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Tweens and ionic detergents in the hydrolytic activity of *Pseudomonas mendocina* 3121-1 lipase

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A more detailed analysis of *Pseudomonas mendocina* 3121-1 lipase catalytic activity in hydrolysing non-ionic detergents Tweens was the goal of the present study. Tweens as lipase substrates were used in aqueous media at the following final concentrations: half of the critical micelle concentration (CMC), close to CMC, five-fold above CMC and by far exceeding CMC (11–44 mM). The intensity of the hydrolysis of the detergents increased with increasing fatty acid chain length in the Tween structure. The reaction rate also depended on the detergent concentration and changed (increased) at the point when it exceeded the CMC, suggesting that Tweens at those concentrations are not well suitable for stabilizing the *Ps. mendocina* lipase substrate emulsion.

The role of ionic detergents in the lipase-catalysed hydrolysis of soluble substrate p-nitrophenyl butyrate (p-NPB) was also investigated. The hydrolytic activity was shown to be rapidly declined by sodium dodecylsulphate (SDS) during the first 10 min, slower inactivated by dodecyltrimethylammonium bromide (DTMAB) and only slightly affected by sodium deoxycholate (SDCh). The inactivation rate constants (k_{inact}, min^{-1}) were calculated to be 0.29, 0.07 and 0.006 for SDS, DTMAB and SDCh, respectively.

Key words: bacterial lipase, Tweens, ionic detergents, critical micelle concentration, hydrolytic activity

INTRODUCTION

Lipases (EC 3.1.1.3) are hydrolytic enzymes involved in the conversion of lipids but also catalyzing the synthesis of various fatty acid esters under certain unconventional conditions. A number of reactions catalyzed by lipases are still of interest for application in biotechnology. Substrate specificity of lipases is another factor attracting the attention to those biocatalysts [1–3].

Lipases usually are distinguished as a specific class of esterases. Such classification has been related to their interfacial activation characterised by a rapid increase of the catalytic activity above CMC of the substrate. Nowadays, lipases are characterized as enzymes catalyzing the conversion of esters of medium- and long-chain fatty acids (FA) most effectively, while esterases are usually inactive towards these substrates [1]. Tweens (polyoxyethylene-sorbitan FA esters), formerly known as detergents only, have been reported to be hydrolyzed by lipases similarly to natural substrates [4–6]. Nevertheless, some uncertainty still remains as regards distinguishing Tweens as substrates and as detergents for lipolytic assay of lipases, including those of the *Ps. mendocina* strain.

The presence of ionic detergents is essential in the lipolytic process in 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 [7]. However, only few reports were found concerning the effect of ionic detergents in the presence of soluble substrates that, however, form micelles [8, 9].

The lipolytic enzyme from the *Ps. mendocina* 3121-1 variant exhibiting both esterase and lipase activity had been isolated and purified at the Institute of Biochemistry (Vilnius, Lithuania) [10]. Despite the increasing interest to the lipase and also our previous results [11–13] concerning the catalytic properties and substrate specificity of the lipase from *Ps. mendocina*, data on the enzyme specificity towards Tweens are insufficient.

The purpose of the present study was a more detailed analysis of *Ps. mendocina* 3121-1 lipase hydrolytic activity on various Tweens and of p-NPB hydrolysis under the influence of ionic detergents.

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MATERIALS AND METHODS

Materials

Lipase from *Ps. mendocina* 3121-1 was provided by the Institute of Biochemistry, Vilnius, Lithuania. p-NPB, stabilized olive oil emulsion (Sigma substrate), Tris, SDS, SDCh, DTMAB, Tweens 20, 40, 60, 80, 85 were from Sigma. Acetic, ortho-boric and ortho-phosphoric acids were from Lachema. Other acids, salts and NaOH of the highest purity available were from Reachim.

Methods

The standard titrimetric assay for the hydrolytic effect on Tweens [14]. Tween 20, 40, 60, 80 and 85 as the lipase substrates were used in aqueous media at the following final concentrations: twice below CMC, close to CMC, five-fold exceeding CMC and by far exceeding CMC (11–44 mM). The mixture containing the substrate and 50 mM Tris-HCl buffer, pH 7.9 (30 °C) (final volume 8 ml) was adjusted to pH 8.0 by 1N NaOH, and such pH was maintained for 3 min by titration with 43 mM of NaOH solution (blank). Then 1 ml of the lipase solution (4 μg/ml) in the buffer was added, and the lipolytic reaction was tested for 1–30 min by titration as mentioned above (sample). One unit of the lipolytic activity corresponds to the amount of the enzyme releasing 1 mmol of FA per minute under standard conditions.

The effect of Tween 85 concentration on the hydrolytic activity. The activity was determined by the standard titrimetric method at a final Tween 85 concentration of 2–108 mM.

Duration of Tween hydrolysis reaction. The hydrolytic activity was determined by the standard titrimetric method at the final Tweens 20, 40, 60, 80 and 85 concentrations of 44 mM. The reaction was tested for 5 min, and the titrant volume was detected each minute to determine the rate of FA release.

p-NPB hydrolysis by the lipase. The hydrolytic activity in the reaction mixture containing 2.325 ml of 100 mM the Britton–Robinson buffer (universal buffer), pH 9.0, 25 μl of p-NPB solution in propan-2-ol and 100 μl of lipase solution in the buffer was measured spectrophotometrically. The Britton–Robinson buffer is composed of acetic, ortho-boric and ortho-phosphoric acids at a ratio of 1 : 1 : 1 providing a buffering capacity over a wide range of pH. Final concentrations in the mixture were the following: of substrate, 0.1 mM, of organic solvent, 1% (v/v) and of the enzyme, 0.08 μg/ml. The reaction was carried out at 30 °C for 6 min [13]. The absorbance was measured at 400–410 nm. The spontaneous hydrolysis of p-NPB was considered in blank measurements and was eliminated from the absorbance data of the sample. The molar extinction coefficient for p-NPB (0.15 mM) was determined to be 1.03. One unit of the hydrolytic activity corresponds to the amount of enzyme releasing 1 μmol of p-nitrophenol per min under standard conditions.

The effect of ionic detergents on p-NPB hydrolysis by the lipase. SDS and SDCh as anionic detergents and DTMAB as cationic one were tested. The experiment was carried out according to the following scheme: 1. The lipase was pre-incubated for 1 min with each detergent at a concentration of 2.5–10.0 mM, and the residual hydrolytic activity was determined as described above. On analogy, the detergent was added to the reaction mixture starting the reaction. 2. Detergents were added 1 min after the initiation of the reaction. 3. The substrate was pre-incubated with each detergent prior to the addition of the enzyme. 4. The lipase was pre-incubated at a temperature of 20 °C with the detergent concentration of 2.5 mM (for 1–20 min with SDS, for 5–30 min with both DTMAB and SDCh), and the residual hydrolytic activity was determined as described. The rate constants of inactivation by the detergents were calculated according to the logarithmic equation $\ln A_0 / A = f(t)$.

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

RESULTS AND DISCUSSION

The earlier investigations of *Ps. mendocina* 3121-1 lipase have 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 molecular mass 30 kDa. The identification of certain structural features by examining the hydrolysis of p-NPB catalyzed by the lipase showed the importance of Arg residue, the involvement of His in the catalysis and confirmed the essential role of the Ser residue. Moreover, the Cys residue was found to be essential neither to the catalytic action nor to the structural features of the lipase, although another oxidizable amino acid residue was suggested to be important [11, 12].

Despite the fact that soluble substrates are usually used for the hydrolytic reaction measurements, "true" substrates of lipases are various oils and fats. Water-soluble lipases act at the water–oil interface, i. e. are active only in the presence of emulsified water-insoluble substrates stabilized by various detergents [3, 13]. An essential property determining their usability for that purpose should be inertness with respect to the enzyme, because detergents are expected not to bind to protein, to show no inactivating effect and to be not hydrolysed by the biocatalyst. Tweens, Tritons or ionic detergents meet the mentioned requirements and are usually used as lipase substrate stabilizers [3, 14].

We showed *Ps. mendocina* 3121-1 lipase to hydrolyse Tweens at a concentration of 0.8–20% and found the enzyme to display the most effective lipolytic activity upon long-chain triacylglycerols (TAG), especially triolein (TO) as a substrate composed of unsaturated fatty acid [15]. Consequently, *Ps. mendocina* 3121-1 lipase could be attributed to lipolytic enzymes specific of long-chain unsaturated TAGs. Most of lipases have been shown to be specific for TAGs of a moderate chain length [8, 16–19]. According to such results, we investigated the hydrolysis of Tween 85 (polyoxyethylenesorbitan trioleate) in a wide range of the detergent concentrations.

The lipase was found to hydrolyse Tween 85 most efficiently at a concentration of 40–60 mM [15]. The specificity and the activity of lipases for acylglycerols was shown to decrease noticeably in the direction triglycerol > diglycerol > monoglycerol [16]. It had been already noted that lipase was the most active on glycerol trioleate structurally close to Tween 85. We examined also the hydrolytic activity of *Ps. mendocina* 3121-1 lipase upon Tweens of various FA chain length. Moreover, it was important to evaluate the optimal duration of the reaction as it was noted that other lipases catalyse lipolysis at a very diverse time length [7, 9, 16]. We used Tweens 20 (monolaurate), 40 (monopalmitate), 60 (monostearate), 80 (monooleate) and 85

(trioleate) as substrates at the optimal concentration of 44 mM, determined for Tween 85 [15]. The reaction rate was analysed from 1 to 30 min and was determined to drop extremely as soon as after 5 min (Fig. 1, A) indicating the catalytic process of *Ps. mendocina* 3121-1 lipase to be more rapid than of some other lipases [7, 16, 20]. The optimal duration of the reaction (3 min) was chosen for further experiments.

We found that the hydrolytic effect of lipase on Tweens depended on the FA chain length in the detergent and was highest for Tween 85 (Fig. 1 B).

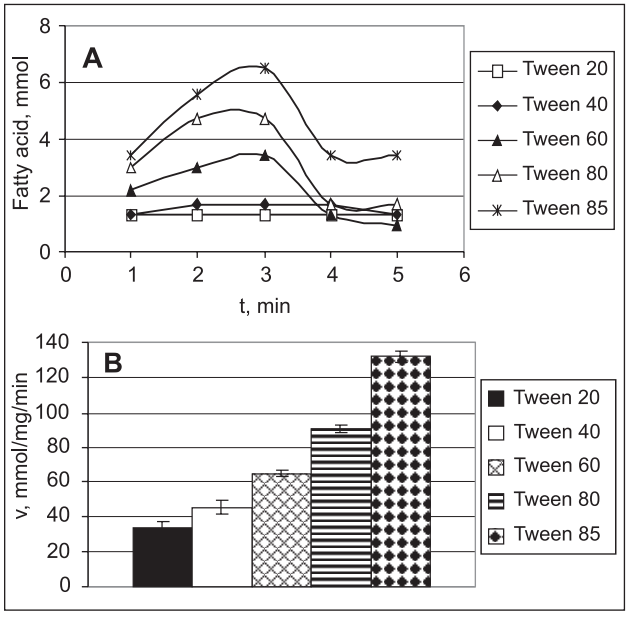

Fig. 1. The hydrolytic activity of lipase on various Tweens. **A.** The duration (1–5 min) of the hydrolysis. **B.** The hydrolysis at the optimal time. The activity was determined by the standard titrimetric method at Tween concentration of 44 mM

In contrast, Janda [21] noted Tweens 20, 40, 60 and 80 not to be used effectively as substrates for *Thermomyces lanuginosus* lipases. Dos Prazeres and co-authors [9] showed the activity of *Fusarium oxysporum* lipase to be lowered in the presence of various Tweens. Brunke et al. [22] found fungal lipase activity to be depressed by the detergents.

As mentioned above, lipases act at the water–oil interface, consequently, the catalytic activity should be dependent on Tween concentration and physical state (the change of the latter is most usually characterized by CMC as a point where micellar aggregates of the substrate start forming) (Table 1). Tweens 20, 40, 60, 80 and 85 as lipase substrates were used in aqueous media at final concentrations: two-fold below CMC, close to CMC, five-fold above CMC and by far exceeding CMC (11–44 mM). The results are presented in Fig. 2.

It is evident that the efficiency of the hydrolysis changed at the point when the Tween concentration exceeded CMC. So, it could be concluded that Tweens were not suitable for the stabilization of lipase substrate emulsion as these were hydrolysed by the enzyme. On the other hand, here these detergents should be used at a concentration below CMC, which is rather low for Tweens. Supporting the suggestion, Tween 80 was shown to decrease lipolytic activity almost by half at a 5-fold higher concentration (2 to 10 g/l) [23].

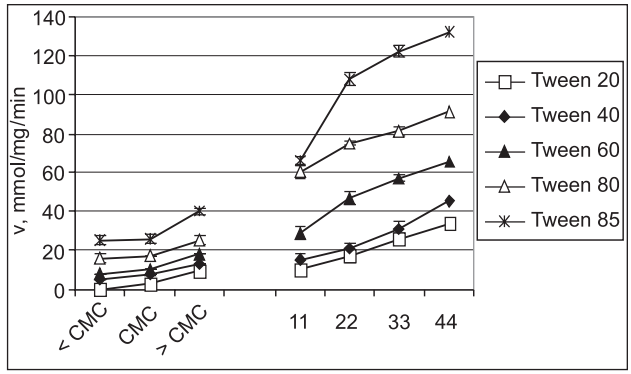

Fig. 2. The effect of Tween physical state in the reaction mixture on the lipase hydrolytic activity. The activity was determined by the standard titrimetric method at the following final Tween concentrations: two-fold below CMC, close to CMC, five-fold above CMC and far exceeding CMC (11–44 mM)

Table 1. **Critical micelle concentrations (CMC) and hydrophilic / lipophilic balance (HLB) of Tween detergents [14]**

Detergents	CMC (mmol/l)	HLB
Tweens		
20	0.049–0.060	16.7
40	0.020–0.025	15.6
60	0.018–0.022	14.9
80	0.010–0.016	15.0
85	n.a.	11.0

Lipase activity variation upon different Tweens could be explained by the difference of their hydrophilic / lipophilic balance (HLB) – the measure of hydrophobicity [14] (Table 1). Thus, it should be noted that the activity of *Ps. mendocina* 3121-1 lipase upon Tweens enhanced with increasing the hydrophobicity of those detergents.

We also investigated the role of anionic (SDS and SDCh) and cationic (DTMAB) detergents in the hydrolytic reaction upon p-NPB catalysed by *Ps. mendocina* 3121-1 lipase. Results are summarized in Table 2.

It is evident that SDCh reduced the catalytic activity at a concentration below CMC (CMC for SDCh 4–6 mmol/l) and enhanced it at a concentration above CMC. In both cases, the effect depended on the conditions of the interaction with the enzyme. The other anionic detergent, SDS, reduced the catalytic activity enhancing its concentration, especially when present directly in the reaction mixture or pre-incubated with the substrate, and only a negligible residual activity was determined at an SDS concentration of 10 mM (CMC for SDS 8.2 mmol/l), contrary to SDCh. The highest residual catalytic activity in the presence of detergents at a concentration of 2.5 mM was detected with cationic DTMAB, and the effect also depended on the conditions of the interaction with the enzyme like in the case of SDCh. So, it could be concluded that the effect of ionic detergents on the *Ps. mendocina* 3121-1 lipase catalysed hydrolysis of p-NPB was determined not only by their charge, but also by their concentration and the progression of the interaction with the enzyme.

SDS was often noted by other authors to more or less depress the activity of lipolytic enzymes [8, 9, 24]. Sodium cholate

Table 2. **The role of ionic detergents in lipase hydrolytic activity. The activity was measured at 30 °C and pH 9.0. Reaction mixture components were used at the following final concentrations: 0.1 mM of substrate, 0.08 µg/ml of enzyme and 1% (v/v) of propan-2-ol. The experiment was carried out in three steps: 1 – the activity was detected in the presence of the detergent directly in the reaction mixture (t = 0) or after pre-incubation of the biocatalyst with it for 1 min; 2 – the detergent was added 1 min after the initiation of the reaction; 3 – the enzyme was added after pre-incubation of the substrate with the detergent for 1 min**

Detergents	v, µmol/mg/min			
	Step 1, t = 0	Step 1, t = 1 min	Step 2	Step 3
Without	128 ± 6	130 ± 5	118 ± 6	120 ± 5
2.5 mM				
SDS	10 ± 2	4 ± 1	8 ± 2	0
DTMAB	95 ± 5	78 ± 5	60 ± 4	47 ± 3
SDCh	49 ± 3	51 ± 6	26 ± 4	24 ± 5
10 mM				
SDS	5 ± 1	0	0	0
DTMAB	5 ± 1	0	0	0
SDCh	141 ± 6	159 ± 5	151 ± 4	128 ± 3

and taurocholate moderately inhibited the alkaline lipase of *Fusarium globulosum* [25].

In our case, the lipase was shown to be inactivated by SDS most strongly. At a detergent concentration of 0.6 mM (data not shown), i. e. 10-fold lower than CMC, SDS was shown to reduce the hydrolytic activity by half if added directly to the reaction mixture and even 4-fold if pre-incubated with the enzyme. Furthermore, the lipase was completely inactivated when p-NPB was pre-incubated with the detergent at a concentration of 2.5 mM, and a negligible catalytic activity remained only when the detergent was added straight at the reaction initiation moment at a concentration of 10 mM (exceeding CMC). SDS was noted to inactivate lipase forms *Asp. terreus* and *B. species* [8, 24] but not to affect invertase even at a concentration of 90 mM in an aqueous environment and even to stimulate the enzyme in the presence of 60% (v/v) of organic solvent [26]. We used p-NPB as a substrate at a concentration of 0.1 mM (lower than CMC) and 1% (v/v) of the organic solvent propan-2-ol. The formation of a lamelar structure is known to occur, if the ratio of the solvent / to the detergent / aqueous phase is insufficient for micelles. This fact should explain the higher hydrolytic activity of the lipase at SDS concentrations lower than CMC versus the catalysis when the concentration exceeded CMC. Possibly, the anionic detergent SDS affected the enzyme at a concentration of 0.6 mM, "poisoned" the substrate at 2.5 mM and completely blocked the catalytic process at a concentration of 10 mM.

On the other hand, the effect of another anionic detergent, SDCh, was shown to be opposite. The detergent inactivated the lipase at a concentration of 2.5 mM, especially when added directly or after pre-incubation with the substrate, and activated the enzyme in all cases at a concentration of 10 mM. The effect of bile acids and salts should occur via stabilization of lipase substrates, improved enzyme adsorption at the interface, partial protection from denaturation and the removal of FA formed in the catalytic reaction. Moreover, the detergents were found to act as competitive lipase inhibitors [27]. In our experiment, most probably in the presence of SDCh at a concentration of 2.5 mM (lower than CMC), the removal of reaction products should be inconvenient, thus "poisoning" the substrate and hindering the enzyme binding. On the other hand, the drop of the lipase activity in the presence of SDCh during the reaction or when the enzyme was pre-incubated with the detergent could guide SDCh to act as a lipase inhibitor. But the activating effect of SDCh at a concentration of 10 mM (higher than CMC) could be related to an effective removal of reaction products as mixed micelles, if added to the reaction mixture, and to the formation of an enzyme conformation optimal for the substrate binding if pre-incubated with the lipase [14].

In the presence of the cationic detergent DTMAB at a concentration of 2.5 mM, the residual activity of *Ps. mendocina* 3121-1 lipase was shown to be higher than in the case of both anionic detergents under the same conditions. The most noticeable inactivating effect of DTMAB was detected after pre-incubation with the substrate on analogy with SDS and SDCh. The similar effect of DTMAB on SDS at a concentration of 10 mM should be associated with the similar structure of both detergents rather than with the different charge.

In many cases, anionic detergents were found to affect lipases more strongly than cationic ones [8, 14]. The inactivation of the enzymes by anionic detergents could be associated with repulsion of protein molecules from the interface of the substrate [8]. Consequently, it should be suggested the role of three study detergents in *Ps. mendocina* 3121-1 lipase hydrolytic activity on p-NPB to be dependent not only on the charge and the nature of the detergent, but also on the concentration and the order of the detergent interaction with other reaction components.

The lipase was also pre-incubated with each detergent at a concentration of 2.5 mM for 1–30 min at 20 °C (Fig. 3). It should be noted that the lipase was rapidly inactivated by SDS

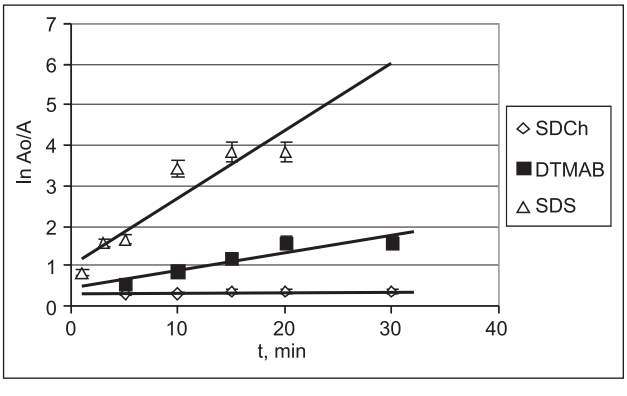

Fig. 3. The lipase-catalysed hydrolytic reaction affected by ionic detergents. Inactivation rate constants of the reaction upon p-NPB solution in propan-2-ol, k_{inact}, min^{-1}: SDS – 0.290; DTMAB – 0.070; SDch – 0.006

during the first 10 min, slower inactivated by DTMAB, and only slightly affected by SDCh. The inactivation rate constants (k_{inact}, min^{-1}) was calculated using a logarithmic equation and was found to be 0.290, 0.070 and 0.006 for SDS, DTMAB and SDCh, respectively. So, the effect of ionic detergents was shown to be conditioned not only by their charge, but also by their nature.

In conclusion, our results showed that:

1. Tweens 20, 40, 60, 80 and 85 were hydrolysed by *Ps. mendocina* 3121-1 lipase and should be considered as lipase substrates rather than as emulsifiers, at least at their concentration exceeding CMC.

2. The effect of ionic detergents on lipase hydrolytic activity depended on their charge, nature and also on the order of the detergents' interaction with other components of the reaction mixture.

3. The concentration of detergents is the crucial factor as the physical phase (solution or micelle) is essential for their action.

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TWEEN'Ų IR JONINIŲ DETERGENTŲ POVEIKIS HIDROLIZINIAM *PSEUDOMONAS MENDOCINA* 3121-1 LIPAZĖS AKTYVUMUI

Santrauka

Šiame straipsnyje skelbiamų tyrimų tikslas buvo nuodugniai ištirti *Pseudomonas mendocina* 3121-1 lipazės katalizinį aktyvumą hidrolizuojant nejoninius detergentus – Tween'us. Naudotos tokios Tween'ų, kaip lipazės substratų, vandeninių tirpalų koncentracijos: dukart mažesnės už kritines micelių koncentracijas (CMC), artimos CMC, penkiskart didesnės už CMC ir gerokai viršijančios CMC (11–44 mM). Detergentų hidrolizės gilumas priklausė nuo Tween'ų struktūrinių elementų – riebalų ir rūgščių – grandinės ilgio. Reakcijos greitis taip pat yra susijęs su detergentų koncentracija ir kito (ženkliai didėjo) joms viršijus CMC, tai rodo, kad, esant tokioms jų koncentracijoms, Tween'ai negali būti naudojami kaip *Pseudomonas mendocina* 3121-1 lipazės substratų (emulsijų) stabilizatoriai.

Dar buvo ištirtas joninių detergentų poveikis *Pseudomonas mendocina* 3121-1 lipazės katalizuojamam tirpaus substrato – butano rūgšties p-nitrofenilo esterio (p-nitrifenilbutirato, pNPB) – skaldymui. Nustatyta, kad joniniai detergentai skirtingai veikia fermento hidrolizinį aktyvumą: natrio dodecilsulfatas (SDS) gerokai jį sumažina per pirmas 10 min; dodeciltrimetilamonio bromidas (DTMAB) fermentą inaktyvina lėtai, o natrio deoksicholato (SDCh) įtaka menka. SDS, DTMAB ir SDCh inaktyvacijos greičio konstantos (k_{inakt}, min^{-1}) yra 0,29, 0,07 ir 0,006 atitinkamai.