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Pseudomonas mendocina 3121-1 lipase-catalysed lipolysis. The effect of detergents

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The role of various detergents in the lipolysis of olive oil emulsion catalyzed by Pseudomonas mendocina 3121-1 lipase was investigated.

Sodium dodecylsulphate (SDS) was determined to inactivate the lipolysis of emulsified substrate, whereas dodecyltrimethylammonium bromide (DTMAB) activated the process independently of the addition moment, and sodium deoxycholate (SDCh) strongly activated the lipolysis when added after the reaction had been started.

The effect of Tweens on the lipolysis showed their synergetic character as compared to the lipolysis of emulsified substrates only and to the hydrolysis of non-ionic detergents. The effect was shown to be different from that of Triton X-100.

The effect of guanidine hydrochloride (Gdn-HCl) on the lipolysis was found to be of different character as compared with the hydrolysis of the soluble substrate.

Key words: bacterial lipase, detergents, lipolysis, substrate specificity

INTRODUCTION

Most of reactions catalyzed by lipases (EC 3.1.1.3) are of great interest for application in biotechnology because control of various parameters of the catalytic process enables to achieve the high yield of pure product. One of the first processes lipases have been succesfully used in was the preparation of additives to detergents. Substrate specificity of lipases is another factor attracting attention to these biocatalysts. Some of lipases are non-specific and can be used in the conversion of various substrates. However, most of them have been shown to possess a strong selectivity for substrates of a certain structure [1–4].

At the Institute of Biochemistry (Vilnius, Lithuania), the lipolytic enzyme from *Pseudomonas mendocina* 3121-1 (*Ps. mendocina*) variant possessing both esterase and lipase activity has been isolated and purified [5]. Although detergents of various origin are applied for the stabilisation of lipase substrate emulsions, the effect of non-ionic and ionic ones on the *Ps. mendocina* lipase lipolytic activity remains uncertain. Sodium dodecylsulphate (SDS) and deoxycholate (DTMAB) as anionic detergents and dodecyltrimethylammonium bromide (SDCh) as a cationic one are usually used for substrate stabilisation. On the other hand, detergents can affect the hydrolysis of soluble substrates, even in a way different from that of emulsified ones. Tweens (polyoxyethylensorbitan fatty acid esters), formerly known as detergents only, also have been reported to be hydrolyzed by various lipases, including *Ps. mendocina* [6, 7]. Nevertheless, data concerning the effect of detergents in dependence of their nature on the lipolysis of natural or artificial *Ps. mendocina* lipase substrates are still lacking.

The purpose of the present study was to provide a more detailed analysis of *Ps. mendocina* lipase lipolytic activity under the influence of non-ionic and ionic detergents as well as of Gdn-HCl added to the reaction mixture.

MATERIALS AND METHODS

Materials. Lipase from *Ps. mendocina* was provided by the Institute of Biochemistry, Vilnius, Lithuania. Stabilized olive oil emulsion (Sigma substrate), Tris, Tweens 20, 40, 60, 80, 85, SDS, SDCh, DTMAB, tributyrin (TB), tricaprin (TC), triolein (TO) were from Sigma and gum-arabic was from Merck. Gdn-HCl was from Aldrich. Acetic acid, boric and ortho phosphoric acids were from Lachema. Other acids, salts and NaOH of the highest purity available were from Reachim.

Methods

Lipolysis of Sigma substrate and triacylglycerols (TAGs) by lipase. The lipolytic activity was determined by the modified method described previously [8]. The reaction mixture containing 4 ml of Sigma substrate or TAGs emulsion (prepared by ultrasonic treatment in 8% (w/w) gum-arabic aqueous solution) and 4 ml of 50 mM Tris-HCl buffer, pH 7.9 (30 °C) was adjusted to pH 8.0 with 0.5 M NaOH. The mixture (blank) was titrated using 43 mM NaOH maintaining a constant pH for 3 min. The final concentration of the olive-oil emulsion was 11% (v/v). Then 1 ml of the enzyme solution in the buffer (final concentration 4 μg/ml) was added to the titrated blank. The sample was titrated

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for 5 min. One unit of the lipolytic activity corresponds to the amount of enzyme that releases 1 mmol of fatty acid per min under the indicated conditions.

The effect of non-ionic detergents on the lipolysis of Sigma substrate and TAGs emulsion by lipase. The lipolytic activity of the lipase was determined at an 11% (v/v) of the emulsion and 11–44 mM of Tween 20, 40, 60, 80, 85 and Triton X-100 concentrations or at 6–22% (v/v) of the emulsion and 22 mM of Tween 85 concentrations. Also, the effect of Tween 80 on the lipolysis of TB and TO as well as of Tween 85 on TB, TC and TO was investigated. The catalytic activity was detected at 11 mM Tween and at 11 mM and 44 mM TAGs concentrations. The reaction without Tweens and the hydrolysis upon Tweens only was explored as control.

The effect of ionic detergents on the lipolysis of Sigma substrate by lipase. The lipolytic reaction was investigated by the standard method in the presence of 11% (v/v) of emulsion and 10 mM of the ionic detergents mentioned. The experiment was carried out according to the following scheme: 1. The lipase was pre-incubated for 1 min with each detergent at a 2.5–10 mM concentration and the residual hydrolytic activity was determined as described above. Analogously, the detergent was added to the reaction mixture starting the reaction at zero time. 2. Detergents were added 1 min after the initiation of the reaction. 3. The substrate was pre-incubated with each detergent prior to adding the enzyme. 4. The lipase was pre-incubated at 20 °C with 2.5 mM of the detergent (with SDS for 1–20 min, with DTMAB and SDCh for 5–30 min) and the residual hydrolytic activity was determined as described above.

The effect of Gdn-HCl on the lipolysis of Sigma substrate by lipase. The lipolysis was detected by the standard method at an 11% (v/v) emulsion concentration. Gdn-HCl at a concentration of 100 mM was used in the following experiments: 1. Gdn-HCl was added to the reaction mixture directly or after pre-incubation of the enzyme at 20 °C for 5, 10 and 20 min. 2. Gdn-HCl was added 2 min after the initiation of the lipolysis. 3. The enzyme was added after pre-incubation of the substrate with Gdn-HCl for 2 min.

Statistics

Four measurements were carried out in each experiment, and the results are presented as means ± S.E.M.

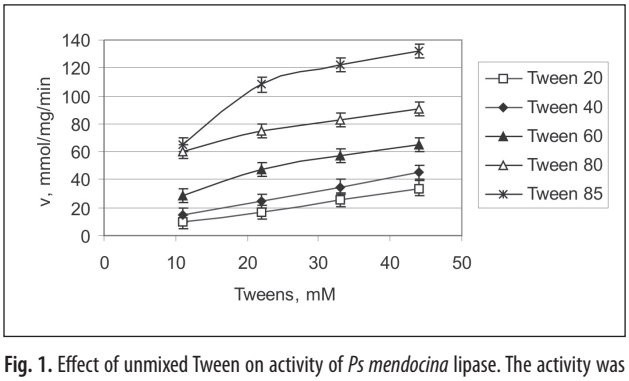

Fig. 1. Effect of unmixed Tween on activity of *Ps mendocina* lipase. The activity was determined by the standard titrimetric method in the presence of 11–44 mM Tween

RESULTS AND DISCUSSION

The previous investigation of *Ps. mendocina* lipase has indicated unusual features of the enzyme not typical of other lipases from different variants of the strain characterized in the literature. The lipase was shown to be composed of two identical subunits, each of 30 kDa molecular mass. The identification of certain structural features by examining the hydrolysis of p-nitrophenyl butyrate catalyzed by *Ps. mendocina* lipase showed the importance of Arg residue, the involvement of His in the catalysis and confirmed an essential role of the Ser residue. Moreover, Cys residue was found to be essential neither to the catalytic action nor to structural features of the lipase, although another oxidizable amino acid residue should be important [6, 9].

Although soluble substrates are usually used for the hydrolytic reaction measurements, "true" substrates of lipases are various oils and fats. Lipases that are water-soluble act at the water–oil interface, i. e. are active only on emulsified water-insoluble substrates stabilized by various detergents [1, 8]. The main properties determining their usability for that purpose would be inertness with respect to the enzyme (no binding to protein, no inactivating effect and no hydrolysis by the biocatalyst). Tweens, Tritons or ionic detergents are usually used [8, 10].

We showed that *Ps. mendocina* lipase hydrolysed Tweens [9] and found the lipase to show the most effective lipolytic activity upon long chain fatty acid (FA) glycerol esters, especially TO, i. e. a substrate containing unsaturated FA. Consequently, P*s. mendocina* lipase could be attributed to those specific for long-chain unsaturated TAGs [7]. Most of lipases have been shown to be specific for TAGs of a moderate chain length [11–17].

We found that the hydrolytic activity of *Ps. mendocina* lipase in pure Tweens 20, 40, 60, 80 and 85 depended on the chain length of fatty acid in the detergent (Fig. 1). It is evident that the effect is strongest on the detergent of a long-chain unsaturated FA similarly as in the case of TAGs. The relatively higher activity on pure TAGs [7] than on pure Tweens (Fig. 1) of a similar FA chain length could be related to the structure of the detergents like monoesters (except Tween 85). The specificity and activity of lipases for acylglycerols was noted to noticeably decrease in the sequence: triglycerol > diglycerol > monoglycerol [13].

The effect of Tweens 80 and 85 on the lipolysis of certain TAGs is presented in Tables 1 and 2. When the concentration of both detergents and TAGs was the same (11 mM), the catalytic activity upon pure Tween 80 was found to be higher than upon unmixed TB, and the effect on pure Tween 85 exceeded that upon unmixed TB and TC (Tables 1, 2). The effect on TO and on both detergents was shown to be similar. In contrast, a synergetic effect on the lipolysis of the mixture of detergents and TAGs mentioned was noted at the 11 mM concentration.

Table 1. **The role of Tween 80 in the lipolytic activity on TB and TO emulsions. The activity was determined by the standard titrimetric method in the presence of 11 mM Tween. The hydrolytic activity on pure Tween 80 was 72 ± 4 mmol/mg/min**

v,	Tributyrin		Triolein	
mmol/mg/min	11 mM	44 mM	11 mM	44 mM
Without Tween	44 ± 3	121 ± 5	76 ± 4	253 ± 6
With Tween	95 ± 5	142 ± 6	129 ± 3	351 ± 5

Table 2. **The role of Tween 85 in the lipolytic activity on TB, TC and TO emulsions. The activity was determined by the standard titrimetric method in the presence of 11 mM Tween. The hydrolytic activity on pure Tween 85 was 77 ± 3 mmol/mg/min**

v, mmol/mg/min	Tributyrin		Tricaprin		Triolein	
	11 mM	44 mM	11 mM	44 mM	11 mM	44 mM
Without Tween	44 ± 3	121 ± 5	40 ± 1	146 ± 2	76 ± 4	253 ± 6
With Tween	129 ± 4	152 ± 4	124 ± 5	186 ± 5	158 ± 3	511 ± 3

On the other hand, when the concentration of TAGs 4-fold exceeded that of Tweens in the mixture, the synergetic effect seemed to be deviated, in particular with Tween 85. Most probably the specificity of *Ps. mendocina* lipase toward TAGs of long chain FA [7] was also valid: in the presence of 11 mM Tweens the lipolytic effect on TB and TC was under the effect while upon TO exceed the effect near 1.5 times (Tables 1, 2).

Interestingly, at a higher concentration (44 mM) of pure TAGs and Tweens the catalytic effects of the lipase on the detergents in most cases was lower than upon TAGs (Fig. 1, Tables 1, 2). It has been noted that the lipase was most active on TO structurally close to Tween 85.

The results of the lipolysis of a "true" lipase substrate – olive oil emulsion (without Tween) and of the effect of Tweens mixed with the emulsion are presented in Figs. 2 and 3. It is evident that at each concentration of the detergent the lipolytic activity increased similarly to the efficiency of the hydrolytic activity on these detergents (Fig. 1). The titrimetric method allowed to detect the total amount of fatty acids released, but the experimental results did not indicate the individual contribution of the hydrolysis of Tween and the lipolysis of olive oil. Anyway, it is worth noting that the addition of Tweens enhanced the total catalytic activity of *Ps. mendocina* lipase not only because of their detergency, but also because of the catalytic effect of the enzyme on them.

Addition of a non-ionic detergent of different nature – Triton X-100 – caused the opposite effect in comparison with Tweens (Fig. 4). Tritons are known to be more effective as stabilizers of lipase substrate emulsion or to show a higher emulsification capacity than Tweens [10]. Also, this compound was found either

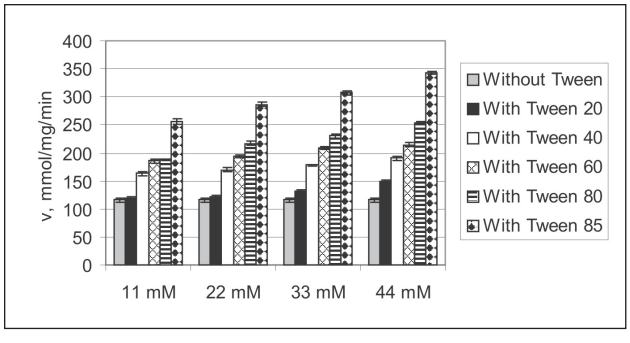

Fig. 2. Effect of Tweens at various concentrations mixed with Sigma substrate. The lipolytic activity was determined by the standard titrimetric method in the presence of 11% (v/v) of Sigma substrate and 11–44 mM of Tween

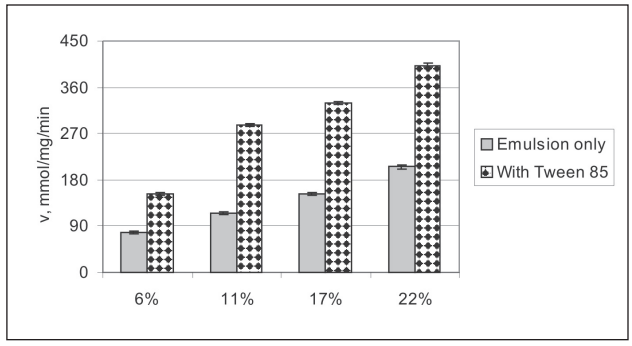

Fig. 3. Effect of Tween 85 mixed with Sigma substrate at various concentrations. The activity was determined by the standard titrimetric method in the presence of 6–22% (v/v) emulsion and 22 mM of Tween 85

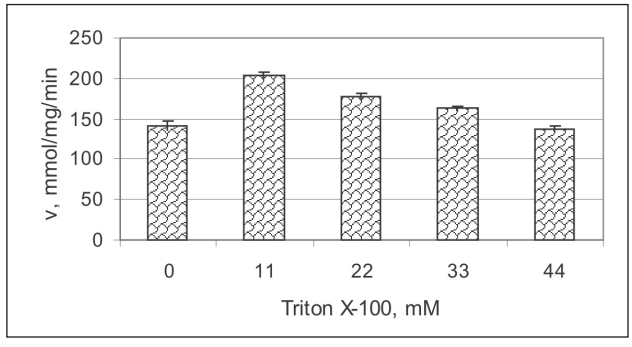

Fig. 4. Effect of non-ionic detergent Triton X-100 upon lipolytic activity. The activity was determined by the standard titrimetric method in the presence of 11–44 mM of Triton X-100

to inactivate or to activate the lipolytic enzymes [8, 11, 18]. We showed that, contrary to Tweens, Triton X-100 was not hydrolysed by Ps. mendocina lipase to be suitable for the substrate emulsification. The higher the detergent concentration, the lower was the lipolytic activity on Sigma olive oil emulsion (Fig. 4).

The presence of ionic detergents is essential during the lipolysis of insoluble emulsified substrates as they stabilize emulsions, prevent the accumulation of released fatty acids, activate lipases at certain concentrations or affect the enzyme binding at the interface [3, 10].

We investigated the role of ionic detergents on the lipolysis of olive oil emulsion catalyzed by *Ps. mendocina* lipase. The appearance of the charge at the interface, caused by the presence of those detergents, can either facilitate or burden the catalytic process. Such effect is especially evident in heterogeneous media. The results are presented in Fig. 5. One can see that the anionic detergent SDS reduced lipolytic activity most noticeably after the enzyme pre-incubation, while the other anionic detergent – SDCh (salt of bile acid) – enhanced the activity when added during the reaction but reduced it after the substrate pre-incubation. The cationic detergent DTMAB activated the lipase in all three cases and most noticeably after the enzyme pre-incubation. So it could be suggested that SDS affected the biocatalyst, SDCh most effectively removed the reaction products from the

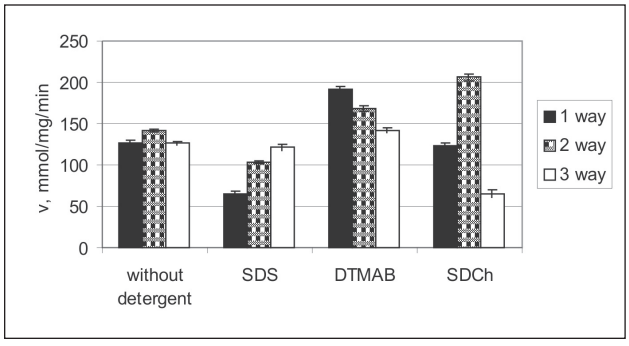

Fig. 5. Effect of ionic detergents on lipolytic activity. The activity was determined by the standard titrimetric method in the presence of 11% (v/v) of emulsion and of 10 mM detergents. Way 1 – the enzyme was pre-incubated with the detergent for 1 min before the lipolysis; way 2 – the detergent was added 1 min after the initiation of the reaction; way 3 – the substrate was pre-incubated with the detergent for 1 min before adding the enzyme

interface but probably destabilized the substrate, and DTMAB might form an active conformation of the enzyme and create a surface charge favourable for the catalytic process. The inactivating effect of SDS on *Ps. mendocina* lipase could be explained by the change of enzyme conformation, and that of SDCh – by the repulsion of the enzyme from the interface. The effect of cationic detergents was noted to be contrary to that of anionic ones [8, 11]. The activation of *Ps. mendocina* lipase by DTMAB could confirm such tendency.

Structural studies of various lipases suggested a hydrophobic lipid-binding site to be opened up by movement of the "lid" from the active site at the oil–water interface. However, even in the absence of the interface, there might be a subtle equilibrium between the two conformations of the enzyme. It was believed that the opening of the "lid" should be essential but not sufficient to explain the interfacial activation. *Ps. mendocina* lipase has been reported as an enzyme lacking the "lid" and not displaying an interfacial activation [20]. Our investigations of the effect of substrate concentration on the hydrolytic reaction of various p-nitrophenyl (p-NP) fatty acid (C3–C18) esters showed a moderate interfacial activation when hydrolyzing p-NP caprylate (unpublished data). Interfacial activation of lipases is related to the "lid", although the relation is not common and direct. Arg residue has been found to be important to stabilize the open-lid conformation [21]. Considering a relatively high amount of Arg (14 residues per molecule) in *Ps. mendocina* 3121-1 lipase [5], the effect of guanidine, an arginine side-chain analogue, derivatives (hydrochloride (Gdn-HCl), carbonate and diaminoguanidine) on the hydrolytic activity of the enzyme was investigated [6]. The study indicated the inactivation by Gdn-HCl to be more rapid than by the other two derivatives. Moreover, even a 20 mM concentration of all derivatives showed more or less inactivating effect. Rhizomucor miehei lipase has been shown to be strongly inactivated by Gdn-HCl, while those from Humicola lanuginose and porcine pancreas were moderately affected [22]. Lipases from three *Candida* strains, *B. cepacia* and *Ps. fluorescens*, have been found to be not inhibited [21]. *R. miehei* lipase was strongly inhibited by Gdn-HCl and less affected by 1-ethylguanidine.

Examination of the amino acid sequences of the "lid" regions of several lipases has shown the presence and importance of Arg residue only for those inhibited by guanidine [21, 22]. Thus implies that any of 14 Arg residues might be important for the catalytic process of *Ps. mendocina* 3121-1 lipase, most probably structurally. Arg also could be directly involved in the catalysis, but a more rapid inactivation by guanidine could be detected in this case [6]. We concluded that *Ps. mendocina* lipase was noticeably inactivated by Gdn-HCl, indicating the importance of Arg residue for the catalytic process with a soluble substrate.

The role of Gdn-HCl in the lipolysis of olive oil emulsion was also examined (Table 3). The most noticeable decrease of the lipolytic activity was observed after the substrate pre-incubation with Gdn-HCl. We could suggest that Arg residues play a different role in the catalytic process in the presence of substrate solution [6] and emulsion. It is possible that Arg ise essential for *Ps. mendocina* lipase adsorption and stabilization at the interface [20], and pre-incubation of the substrate with Gdn-HCl hindered somehow this process.

CONCLUSIONS

Summarizing the role of various non-ionic and ionic detergents in the lipolysis catalyzed by *Ps. mendocina* 3121-1 lipase it should be noted that: 1. Tweens were shown to serve as substrates of the lipase, and their effect on the lipolysis of other substrates was synergetic. 2. Triton X-100 showed an effect different from that of Tweens. 3. The cationic detergent DTMAB caused an enhancement of the lipolysis when pre-incubated with the enzyme, the substrate or added during the reaction, while the effect of anionic detergents (SDS and SDCh) strongly depended on the order of their interaction with the components of the reaction mixture.

Table 3. **The role of Gdn-HCl in the lipolysis of Sigma substrate. The activity was determined by the standard titrimetric method in the presence of 100 mM Gdn-HCl. 1 – the reaction was observed with Gdn-HCl directly in the reaction mixture (t = 0) or after the enzyme pre-incubation with it for 5, 10 and 20 min at 20 °C; 2 – Gdn-HCl was added 2 min after the initiation of the lipolysis; 3 – the enzyme was added 2 min after pre-incubation of the reaction mixture with Gdn-HCl**

	v, mmol/mg/min					
	Assay 1				Assay 2	Assay 3
	t = 0	After 5 min	After 10 min	After 20 min		
Without Gdn-HCl	114 ± 3	114 ± 3	107 ± 1	101 ± 4	121 ± 3	111 ± 4
With Gdn-HCl	85 ± 3	85 ± 3	66 ± 2	61 ± 1	97 ± 1	41 ± 3

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PSEUDOMONAS MENDOCINA 3121-1 LIPAZĖS KATALIZUOJAMA LIPOLIZĖ. DETERGENTŲ POVEIKIS

Santrauka

Ištirta *Pseudomonas mendocina* 3121-1 lipazės katalizuojama tikrojo lipazių substrato – alyvuogių aliejaus emulsijos – lipolizė veikiant įvairiems detergentams.

Nejoniniai Tween'ų tipo detergentai mišinyje su tikruoju substratu veikia sinergetiškai, lyginant ir su grynų emulguotų substratų lipolize, ir su grynų detergentų hidrolize. Nustatyta (neskelbti duomenys), kad *Pseudomonas mendocina* 3121-1 lipazė skaldo grynus nejoninius Tween'ų tipo detergentus, o reakcijos efektyvumas priklauso nuo jų sudėtyje esančios riebalų rūgšties grandinės ilgio bei koncentracijos. Tritono X-100 – lipazės nehidrolizuojamo detergento – poveikis emulguotų substratų hidrolizei skiriasi nuo Tween'ų poveikio.

Joninių detergentų – natrio dodecilsulfato (SDS), dodeciltrimetilamonio bromido (DTMAB) bei natrio deoksicholato (SDCh) – įtaka lipazės kataliziniam procesui labai priklauso ne tik nuo jų krūvio bei koncentracijos, bet taip pat nuo substrato prigimties ir nuo šių detergentų sąveikos su reakcijos mišinio komponentais eiliškumo. Nustatyta, kad SDS inaktyvuoja fermentą lipolizės metu, DTMAB aktyvuoja procesą nepriklausomai nuo jo sąveikos su reakcijos mišinio komponentais sąlygų, o SDCh stipriai greitina (aktyvuoja) procesą, jei pridedamas jau prasidėjus reakcijai.

Daugelio lipazių katalizei didelės įtakos turintis guanidino hidrochloridas (Gdn-HCl) *Ps. mendocina* 3121-1 lipazės katalizinį aktyvumą emulguotų substratų atžvilgiu veikia skirtingai nei tirpių sintetinių substratų.