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ORIGINAL PAPER

Acidolysis of Tripalmitin with Oleic Acid Catalyzed by a Newly Isolated Thermostable Lipase

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Abstract The catalytic efficiency of a lipase from *Bacillus stearothermophilus* MC7 (lipase MC7) was evaluated in acidolysis of tripalmitin with oleic acid to yield dioleoyl-palmitoylglycerol, a structured triglyceride used in health food. The immobilized enzyme exhibits good operational thermostability with a half-life of 50 days at 60 °C in a solvent-free system. The degree of conversion exceeded 50% after 48 h. The side reaction of hydrolysis was suppressed. However, the monosubstituted product was prevalent in the product mixture. Tested in a broad range of solvents, lipase MC7 showed tolerance towards medium polarity.

Keywords *Bacillus stearothermophilus* MC7 · Thermostable lipase · Structured lipid · Non-solvent acidolysis

Introduction

Lipases have found various applications in the detergent, food, flavour, pharmaceutical, leather, textile, cosmetic, and paper industries. Their unique characteristics include substrate specificity, stereospecificity, regioselectivity, heterogeneous catalysis. Most of the industrial processes in which lipases are employed function at temperatures exceeding 45 °C in an almost anhydrous environment. So there is a continuous search for sources of highly active lipases with specific stability to pH, temperature, ionic strength and organic solvents [1, 2].

The object of our investigation is a lipase produced by the thermophilic bacteria *Bacillus stearothermophilus* MC7 (lipase MC7), isolated from a hot spring in Bulgaria [3, 4]. Its catalytic properties have not been investigated in detail yet. We chose to test its activity in the synthesis of a structured lipid, which is one of modern applications of lipases [5, 6]. OPO (1,3-oleyl-2-palmitoyl-glycerol) is used as a substitute for human milk lipids [7, 8]. Industrially OPO is produced by acidolysis of tripalmitin (PPP) with oleic acid with *Rhizopus delemar* and *Rhizomucor miehei* lipases as catalysts [9]. The main problems of the method are hydrolysis and the isomeric products [10]. These can be overcome by using active and selective enzymes or suppressing the acyl migration in organic media [11–13]. Yet, for food products the use of solvents is undesirable, so new biological sources are still being investigated in search of active, stable and specific lipases to catalyze non-solvent high-temperature acidolysis. In this paper we study the catalytic activity of immobilized lipase MC7 in acidolysis of PPP with oleic acid as a function of temperature and reaction medium.

Materials and Methods

Materials

Glyceryl tripalmitate (tripalmitin, PPP) (99% purity), oleic acid (98% purity) and all solvents used were purchased from Fluka (Germany). The solvents were dried over a 4-Å molecular sieve. DEAE-cellulose was purchased from Serva, Germany. The lipase from *B. stearothermophilus* MC7 (lipase MC7) was isolated and partially purified (ultrafiltration, purification factor 1.56) as described by Kambourova

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et al. [3, 4]. Aminopropyl SPE packing (cat. No 211516) and EXTRACT-CLEAN filter columns (8.0 mL, 50/PK, cat. No 211108) used for sample separation were purchased from Alltech Associates Inc. (USA). Analytical grade solvents for TLC were purchased from Merck (Darmstadt, Germany) and used without additional purification. Standard mono-, di- and triacylglycerols were from Sigma (St. Louis, MO, USA).

Immobilization of Lipase MC7 onto DEAE-Cellulose

A measure of 100 mg of DEAE-cellulose was soaked in 2 mL sodium phosphate buffer (50 mM, pH = 8.0) for 3 h. The activated carrier was then filtered, mixed with 2 mL of the enzyme solution (total activity 1,400 U mL^{-1}, specific activity 350,000 U g^{-1} protein) and kept for 12 h at 4 °C. The immobilized preparation was filtered and vacuum-dried at 20 °C. The lipase activities of the native and immobilized preparations were estimated using a spectrophotometric assay with 4-nitrophenyl palmitate as a substrate. One lipase unit (U) is equal to the quantity of enzyme liberating one μmol 4-nitrophenol per minute ($\varepsilon_{410\ nm} = 1.46 \times 10^5\ cm^{-1}\ M^{-1}$) [14].

Lipase-Catalysed Acidolysis Reaction

Tripalmitin (200 mg; 250 μmol) and oleic acid (280 mg; 1,000 μmol) were mixed in 2 mL of organic solvent at 60 °C. A measure of 100 mg (26,600 Ug^{-1} carrier) of the catalyst (lipase MC7 immobilized on DEAE-cellulose) was added. The non-solvent reaction was carried out with the same amount of substrates and enzyme. The separating water was removed by a flow of nitrogen. Samples (40 mg) were periodically taken from the reaction mixture and analysed. Operational thermostability was determined by transferring the immobilized biocatalyst to a fresh substrate mixture every 48 h.

Solid Phase Extraction

The reaction sample, dissolved in dichloromethane, was applied to a pre-conditioned silica aminophase. First, the glyceride fraction was eluted with dichloromethane/2-propanol (2:1, vol/vol). Second, the FFA fraction was eluted with diethyl ether/acetic acid (98:2). The fatty acids were then methylated according to [15].

Gas-Chromatographic Analysis of FFA Fraction

The derivatized fatty acids were analysed using a Shimadzu GC 17A gas chromatograph, equipped with a column SP WAX 52 CB (30 m × 0.25 mm; 0.25 μm, Supelco) and fitted with a flame ionization detector. The temperature was maintained at 165 °C for 10 min and then raised by 4 °C/min to 230 °C. The temperature of the injector was 260 °C and of the detector was 280 °C. Nitrogen was used as the carrier gas.

Thin-Layer Chromatography of the Glyceride Fraction

Mono-, di- and triacylglycerols (TAGs) were separated and isolated by thin-layer chromatography on silica gel G on glass plates using hexane/acetone (100:6, vol/vol) as developing solvent.

The triacylglycerol classes were separated and identified according to the overall degree of unsaturation by silver ion thin-layer chromatography (Ag+-TLC) [16]. Silica gel G on glass plates was impregnated by dipping into 0.5% solution of $AgNO_3$ in methanol. The triacylglycerol fraction was dissolved in hexane. The mobile phase used was hexane/acetone (100:4, vol/vol). For the identification a standard mixture of triacylglycerols was used, containing all possible combinations of saturated, mono- and dienoic fatty acid moieties [17].

The quantitative ratio of tripalmitin (PPP), oleyl-dipalmitin (OPP), di-oleyl-monopalmitin (OOP) and triolein (OOO) in the reaction samples was determined by Ag+-TLC/densitometry. For that purpose the developed chromatographic plates were visualized by successive saturation with bromine and sulfuryl chloride vapours, heating at 200 °C and carbonization. A Shimadzu CS-930 densitometer was used at 450 nm. The quantity of each of the TAG classes was calculated as a percentage of the total peak area. All data are reported as the mean of at least three replications ± standard deviation. The positions of the acyl residues in the triglyceride were not identified.

The degree of tripalmitin conversion (DC_{PPP}) is defined as per cent of the initial quantity of tripalmitin undergone transformation:

$$DC_{PPP} = \frac{[PPP]_0 - [PPP]_t}{[PPP_0]} \times 100,$$

where $[PPP_0]$ and $[PPP_t]$ are concentrations of tripalmitin at the beginning and at a given time of the reaction, respectively.

Results and Discussion

Higher operation temperatures in the synthesis of structured triglycerides ensure better homogenization and a minimal risk of bacterial contamination of the mixture; shorter

reaction time and thus, a lower number of isomeric products [1]. Hence biocatalysts operating in non-solvent high-temperature reaction systems are needed. Lipase MC7 demonstrates comparably high thermal stability: the native enzyme has a half-life of 30 min at 70 °C in aqueous solution and 30 min at 85 °C in the presence of 1% substrate [3, 4]. Under the conditions of the studied non-solvent synthesis of OPO (Fig. 1) the thermostability of the immobilized preparation is even higher: half-life of 50 days at 60 °C, which is comparable to commercial *Rhizopus oryzae* lipase immobilized on Dowex with $\tau_{1/2} = 60$ days (50 °C) in hexane. Nagao et al. [11] reported a mutant *Fusarium heterosporum* lipase R275A with the impressing half-life of 375 days at 50 °C in hexane.

There are some examples of high-yield and -purity synthesis of the target OPO by acidolysis of PPP using active and selective enzymes in hexane [11, 12]. But there is little information on conducting this reaction in solvent-free system, which has many advantages like maximum substrate concentration, no toxic and expensive solvents, and reduced down-stream processing [18]. The immobilized lipase MC7 showed high activity in non-solvent acidolysis (Table 1). The degree of conversion exceeded 50% after 48 h without preincubation. Tri-, di-, and monoacylglycerols were 84.8, 13.6, and 1.6% of the glyceride fraction. The low degree of hydrolysis was probably due to the effective removal of the separating water by the continuous flow of nitrogen. The yield of structured lipids with nutritional value (at least one oleoyl residue at sn-1,3 and palmitoyl residue at sn-2) was over 90% of product triacylglycerols, but the monosubstituted product was prevalent. The content of the unwanted OOO was low (Table 1).

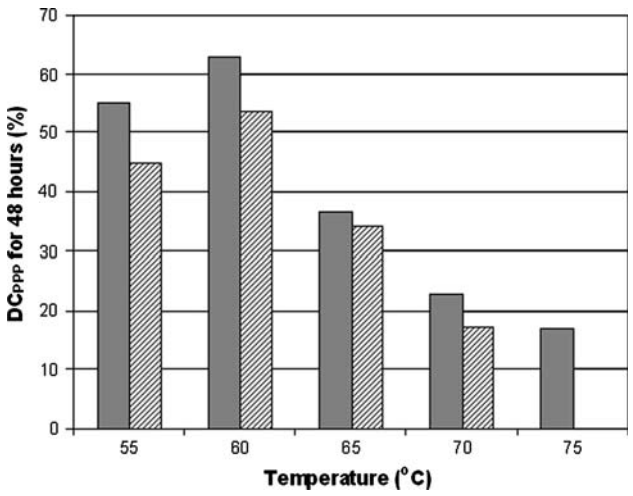

Fig. 1 Influence of temperature on the degree of conversion of tripalmitin by immobilized lipase MC7. Reaction conditions: tripalmitin (200 mg); oleic acid (280 mg); catalyst (100 mg, 26,600 U/g) were mixed: (*filled bars*) in 2 mL i-octane; (*striped bars*) without solvent

Table 1 Degree of conversion and ratio of TAG products as a function of solvent polarity

Solvent	log *P* [19]	DC_{PPP} (%)	Product TAGs ratio (%)		
			PPO[a]	POO[a]	OOO
No solvent	–	53.5	66.80	25.29	7.88
n-hexadecane	8.8	41.8			
n-dodecane	6.6	50.5			
n-decane	5.5	34.0			
i-octane	4.5	62.9	54.17	32.28	13.54
n-heptane	4.0	54.0			
n-hexane	3.5	48.0	68.37	24.50	7.12
Toluene	2.5	17.1			
Tetrahydrofuran	0.5	10.6			
DMSO	−1.3	7.6			

Reaction conditions: tripalmitin (200 mg); oleic acid (280 mg); catalyst (100 mg, 26,600 U/g); $T = 60$ °C; 2 mL of solvent or without solvent

[a] Does not indicate the positions of the acyl moieties in the molecule

The acidolysis activity of immobilized lipase MC7 was assayed in a series of solvents of different polarity. Polar solvents ($\log P < 3.5$) strongly inhibited the enzyme. DC_{PPP} was highest in *i*-octane, hexane and heptane and comparable to that in the non-solvent reaction. However, when hexane and heptane were used as reaction media, an unidentified peak with Rt = 24.5 min appeared in the gas-chromatogram of the FFA-fraction. We assumed this was a product of the oxidation of the oleic acid as a result of the prolonged heating (60 °C for 48 h). At the end of the synthetic reaction its quantity was 3.7% for hexane and 13.6% for heptane. In the case of *i*-octane, only at higher temperature (70 °C, 48 h) this product exceeded 10%, whereas in the non-solvent reaction it was not detected. The enzyme stability in organic solvents can be advantageous for various nonfood applications such as: detergent and biodegradable polymer production, cosmetics, racemic mixture resolution, sewage treatment, pharmaceuticals.

This synthetic reaction is a good model revealing the ability of lipase MC7 to work in a non-solvent system at high temperature for long reaction time. Lipase MC7 also showed tolerance towards solvent polarity, which can be advantageous for laboratory synthesis of different esters.

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