO = 1:3.5 (37°C, 72 h); *3*, TMP/MetO = 1:3.5 (47°C, 48 h); *4*, TMP/MetO = 1:3.5 (47°C, 72 h); *5*, TMP/MetO = 1:4.5 (37°C, 48 h); *6*, TMP/MetO = 1:4.5 (37°C, 72 h); *7*, TMP/MetO = 1:4.5 (47°C, 48 h); *8*, TMP/MetO = 1:4.5 (47°C, 72 h). Symbols are the same as in Fig. 1

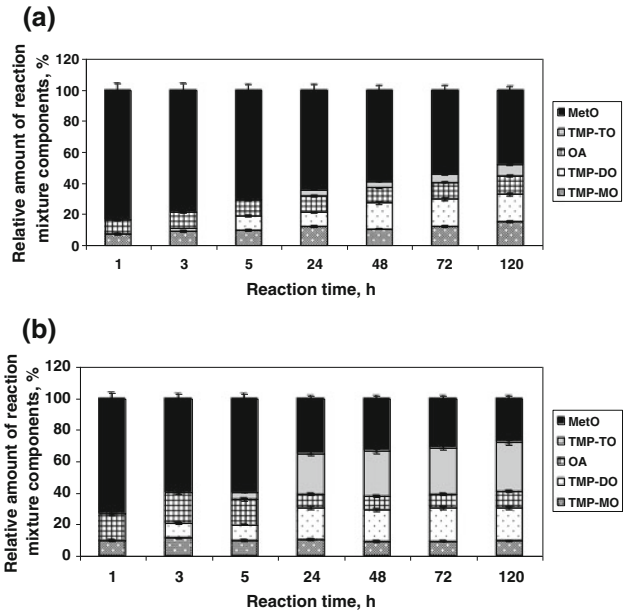

Fig. 3 Time course of Lipoprime 50T-catalyzed methyl oleate transesterification with TMP in *tert*-butanol (**a**) and aqueous solution (**b**). Reaction conditions: molar ratio of TMP to methyl oleate 1:3.5, reaction temperature 60°C. Symbols as in Fig. 1

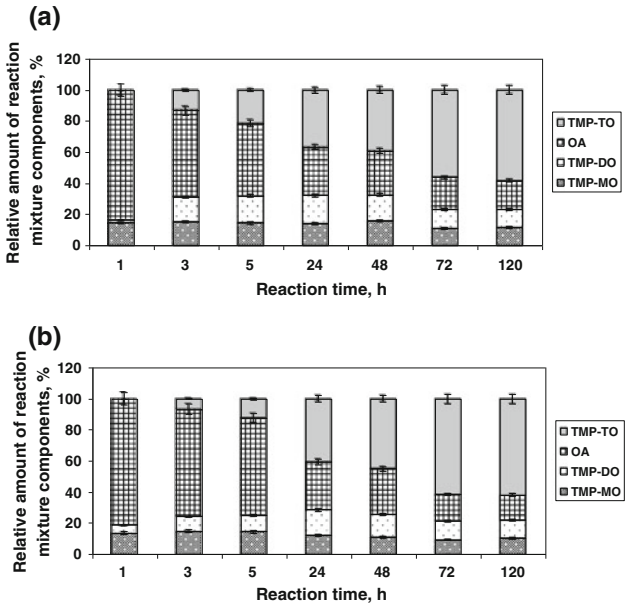

Fig. 4 Time course of Lipoprime 50T-catalyzed oleic acid esterification with TMP in *tert*-butanol (**a**) and aqueous solution (**b**). Reaction conditions: molar ratio of TMP to oleic acid 1:3.5, reaction temperature 60°C. Symbols as in Fig. 1

compared. Aqueous solution was used as a reaction medium to ensure an environmentally friendly process without any additional organic solvents that are also undesirable from the point of view of practical application. *tert*-Butanol was chosen as a reaction medium as it was reported to be a good solvent for lipase-catalyzed transesterification reactions due to its ability to eliminate the negative effects of excess short-chain alcohols on lipases. The negative effects caused by methanol and glycerol could therefore hopefully be eliminated by the ability of *tert*-butanol to dissolve both these alcohols [6, 8, 12]. Analogous experiments were carried out with OA and TMP to compare the effectiveness of both transesterification and esterification reactions and to evaluate the effect of methanol on the overall process. The results of transesterification and esterification reactions are shown in Figs. 3 and 4, respectively.

It was noted that both lipase-catalyzed processes in both solvents—*tert*-butanol and aqueous solution—were clearly time-dependent. Transesterification was more efficient in aqueous solution (Fig. 3b) than in *tert*-butanol (Fig. 3a),

Table 1 Effect of reaction conditions on the total conversion of trimethylolpropane (TMP) to trimethylolpropane trioleate

Substrates	Molar ratio	Temperature (°C)	Reaction medium	Trioleate yield (%)[a]		
				24 h	48 h	72 h
TMP/methyl oleate	1:3.5	37	Aqueous solution	0	1	14
TMP/methyl oleate	1:3.5	47	Aqueous solution	10	30	31
TMP/methyl oleate	1:3.5	60	Aqueous solution	26	29	30
TMP/methyl oleate	1:3.5	60	*tert*-Butanol	3	4	5
TMP/methyl oleate	1:4.5	37	Aqueous solution	0	2	4
TMP/methyl oleate	1:4.5	47	Aqueous solution	5	30	30
TMP/oleic acid	1:3.5	60	Aqueous solution	41	45	62
TMP/oleic acid	1:3.5	60	*tert*-Butanol	37	39	56

[a] Results are the mean of three repeats

whereas in the case of the esterification reaction no significant difference was observed between the reaction media (Fig. 4). The reason for these observations could be the difference in the reaction mechanisms. In the lipase-catalyzed transesterification reaction, a certain quantity of water is necessary for the enzyme to hydrolyze MetO, whereas the synthesis of TMP esters needs no additional water. The esterification reaction afforded a maximum total conversion to TMP mono-, di-, and trioleates of around 83% and to TMP-TO of about 62% with a TMP to OA molar ratio of 1:3.5, at a reaction temperature of 60°C in aqueous solution within 72 h, and a little further increase within 120 h (Fig. 4b). Although the yields are not high enough for industrial application, the process has the potential to be optimized in the near future as these yields are very close to those obtained under vacuum during analogous preliminary experiments described by Linko and co-workers [7]. A summary of the results is shown in Table 1.

The transesterification reaction was determined to be clearly temperature-dependent within 24 h in aqueous solution. The conversion of MetO to TMP-TO increases with increasing temperature. After 48 h or more, the yield of TMP-TO was nearly the same at both 47 and 60°C. The processes were determined to be least effective at 37°C; only negligible quantities of desired TMP-TO were obtained within 72 h. *tert*-Butanol was determined to be a poor solvent for the studied transesterification reactions. The yields of the product of esterification were determined to be almost the same in both reaction media.

Conclusions

The ability of the commercial lipolytic enzyme Lipoprime 50T to catalyze the synthesis of biodegradable oleic acid trimethylolpropane ester was investigated. Changes in the reaction mixture composition were followed by thin-layer chromatography and computer analysis methods. The yields of the esterification reaction product were determined to be almost the same in both aqueous solution and *tert*-butanol as a reaction medium. Esterification was determined to be more efficient than transesterification. Under the optimal conditions (aqueous solution, temperature 60°C, trimethylolpropane to oleic acid molar ratio 1:3.5, and reaction time 72 h), the highest TMP trioleate yield of around 62% and the overall yield of TMP mono-, di-, and trioleates of about 83% were obtained.

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