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The specificity of *Pseudomonas mendocina* 3121-1 lipase. Hydrolysis

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The substrate specificity of a lipolytic enzyme from *Pseudomonas mendocina* 3121-1 regarding various oils (linseed, maize, olive, sunflower and soy-bean), triacyl glycerols (TAGs) (triacetin, tributyrin, tricaprin, tripalmitin, tristearin and triolein) and Tween type (20, 40, 60, 80 and 85) non-ionic detergents was investigated. *Ps. mendocina* lipase showed the highest lipolytic activity with regard to long chain TAGs, especially triolein, *i.e.* a substrate containing unsaturated fatty acid, and to olive and maize oils. The Tweens were used both as surfactants and substrates. Lipase hydrolysed all Tweens, and the efficiency of the hydrolysis was found to depend on their concentration and structure. This indicated that the Tweens examined could not be used for substrate emulsion stabilization, at least at a concentration of 11–44 mM. The enzyme showed the highest activity on Tween 85, *i.e.* polyoxyethylensorbitan trioleate, whose structure is close to that of triolein. The catalytic activity towards Tweens was found to be lower than towards triacylglycerols (TAGs) at the same concentration. In the presence of both olive oil emulsion and Tween 85 aqueous solution of a relatively low (0.8% (w/w) concentration the detergent seemed to act as a surfactant rather than a a substrate, because the hydrolysis in the system increased to a higher degree than the decomposition of Tween. Tween 85 was found to be the best substrate among all the detergents studied, and a 10% (w/w) concentration was found to be optimal for the hydrolysis by lipolytic enzyme from *Pseudomonas mendocina* 3121-1.

Key words: *Pseudomonas mendocina* lipase, hydrolysis, fats, Tweens

INTRODUCTION

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are enzymes which have been classically employed to carry on hydrolysis with concommitant production of free fatty acids. The fat splitting ability of lipases allows a wide range of applications for these enzymes in industrial products and processes. However, these enzymes also display catalytic activity towards a large variety of alcohols and acids in ester synthesis reactions and in modification of fats and other lipids via hydrolysis, esterification and transesterification [1–3]. Lipases hydrolyze triglycerides according to their specificity, which has been defined by Jensen et al. [4], as a comparative difference in the rates of catalysis of certain reactions. If the substrate contains three different fatty acids, they can be hydrolyzed at three different rates. Similarly, the regioselectivity of triacylglycerol lipases for the fatty acids esterified at definite positions of TAGs, *e.g.*, sn-1,3-positions, has been utilized to enrich those fatty acids via kinetic resolution through selective hydrolysis or transesterification.

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Most of lipases have been shown to be specific for TAGs of moderate chain length [5–7]. *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) [8], *Mucor miehei*, *Geotrichum candidum* [9], *Ps. spec.* ATCC [10] and some other lipases could be attributed to those specific for long-chain unsaturated triacylglycerols.

The most commercially important field of application of hydrolytic lipases is their addition to detergents used mainly in household and industrial laundries and household dishwashers. These enzymes can reduce the environmental load of detergent products, since they save energy by enabling a lower wash temperature to be used; allow the content of other, often less desirable, chemicals in detergents to be reduced; are biodegradable, leaving no harmful residues; have no negative impact on sewage treatment processes, and not present no harm to aquatic life. An estimate of 1000 tons of lipases are added to the approximately 13 million tons of detergents produced each year. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, originated from the fungus *Thermomyces lanuginosa* (formerly *Humicola lanuginosa*) and was expressed in *Aspergillus oryzae* [11].

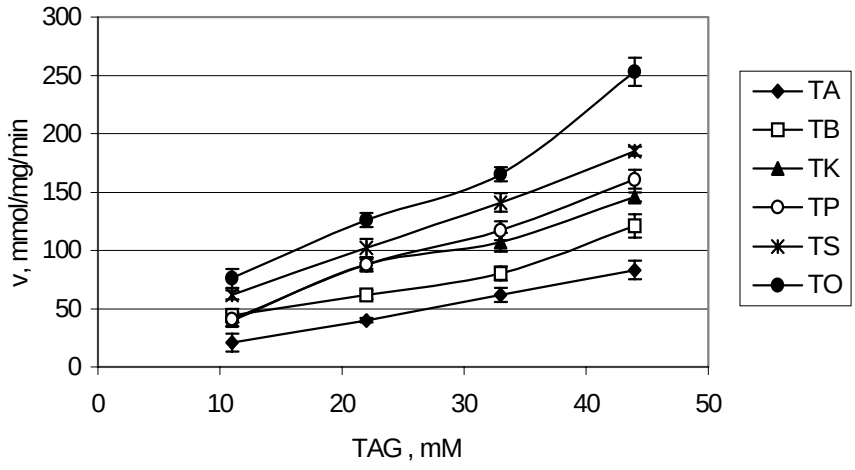

Fig. 1. The role of chain length of fatty acid in triacylglycerol (TAG) in lipolytic activity. The activity was determined by the standard titrimetric (continuous) method. Triacetin (TA) solution, mechanically stirred tributyrin (TB) emulsion and tricaprin (TC), tripalmitin (TP), tristearin (TS) and triolein (TO) emulsions prepared by ultrasonic treatment in 8% (w/w) gum-arabic aqueous solutions were used as substrates

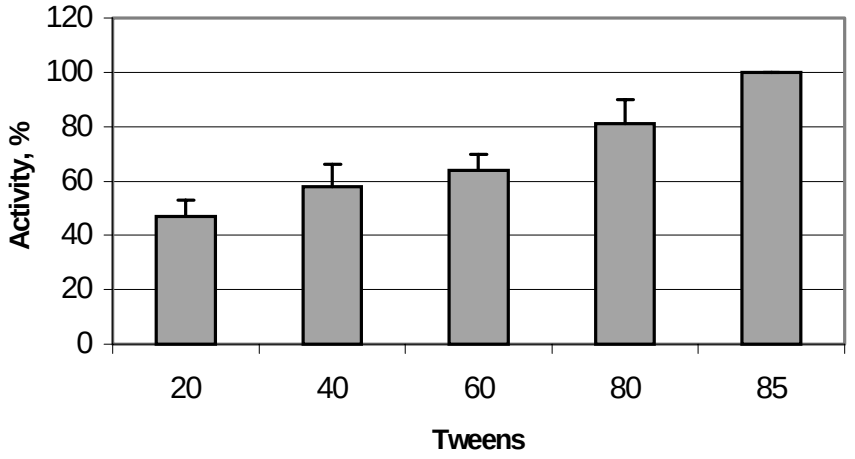

Fig. 2. Effect of Tweens structure on lipolytic activity of *PmL*. The activity was determined by the standard titrimetric method in the presence of Tween at 10% (w/w) final concentration. Maximal activity (upon Tween 85) corresponds to 144 ± 4 mmol/mg · min

Table 1. **Lipolytic effect of *Ps. mendocina* lipase on various substrates**

Substrate (10%, v/v)	Lipolytic activity (%)
Olive oil gum arabic emulsion	98 ± 2
Olive oil PVA emulsion	66 ± 4
Triacetin (aqueous solution)	42 ± 3
Soy bean oil-gum arabic emulsion	90 ± 3
Sunflower oil gum arabic emulsion	72 ± 5
Linseed oil gum arabic emulsion	69 ± 6
Maize oil-gum arabic emulsion	96 ± 2
Triolein gum arabic emulsion	100

The object of the present paper is *Pseudomonas mendocina* 3121-1 lipase. The enzyme was found to exhibit the substate specificity and other features different from those of lipolytic enzymes from *P. mendocina* described in the literature [12, 13].

The purpose of this review is to cover the specificity of *Pseudomonas mendocina* lipase 3121-1 in view of the splitting ability of oils, triacylglycerols and Tweens.

MATERIALS AND METHODS

Pseudomonas mendocina lipase (*PmL*) 3121-1 was purified and kindly provided by Institute of Biochemistry. The concentration of the enzyme was determined spectrofotometrically. p-Nitrophenylbutyrate (pNPB), triacetin (TA), tributyrin (TB), tricaprin (TC), tripalmitin (TP), tristearin (TS), triolein (TO) (C 18:1 [cis]-9 were received from Sigma; maize, sunflower, soybean and linseed oils were food stuffs; gum arabic and Tris were from Serva; 2-propanol, ethanol, n-hexane, petrol- and diethyl ethers, acetic, boric, hydrochloric and phosphoric acids were from Lachema and silica gel G-25 plates from Merck.

The activity was measured spectrophotometrically by following the increase in absorbance at 405 nm, which accompanies the hydrolysis of the synthetic substrate pNPB as described in an accompanying paper [13]. All measurements were performed in 100 mM Briton-Robson (universal) (UB), pH 7.0–9.0 or in 50 mM Tris-HCl, pH 7.5 buffers. All solutions were prepared in twice-distilled water.

Cuvettes were pre-heated at 30 °C for 10 min and the assay was started by addition of 0.1 ml of the enzyme. One unit of lipase hydrolytic activity (HA) corresponds to the amount of the enzyme releasing 1 μmol of p-nitro phenol per minute under standard conditions.

The titrimetric assay [13, 14] for the determination of lipolytic activity upon emulsified substrates and Tween solutions: the final concentration of oil

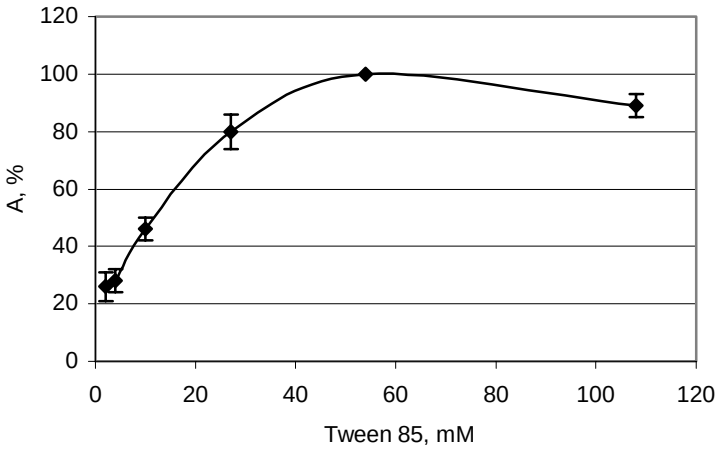

Fig. 3. Effect of Tween 85 concentration on lipolytic activity of *PmL*. The activity was determined by the standard titrimetric method in the presence of 2–108 mM Tweens concentration. 100% activity corresponds to 144 ± 4 mmol/mg · min

Table 2. **Effect of 0.8% (v/v) concentration of Tweens on lipase-catalysed lipolysis of "true" substrate**

Tweens	"True" substrate: emulsion A (10% (v/v) olive oil 8% (v/v) gum arabic)	Activity, %		
		1	2	3
20	+	111 ± 5	132 ± 4	7 ± 1
40	+	107 ± 5	124 ± 3	5 ± 1
60	+	103 ± 2	108 ± 2	3 ±1
80	+	136 ± 4	133 ± 4	11 ± 2
85	+	130 ± 3	129 ± 3	18 ± 3
–	+	100	100	100

1. 1 ml of Tween and 0.25 ml of enzyme solutions were incubated at 37 °C for 20 min, then 1 ml of emulsion A was added and after 3 min the reaction was ended by adding 6.5 ml of ethanol
2. 1 ml of Tween solution and 1 ml of emulsion A were incubated at 37 °C for 20 min, then 1 ml of enzyme solution was added and after 3 min the reaction was ended
3. 1 ml of Tween and 1 ml of enzyme solutions were incubated at 37 °C for 3 min. The reaction was ended by adding 6.5 ml of ethanol and then 1 ml of emulsion A was added

was 10% (v/v), gum arabic and PVA 8.0% (v/v), triacylglycerols 10–50 mM, Tweens 0.8–20% (v/v).

Tweens aqueous solutions and Sigma stabilized olive oil emulsion or emulsions of TAGs in aqueous gum arabic solutions were used as substrates. The mixture of the substrate and buffer (final volume 8 ml) was adjusted to pH 8.0 by 1N NaOH, and the pH was maintained for 3 min by titration with 50 mM NaOH solution (blank). Then 1 ml of the lipase solution in the buffer was added and the lipolytic reaction was observed for 5 min by titration as mentioned above (sample) [2]. One unit of lipolytic activity corresponds to the amount of the enzyme releasing 1 mmol of fatty acid per minute under standard conditions.

Tween solutions in 50 mM Tris-HCl buffer, pH 7.5 (30 °C) were prepared by heating, and TAGs and oil-gum arabic emulsions were prepared by ultrasonic treatment.

Thin layer chromatography (TLC). A modified method of Yadav et al. was used [3]. The reaction mixture, containing 0.4 ml of TAG or oils, 4 ml of 50 mM Tris-HCl buffer, pH 7.5 (30 °C) or n-hexane and 0.1 ml lipase solution, was incubated at 30 °C under intensive continuous stirring for 0–24 h. The reaction was terminated by adding 25 ml of diethyl ether and the extract was frozen. It was later analysed by TLC (boric acid-impregnated silica gel G-25 plates). The system of solvents for the elution: light fraction of petrol ether/diethyl ether/acetic acid (80:30:1). Chromatograms were developed by iodine vapor. Solutions of oleic acid and TAGs in diethyl ether were used as standards.

RESULTS AND DISCUSSION

Although soluble substrates are usually used for kinetic measurements of lipolytic reaction, "true" substrates of lipases are various oils and fats. Lipases are active only in emulsified oils, TAGs or other water-insoluble substrates and act at the water–oil interface. Lipases break down triglycerides to di- and monoglycerides, glycerol and free fatty acids. We investigated the lipolytic activity of *Pseudomonas mendocina* 3121-1 lipase upon emulsions of pure oils and TAGs in gumarabic aqueous solution. Results concerning the lipolytic activity of *PmL* regarding various oils are summarized in Table 1 and Fig. 1.

The forms of curves clearly show that the lipolytic process did not obey the Michaelis–Menten model. It should be noted that determination of kinetic parameters using emulsified substrates is rather complicated, even indicating them as apparent. Taking into account that lipolysis is affected by many factors, those parameters were not calculated in our experiment. Nevertheless, it could be concluded that *PmL* showed the most effective lipolytic activity upon long chain TAG, especially TO, *i.e.* a substrate containing unsaturated fatty acid. Most of lipases have been shown to be specific for TAG of a moderate chain length [5–7].

Various detergents are often used for stabilization of substrate emulsions. The main properties determining their usability for that purpose would be inertness with respect to the enzyme (do not bind to pro-

tein, show no inactivating effect and are not hydrolyzed by a biocatalyst). Tweens, Tritons or ionic detergents are usually used [15, 16]. However, Tweens (polyoxyethylensorbitan fatty acid esters) contain a bond that potentially could be hydrolyzed by lipases. We have shown that *PmL* hydrolyzes various Tweens (Fig. 2). The hydrolytic effect of lipase on Tweens depended on the chain length of fatty acid in the detergent (Tweens: 20 – monolaurate, 40 – monopalmitate, 60 – monostearate, 80 – monooleate, 85 – trioleate) and their concentration. In conclusion, Tweens were found not to be usable for substrate emulsion stabilization as they were hydrolysed by *Ps. mendocina* lipase.

Figure 3 shows the effect of Tween 85 concentration on the hydrolysis with catalysis by *PmL*. A bell-shaped profile was obtained, in which a maximum exists at a concentration of 10% (w/w). With increasing the concentration, the reaction rates were shown to rise also, again suggesting that Tween 85 could be used for substrate emulgation only at a low concentration (0.4–0.8%).

Table 2 shows the effect of Tween 85 and olive oil-gum arabic emulsion on lipolytic activity of *PmL*. In the presence of both olive oil emulsion and Tween aqueous solution of a relatively low (0.8%) concentration detergents seemed to act as surfactants rather than as substrates, because the hydrolysis in the system increased to a higher degree than the decomposition of Tweens.

The catalytic activity upon Tweens was found to be lower than upon TAGs at the same concentrations. That could be connected with the structure of Tweens as monoesters (except Tween 85), since the specificity and activity of lipases regarding acylglycerols noticeably decreases in the direction: tri- > di- > monoglycerol. The higher effect of lipase on TO than on Tween 85 at the same concentration probably was due to the different physical state of both substrates (TO was emulsified and Tween 85 was solubilized) and to a possible sterical effect, as the molecule of Tween 85 is larger than of TO [17].

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V. Bendikienë, B. Surinënaitë, I. Bachmatova, L. Marcinkevièienë, B. Juodka

PSEUDOMONAS MENDOCINA 3121-1 LIPAZËS SPECIFIÐKUMAS. HIDROLIZË

Santrauka

Pseudomonas mendocina 3121-1 lipazës substratinis specifiðkumas iðtirtas ávairiø aliejø (linø sëmenø, kukurûzø, alyvuogiø, saulëgràþø ir sojø), triacilgliceroliø (TAGø) (triacetino, tributirino, trikaprino, tripalmitino, tristearino ir trioleino) bei nejoniniø Tween’ø (20, 40, 60, 80 ir 85) tipo detergentø atþvilgiu. Nustatyta, kad fermento katalitinis aktyvumas yra didþiausias kaip substratus naudojant ilgagrandþius TAGus (ypaè TO, t. y. substratà, kurio struktûroje yra nesoèioji oleino rûgðtis) bei alyvuogiø ir kukurûzø aliejus. Tween’ai naudoti ir kaip substratai, ir kaip emulsikliai. Nustatyta, kad lipazë hidrolizuoja visus Tween’us, o skaidymo efektyvumas priklauso nuo jø koncentracijos ir struktûros. Ið visø tirtø Tween’ø aktyviausias yra fermentas Tween’o 85 – TO struktûrinio analogo atþvilgiu; optimaliausia jo koncentracija yra 10%. Esant toms paèioms TAGø ir detergentø koncentracijoms, katalitinis aktyvumas yra maþesnis, kai kaip substratai naudojami Tween’ai. Taèiau esant santykinai maþai Tween’o 85 koncentracijai (0,8%), jo kaip emulsiklio savybës pasireiðkia labiau negu kaip substrato. Svarbiausia ðio darbo iðvada – tirtø koncentracijø (11–44 mM) ribose Tween’ai negali bûti naudojami lipazës substratø emulgavimui, nes *Pseudomonas mendocina* 3121-1 lipazës katalizuojamos reakcijos metu yra hidrolizuojami lygiagreèiai su „tikraisiais“ substratais.