Temperature dependence of the catalytic activity of promising nanomaterials based on biopolymers: lipase and chitosan

S Yu Zaitsev¹ and I S Zaitsev²

¹L.K. Ernst Federal Science Center for Animal Husbandry, Dubrovitsy 60, Podolsk Municipal District, Moscow Region, 142132 Russian Federation  
²Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, 117997 Moscow, Russian Federation  

E-mail: s.y.zaitsev@mail.ru

Abstract. A study of the interactions of porcine pancreatic lipase (PPL) with chitosan is the most interesting and important in order to regulate the enzymatic properties of the future nanomaterials based on these biopolymers. A decrease in catalytic activity of pure PPL towards triacetin during prolonged operation and storage is shown: activity decreases by 24.1% within 1 hour and by 54.0% - in the case of the lipase storage in solution for 1 month. The optimum temperature of 40°C was determined for samples of pure PPL, whereas a small shift from 40° to about 45°C - for PPL:Chit complex (50:1). The catalytic activity of free PPL at 40°C was the highest (as compared to lower and higher temperatures) and stable for 20 min. However, the dynamics showed a pronounced decrease to almost 2 times within further 30-60 minutes. In contrast, at 40°C the catalytic activity of PPL in complex with chitosan (50:1) stabilized at almost the same level (although it was lower than the activity of free PPL in the first 30 minutes of measurement). The PPL:Chit complex can be considered as promising catalytic nanomaterials (based on chitosan and lipase) for further applications. Keywords: biopolymers, chitosan, lipase, enzyme immobilization, nanomaterials.

1. Introduction

Enzymes play an important role in numerous metabolic reactions in the body, possessing high selectivity of catalytic action and stereospecificity. These characteristics make their application promising in many industries, especially in the field of biotechnology [1–3]. For example, various types of lipases are used in the production of detergents, dough and cheese flavorings, in the pulp and paper industry, in the oil-fat industry, etc. [4–6]. For the use of enzymes in industry as biocatalysts, they must be stable in operation, cheap and multifunctional in industrial processes. The reaction conditions in industry quite often differ from the natural environment of the enzymes, which can adversely affect their functioning [7–10]. In addition, most enzymes are expensive, so the enzymatic process becomes effective only if the enzyme can be reused. To solve these problems, methods of physical or chemical stabilization are widely used by binding the enzyme to a heterogeneous carrier – immobilization of the enzyme on the carrier [7–10].

Enzyme immobilization is one of the most effective and powerful tools used in industry [4–6]. Immobilization often increases stability, increases activity and selectivity, increases the stability of
enzymes, and also increases the efficiency of separation, purification and reuse of enzymes. These characteristics make the industrial process more efficient. Since the search for new ways to optimize the functioning of enzymes for use in industry is still an urgent task, we devoted our previous research [11–15] to the study of the specific features of the mixtures of porcine pancreatic lipase and chitosan in order to determine the possibilities of using chitosan as an enzyme carrier in industrial processes. Chitosan is a natural cationic polyaminosaccharide [16–19]. It can be used both in pure form and in the form of granules, hydrogels, nanofibres and nanoparticles [11]. In addition, the major advantages of chitosan are biocompatibility, non-toxicity and biodegradability [19–21]. Various methods are used to immobilize the enzyme onto a carrier. One of the simplest and less expensive methods used for immobilization in industry is physical adsorption [13, 22–24]. This method allows you to maintain high catalytic activity of the enzyme and reuse expensive auxiliary materials; in addition, physical adsorption does not affect the conformation of the enzyme molecule [13, 24–27].

This work is devoted to the study of the interaction of porcine pancreatic lipase and chitosan in order to determine the possibilities of using these complexes as promising nanomaterials.

2. Materials and methods

2.1. Materials

Porcine pancreatic lipase (PPL) and the chitosan (Chi) with molecular weight of 200-300 kDa (obtained from Sigma–Aldrich) were used. Triacetylglycerol or “triacetin” (Sigma) was used as a substrate and NaOH (CJSC Caustic) was used as a titrant. For the preparation of salt solutions the following ingredients NaCl and CaCl2 were chosen; whereas KCl was used to fill the electrode (all obtained from Sigma–Aldrich).

2.2. Preparation of Lipase Solutions

To obtain a 0.05 M enzyme solution, a portion of lipase (53.0 mg of PPL) was dissolved in 5 ml of salt solution, then the solution was stirred with a magnetic stirrer for 30 minutes. The PPL solution was then filtered using a funnel and Black Ribbon filter paper.

2.3. Preparation of Polymer Solutions

A preliminary solution of each chitosan (0.05 M) was prepared by dissolving a sample in a 0.1% acetic acid solution. All these solutions were stored in the refrigerator. Subsequently, each preliminary solution was dissolved in distilled water in order to prepare the final solution just before the experiment.

2.4. Substrate Preparation

In order to prepare a 0.05 M solution of substrate for lipase, 545 μl of triacetin was added to 50 ml of a salt solution (equimolar mixture of 0.1 M NaCl and 0.1 M CaCl2). The resulting solution was stirred using a magnetic stirrer for 10 minutes at room temperature. The pH of the resulting solution was ~ 6.1.

2.5. Potentiometric Titration method

Potentiometric method was used to study the catalytic activity of enzymes that based on measuring the potential of an electrode immersed in a solution by titration with 0.01 M NaOH. The magnitude of the potential depends on the concentration of the corresponding ions in the solution. In our work, a laboratory combined pH electrode was used. Standard experimental conditions: 40 °C, pH 7. Measurement of each sample was carried out 3 times within 10 minutes. A water bath and a peristaltic pump were used to maintain a constant temperature. A control measurement of pure lipase was performed daily.

2.6. Adsorption Experiments

Adsorption of lipases on chitosan was performed by addition of 300 μl of lipase to chitosan following ratios: 100:1, 50:1, 25:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:25, 1:50, 1:100. The samples were stored in the
refrigerator. The data obtained were subjected to the statistical treatment by STATISTICA 6.0 (the average errors were below 1%).

3. Discussion and results

3.1. Study of the effect of temperature on the catalytic activity of free lipase

It is well-known that the environmental temperature significantly affects the parameters of the enzymatic reaction. In the study of the catalytic activity of free PPL at different temperatures, the temperature optimum of the free PPL was determined at the first place (taking into account free hydrolysis). At the figure 1, the optimum is reflected at 40°C as the maximum peak at the curve of the dependence of the lipase activity vs. temperature.

![Figure 1. Temperature optimum of the PPL catalysis taking into account free hydrolysis: $V_0$ - catalytic activity (mmol./ml.*min.) vs. temperature (°C), pH = 7.](image)

A decrease in catalytic activity of pure PPL towards triacetin during prolonged operation and storage is shown: activity decreases by 24.1% within 1 hour and by 54% - in the case of the lipase storage in solution for 1 month.

It is important to study the dynamics of the level of PPL catalytic activity at different temperatures (20°C, 30°C, 40°C, 50°C) during 1 hour (table 1).

| Time  | 20°C  | 30°C  | 40°C  | 50°C  |
|-------|-------|-------|-------|-------|
| 1 min | 13.5  | 16.5  | 25.0  | 24.5  |
| 10 min| 14.0  | 16.0  | 24.0  | 23.0  |
| 20 min| 11.0  | 15.0  | 23.0  | 22.5  |
| 30 min| 10.0  | 14.0  | 21.0  | 15.0  |
| 40 min| 11.0  | 13.5  | 18.5  | 8.0   |
| 50 min| 11.5  | 11.0  | 15.0  | 7.0   |
| 60 min| 10.0  | 12.0  | 12.5  | 2.5   |

At relatively high temperature (about 50°C), pure PPL was notable for its high catalytic activity only in the first 20 minutes of measurements; then, it drastically dropped to almost 10 times within an hour. At 40°C, the catalytic activity of free PPL was the highest and stable for 20 min., however, the dynamics
also showed a some decrease to almost 2 times within further 30-60 minutes (although less pronounced as compared to PPL activity at 50°C). In contrast, at 30°C and 20°C, the catalytic activity was relatively low, but more stable for an hour. In addition, similar studies were carried out at 60°C and 70°C (not shown in the Table 1). At these temperatures, the catalytic activity of free PPL was comparable to the level of free hydrolysis at these temperatures, indicating a loss of enzyme activity.

The most important results of our previous studies were the following: chitosan inhibits pancreatic porcine lipase in solution, and the stronger, the higher the concentration of chitosan in relation to the concentration of lipase [11–15]. For example, with a molar ratio of lipase to chitosan of 5:1, the catalytic activity of lipase decreases by 46.8%. In contrast, the PPL: Chit complex in a molar ratio of 50:1, in which lipase had the most stable catalytic activity during prolonged operation and during storage. In this ratio, the catalytic activity of lipase decreases by 0.6% during 1 hour of measurements, and when stored for 1 month - by 39.9% [11–15].

3.2. Study of the effect of temperature on the catalytic activity of lipase:chitosan complex (50:1)
For the PPL: Chit 50:1 complex, the temperature studies similar to free PPL were carried out. In the study of the catalytic activity of the PPL:Chit 50:1 complex at different temperatures, the temperature optimum of the PPL:Chit 50:1 complex was determined (figure 2) taking into account free hydrolysis.

![Figure 2](image)

**Figure 2.** Temperature optimum of the catalytic activity of PPL:Chit complexes (5:1) taking into account free hydrolysis: $V_0$ - catalytic activity (mkmol/ml*min) vs. temperature (°C), pH = 7.

It should be noted (figure 2) that the peak at the PPL:Chit complex (5:1) was broader (especially in the temperature region above 40-45°C) than the free PPL (figure 1). At 50°C, the activity of the lipase-chitosan complex was higher (figure 2) than that of free PPL (figure 1). This indicated the expansion of the temperature optimum of PPL and the stability during its immobilization on chitosan with a molar ratio of lipase to chitosan of 50:1. These data gave certain advantages when using this complex in biotechnological processes.

In a similar manner as above (table 1) the dynamics of the level of PPL catalytic activity in the PPL:Chit complex (50:1) at different temperatures (20°C, 30°C, 40°C, 50°C) during 1 hour (table 2) was studied.
At 50°C, the PPL:Chit complex (50:1), as well as free PPL, had high catalytic activity only in the first 20 minutes of operation, then there was a sharp decrease in activity to almost 4 times within further 30-60 minutes (tables 1 and 2). In contrast, at 40°C the catalytic activity of PPL in complex with chitosan (50:1) stabilized at almost the same level (although it was lower than the activity of free PPL in the first 30 minutes of measurement). At 30°C and 20°C, the catalytic activity of this complex was also stable for 1 hour (table 2). However, the absolute values of the activity at 30°C and 20°C were lower than at 40°C or 50°C, respectively. In addition, the studies at 60°C and 70°C showed low activity and stability at the levels as those similar to free PPL (table 1).

Table 2. The dynamic’s level of the catalytic activity of PPL:Chit complexes (50:1) at different temperatures.

|        | 20°C | 30°C | 40°C | 50°C |
|--------|------|------|------|------|
| 1 min. | 11.5 | 14.5 | 19.5 | 23.0 |
| 10 min.| 11.0 | 14.0 | 19.0 | 22.0 |
| 20 min.| 10.0 | 13.0 | 19.0 | 23.0 |
| 30 min.| 11.0 | 12.0 | 17.0 | 19.0 |
| 40 min.| 10.0 | 11.0 | 18.0 | 15.0 |
| 50 min.| 10.0 | 12.0 | 16.0 |  9.0 |
| 60 min.| 10.5 | 12.5 | 18.0 |  6.0 |

By comparing the results of our study on free PPL with the data of other well-known studies [35, 70, 72], it can be noted that in general they do not contradict each other. There are many studies concerning the temperature optima of free lipases purified by various origins. Most free lipases have temperature optima within a rather small range of 30–45°C [25–27]. The temperature optimum of about 40°C obtained in this study fits into this range.

However, there are many contradictions (among existing studies) regarding the displacement of the temperature optimum of lipases during their immobilization on chitosan. In some studies, no shifts were observed [25–27], which confirmed this study. In other studies, on the contrary, a significant shift in the temperature optimum was observed [27]. It is especially interesting to compare the results of this study with the work of Ali Kln 2006 [27], which used similar experimental conditions with the immobilization of various lipases on soluble chitosan samples. This work described a shift in the temperature optimum from 30°C (40°C) to 45°C during PPL immobilization. A number of studies also indicated an increase in the stability of various lipases during their immobilization on chitosan [25–27]. This indicates the importance and relevance of this research in studying the effect of chitosan on changing the lipase activity.

4. Conclusion
The most interesting is the result of the interaction of pancreatic pork lipase with chitosan in order to regulate the enzymatic properties of these mixtures. A decrease in catalytic activity of pure PPL towards triacetin during prolonged operation and storage is shown: activity decreases by 24.1% within 1 hour and by 54% - in the case of the lipase storage in solution for 1 month. The optimum temperature of 40°C was determined for samples of pure PPL, whereas a small shift from 40° to 45°C - for PPL:Chit complex (50:1). The last complex is promising as catalytic nanomaterials based on chitosan and lipase for further applications.

Acknowledgments
This work was supported by Russian Foundation of Basic Research (RFFI 19-03-00717). The authors are thankful to Abramova O.V. and Savina A.A. for technical support.

References
[1] Javed S, Azeem F, Hussain S, Rasul I, Siddique M H, Riaz M, Afsal M, Kouser A and Nadeem
H 2018 Progress in Biophysics and Molecular Biology 132 23

[2] Treichel H, de Oliveira D, Mazutti M A, Di Luccio M and Oliveira J V 2010 Food Bioprocess Technology 3 182

[3] Gupta R, Gupta N and Rath P 2004 Applied Microbiology and Biotechnology 64 763

[4] Robles-Medina A, Gonzalez-Moreno P A, Esteban-Cerdan L and Molina-Grima E 2009 Biotechnol Adv. 27 398

[5] Sangeetha R 2011 Res. J. Microbiol. 6 1

[6] Sharma R, Chisti Y and Banerjee U C 2001 Biotechnology Advances 19 627

[7] Eremeev N L and Zaitsev S Yu 2016 Mini-Reviews in Organic Chemistry 13 78

[8] Zaitsev SYu 2017 Biological chemistry: from biologically active substances to organs and tissues of animals (Moscow: Capital Print Publishing)

[9] Zhtar Y, Weng Y, Xu H and Mao Z 2012 Applied Microbiology Biotechnology 93 61

[10] Minovska V, Winkelhausen E and Kuzmanova S 2005 J. Serb. Chem. Soc. 70 609

[11] Ivanov E A, Aha B, Volchenkova T A and Zaitsev S Yu 2002 Colloids and Surfaces B: Biointerfaces 23 349

[12] Zaitsev S Yu, Gorokhova I V, Cashtigo T V, Zintchenco A and Dautzenberg H 2003 Colloids and Surfaces A: Physicochem Eng Aspects 221 209

[13] Zaitsev S Yu 2010 Supramolecular Nanosized Systems at the Phase Interface: Concepts and Prospects for Bio-Nanotechnologies (Moscow: URSS-LENAND)

[14] Zaitsev S Yu, Savina A A, Garnashevich L S, Tsarkova M S and Zaitsev I S 2019 BioNanoScience 9 773

[15] Zaitsev S Y, Savina A A and Zaitsev I S 2019 Advances in Colloid and Interface Science 272 1

[16] Cheung R C F, Ng T B, Wong J H and Chan W Y 2015 Marine Drugs 13 5156

[17] Younes I and Rinaudo M 2015 Marine Drugs 13 1133

[18] Pellá M C G, Lima-Tenório M K, Tenório-Neto E T, Guilherme M R, Muniz E C and Rubira A F 2018 Carbohydrate Polymers 196 233

[19] Ruocco N, Costantini S, Guariniello S and Costantini M 2016 Molecules 21 551

[20] Nilsen-Nygard J, Strand S P, Varum K M, Draget K I and Nordgard C T 2015 Polymers 7 552

[21] Kumar M R 2000 Reactive & Functional Polymers 46 1–27

[22] Balaji A B, Pakalapati H, Khalid M, Walvekar R and Siddiqui H 2018 Biodegradable and Biocompatible Polymer Composites 3 3

[23] Zargar V, Aghhari M and Dashti A 2015 ChemBioEng Reviews 2 204

[24] Copeland R A 2000 Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis (New York: Wiley-VCH)

[25] Toscanova L, Montero G, Stoytcheva M, Cervantes L and Gochev V 2014 Biotechnology & Biotechnological Equipment 28 52

[26] Chiou S, Hung T, Giridhar R and Wu W 2007 Preparative Biochemistry & Biotechnology 37 265

[27] Kılıç A, Teke M, Önal S and Telefoncu A 2006 Preparative Biochemistry & Biotechnology 36 153