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Pseudomonas mendocina 3121-1 lipase-catalysed reaction of oleic acid with glycerol

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The specificity of *Pseudomonas mendocina* 3121-1 lipase for oleic acid esterification with glycerol was investigated.

The examination of esterification of oleic acid showed that the most effective conditions for this reaction were obtained at the 1:7 molar ratio of fatty acid (FA) to glycerol at a temperature of 30 ^{0}C.

By thin layer chromatography (TLC) it has been determined that 2-methyl-2-propanol (*tert*-butanol) is not a substrate for *Ps. mendocina* (*PmL*) 3121-1 lipase, because there was no ester formation.

Key words: bacterial lipase, ensymatic esterification, thin layer chromatography

INTRODUCTION

Enzyme-catalysed reactions of lipids are of considerable interest in view of their possible applications in the biotechnology of fats and oils. Lipases (glycerol ester hydrolases; EC 3.1.1.3) have been used successfully as biocatalysts in esterification and ester exchange reactions, such as acidolysis, alcoholysis, and transesterification [1, 2]. Mono- and diglycerides (MG and DG) of fatty acids (FA) are widely used as emulsifiers and stabilizers (E. E. C. code: E 471) in commercial food. These food-grade surfactants are manufactured on an industrial scale by chemical glycerolysis of fats and oils. A high temperature (220–260 ^{o}C) and inorganic base catalysts are used. The products are purified through high-vacuum distillation. Limitations of the chemical processes include formation of undesirable dark color, burnt taste, maximum yield of mono-acylglycerols 40–60% and environmentally undesirable byproducts. Chemical processes are not quite suitable for those heat-sensitive oils and fats as regards their nutritional or biological properties. The World Health Organisation (WHO) directives concerning the composition of additive E 471 mention that: 1) the content of MG and DG must be at least 70% (w/w) of the mixture; 2) the MG content must be <30% (w/w); 3) no more than 3, 7, and 10% of FA, free glycerol, and triglyceride (TG), respectively, are allowed in the mixture [1–4]. Production of specifically structured lipids in which FA content and positional attachment on the glycerol molecule is achieved by design is best accomplished by enzymatic techniques [5].

In this study, we report the results on the enzymatic synthesis of glycerol esters by direct esterification of glycerol with oleic acid using *Pseudomonas mendocina* 3121-1 lipase.

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MATERIALS AND METHODS

Pseudomonas mendocina 3121-1 lipase was purified and kindly provided by the Institute of Biochemistry. p-Nitrophenylbutyrate (pNPB), triolein (TO) (C18:1, [cis]–9) and lipase substrate were received from Sigma; gumarabic and Tris were from Serva; glycerol, 2-propanol, 2-methyl-2-propanol, ethanol, n-hexane, petrol and diethyl ethers, acetic, boric, hydrochloric and phosphoric acids were from Lachema, and silica gel G-25 plates were from Merck.

The standard spectrophotometric assay of the hydrolytic activity of lipase on p-nitrophenylbutyrate (p-NPB) solution in 2-propanol was employed measuring the change of optical density at $\lambda = 400–410$ nm during 3–6 min at 30 or 37 $^\circ$C and pH 7.5 (50 mM Tris-HCl buffer) or pH 7.0–9.0, 100 mM universal (Ub) or Briton–Robson buffer [6–8]. The final concentration of the enzyme was 0.04–1 μg/ml, of the substrate 0.1–0.5 mM and of organic solvent 2% [6]. One unit of the hydrolytic activity corresponds to the amount of the enzyme releasing 1 μmol of fatty acid per minute under standard conditions.

Investigation of esterification reaction. Reaction between oleic acid and glycerol (molar ratio 1:2, 1:3, 1:5 and 1:7) and lipase solution in 50 mM Tris-HCl buffer, pH 7.9 (final concentration of the enzyme 0.07 mg/ml) was performed in hexane and *tert*-butanol [10, 11]. The reaction mixture was incubated for 1–74 hours at 30 $^\circ$C in a thermostated 10 ml glass vessel with constant stirring and temperature control. The reaction progress was followed by extracting 200–500 μl aliquots of reaction mixture at definite time intervals

and analysing them. The reaction was stopped by adding 9.5 ml of methanol / acetone mixture (volume ratio 1:1). The residual amount of oleic acid was determined by titration with 0.1 M NaOH solution in methanol at 20 ˚C. Solution of phenolphthalein in ethanol was used as the indicator. For analysis by the TLC method, samples were diluted with diethyl ether (volume ratio 1:1) and frozen. The analysis was provided as described below.

Thin layer chromatography (TLC) method. A modified method of Yadav et al. [12] was used. Chromatographic analysis was carried out by TLC on silicagel G-25 plates impregnated with boric acid. The system of solvents for the elution: light fraction of petrol ether/diethyl ether/acetic acid (80:30:1 (v/v) according to [12, 13] and 70:30:1 – our modification [6, 10]. The chromatograms were developed with iodine vapour. Solutions of oleic acid, the TAGs mentioned, 1.3/1.2-diolein (DO, content of 1.3-DO 85%, of 1.2-DO 15%) and monoolein (MO) in diethyl ether were used as standards. Chromatograms of the quantitative analysis (%) of reaction products on silicagel-coated glass plates (average of 3–4 assays) were performed using the micro image 4.0 program considering the spot area and intensity.

RESULTS AND DISCUSSION

The enzyme from *Pseudomonas mendocina* 3121-1 was found to exhibit the substrate specificity and other features different from those of lipolytic enzymes from *Ps. mendocina* described in literature [6–8, 14].

The specificity of *Pseudomonas mendocina* 3121-1 lipase for various triacylglycerols (TAGs) was investigated before [6, 13]. It was determined that lipolysis of TAGs with long-chain fatty acids was the most effective. The thin layer chromatography investigation of the catalytic activity upon TAGs composed of long-chain unsaturated fatty acids illustrated that *Ps. mendocina* lipase was regioselective for ester bond at sn-3 position, stereoselective for cis enantiomer of C18:1 fatty acid and specific for TAG composed of long-chain polyunsaturated fatty acids [6, 14].

Examination of esterification of various acids (C12–C18 and C18:1) with alcohols of different structure catalysed by *Ps. mendocina* lipase showed that the reaction was most effective using 1-heptanol, and the enzyme was more specific for oleic acid and long-chain saturated acids. No ester formation was determined using phenylmethanol, but cholesteryl oleate was detected after two weeks of the reaction of oleic acid with cholesterol catalysed by *Ps. mendocina* lipase. It has also been determined that the enzyme is more specific in interesterification reactions: for alcoholysis of methyl-dodecanoate (laurate) and oleate with 1-heptanol and acidolysis of methyllaurate with oleic acid (unpublished data).

In this study, also lipase-catalysed esterification of oleic acid with glycerol was investigated using the bacterial lipase *Ps. mendocina* 3121–1. All reactions were performed in a mixture of organic solvents 2-methyl-2-propanol (*tert*-butanol) and hexane, ratio (w/w) 1:1). *tert*-Butanol was chosen for several reasons: it is not toxic; it is a good solvent for both oleic acid and glycerol (glycerol is slightly soluble in hexane); it has no denaturating effect on lipase activity [11]. Flores et al. [15] have reported that *tert*-butanol is not a substrate for *C. rugosa* lipase. For this reason we examined the effect of *tert*-butanol in the esterification reaction of oleic acid. The results are presented in Fig. 1. No oleic acid esterification with *tert*-butanol was found.

The following scheme of the lipase-catalysed esterification of oleic acid with glycerol using bacterial lipase *Ps. mendocina* 3121-1 under the indicated conditions was used: 1) a temperature of 30 °C; 2) the molar ratio of oleic acid and glycerol: I–1:2, II–1:3, III–1:5, IV–1:7; and 3) reaction duration time: 1–74 h.

To investigate the effect of the ratio of reaction components on the esterification process, the enzymatic reactions were performed at various glycerol concentrations. The time and molar ratio of reaction components courses of product formation by *PmL* are shown in Table.

The first molar ratio investigated (1:2) had not effect on the quantity of product at reaction duration

Table. **Quantity of reaction products (%) depending on molar ratio of reaction components and duration at 30 °C**

The molar ratio of oleic acid:glycerol	The amount of products, %		
	2 h	8 h	50 h
1. 1:2	56.7	58.9	54.7
2. 1:3	67.4	73.6	65.1
3. 1:5	72.6	73.3	73.3
4. 1:7	77.3	85.9	75.4

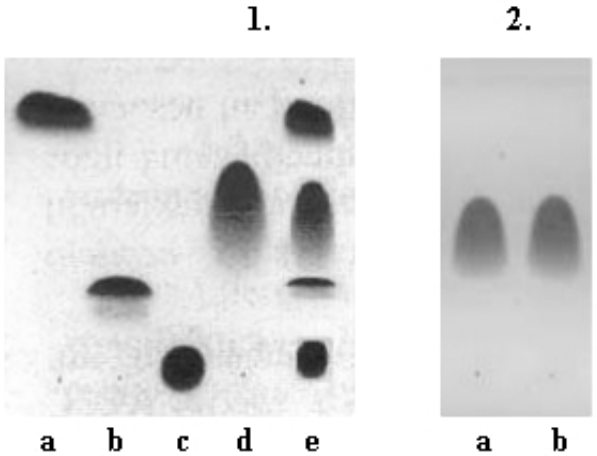

Fig. 1. The specificity of *PmL*. 1: Control: *a* – TO; *b* – DO (1,3-DO-85%, 1,2-DO-15%); *c* – 1-MO; *d* – oleic acid; *e* – mixture of al components; 2: *a* – pure oleic acid; *b* – oleic acid after 50 h incubation at 30^{0}C with *tert*-butanol and *PmL*

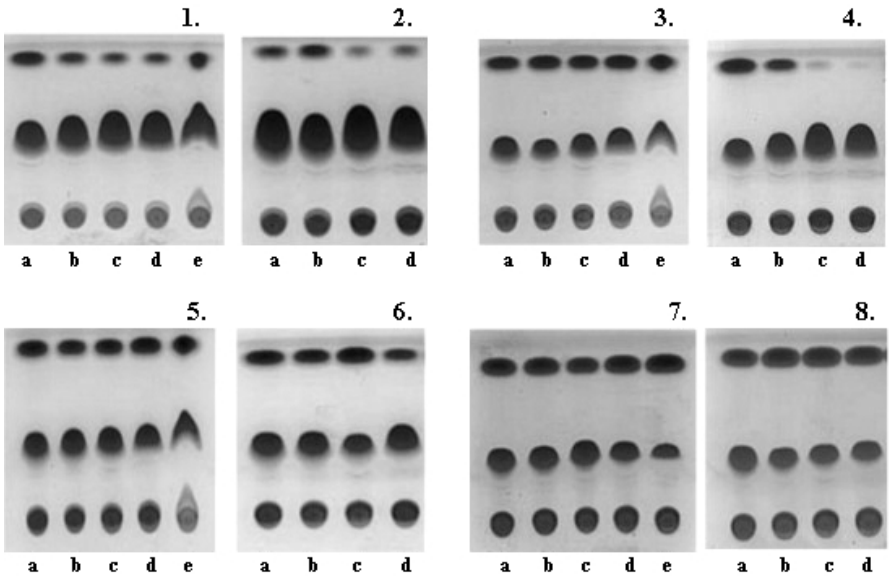

Fig. 2. The progress of lipase-catalysed esterification reaction analysed by TLC. 1. (molar ratio 1:2): *a* – 1 h; *b* – 2 h; *c* – 4 h; *d* – 6 h; *e* – 8 h. 2: *a* – 10 h; *b* – 24 h; *c* – 50 h; *d* – 72 h. 3. (molar ratio 1:3): *a* – 1 h; *b* – 2 h; *c* – 4 h; *d* – 6 h; *e* – 8 h. 4: *a* – 10 h; *b* – 24 h; *c* – 50 h; *d* – 72 h. 5. (molar ratio 1:5): *a* – 1 h; *b* – 2 h; *c* – 4 h; *d* – 6 h; *e* – 8 h. 6: *a* – 1 h; *b* – 2 h; *c* – 4 h; *d* – 6 h; *e* – 8 h. 2: *a* – 10 h; *b* – 24 h; *c* – 50 h; *d* – 72 h. 7. (molar ratio 1:7): *a* – 1 h; *b* – 2 h;*c* – 4 h; *d* – 6 h; *e* – 8 h. 8: *a* – 10 h; *b* – 24 h; *c* – 50 h; *d* – 72 h

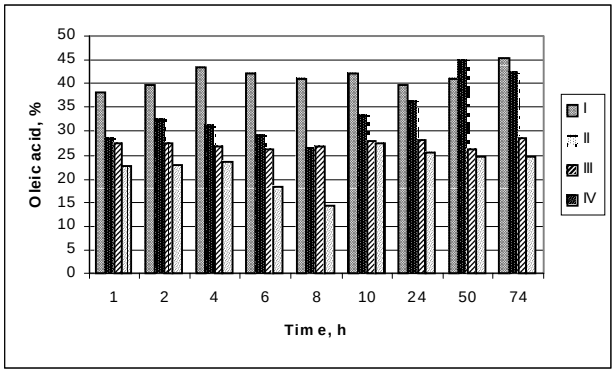

Fig. 3. The FA concentration and time course of esterification at 30 ^{0}C. (Initial concentration of oleic acid is 100%). I – molar ratio 1:2; II – molar ratio 1:3; III – molar ratio 1:5; IV – molar ratio 1:7

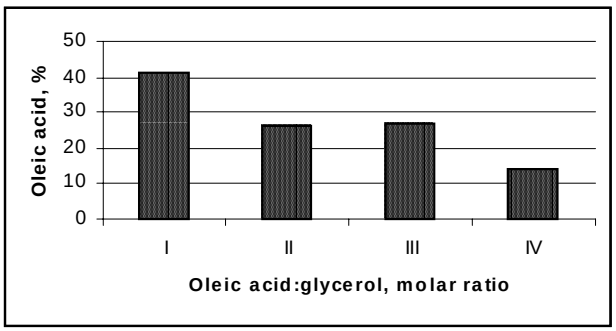

Fig. 4. The amount of oleic acid (%) in *PmL*-catalysed esterification at 30 ^{0}C after 8 h. The oleic acid – glycerol molar ratio: I 1:2; II 1:3; III 1:5; IV 1:7

of 2–50 h. The largest amount of products was detected at III and IV molar ratios oleic acid-glycerol (Figs. 3, 4).

Figures 2–4 show that *PmL* displayed the highest synthetic activity after 8 h. Esterification of oleic acid with glycerol strongly depended on the concentration of glycerol in reaction mixture and on the duration of reaction. The presence of water in reaction mixture moderately influenced the process of esterification and at molar ratio I after 2, 8 and 50 hours of reaction the amounts (%) of the product were 56.7, 58.9 and 54.7, respectively. But the quantitative change was much more significant at molar ratio II, reaching 67.4, 73.6, 65.1%; at molar ratio III – 72.6, 73.3, and 73.3%; at molar ratio IV – 77.3, 85.9; and 75.4, respectively. In this case, a very strong product inhibition of water occurred at molar ratio IV after 50 h, because about 10% (85.9% after 8 h and 75.4% after 50 h) of esters was converted to free oleic acid. The content of water is a very important factor directing the thermodynamic balance of reaction to hydrolysis or synthesis. The content of water was not regulated in this assay and therefore after 50 h the hydrolytic activity of *PmL* was observed. At most products, also after 8 h by various lipases (Novozym 435, Lipozyme IM 60, *Ps. spec.)* catalysed esterification of stearic acid with glycerol was found [16]. The water present in the reaction mixture and produced by esterification reaction might retard the synthesis of esters. Water content has been reported to reduce markedly the rate of immobilised lipase-catalysed esterification or transesterification [17–20].

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PS. MENDOCINA 3121-1 LIPAZĖS KATALIZUOJAMAS OLEINO RŪGŠTIES ESTERINIMAS GLICEROLIU

Santrauka

Ištirtas *Pseudomonas mendocina* 3121-1 lipazės katalizuojamo oleino rūgšties esterinimo gliceroliu specifiškumas. Reakcija vyksta efektyviausiai esant 1:7 oleino rūgšties ir glicerolio moliniam santykiui 30 ˚C temperatūroje, kai reakcija trunka 8 valandas. Nustatyta, kad vanduo skatina grįžtamąją reakciją – susidariusių esterių skilimą, t. y. hidrolizę, kad lipazė nekatalizuoja oleino rūgšties esterifikacijos *tret* butanoliu – vienu iš reakcijos sistemos tirpiklių.