Study of Catalytic Activity of Lipase and Lipase-Chitosan Complexes in Dynamics

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Abstract: Pancreatic porcine lipase (PPL) is the unique enzyme in numerous biochemical processes for human and animals. Stability in time is the critical point for many enzymes in relation to their further applications. The effect of chitosan on the activity of PPL during 2 months was investigated.

Materials and Methods: Potentiometric method was used to study the catalytic activity of enzymes that based on measuring of the potential an electrode immersed in a triacetin (substrate) solution by titration with 0.01 M NaOH. A laboratory pH-stat with combined pH electrode was used for the measurements of this potential in the presence of PPL or PPL-chitosan complexes. Standard experimental conditions: 40 ºC, pH 7.0.

Results: The following PPL activity values (data given in % to the activity of free lipase) vs. time (in 10 min. intervals) were obtained: 100%, 97.4%, 89.9%, 82.9%, 77.9% and 75.9% after 10, 20, 30, 40, 50 and 60 min., respectively. The PPL catalytic activity decreased at about ¼ to ½ of the initial values after 7 or 14 days, 1 or 2 months storage, although there were differences in the decline dynamics. The complex PPL:Chit=50:1 has better stabilizing properties as compared to other complexes; does not strongly inhibit lipase and requires a small amount of carrier (chitosan) for its formation.

Conclusions: The complex PPL:Chit=25:1 is less effective as the PPL:Chit=50:1 complex, but both can be applicable in some industrial processes.

Keywords: Lipase, chitosan, enzyme immobilization, activity, triacetin.

INTRODUCTION

Lipases are the unique enzymes in numerous biochemical and biotechnological processes [1-4]. They possess a high selectivity, stability and stereospecificity [2-6]. These characteristics make their application promising in many fields [4-7]. Stability in time is the critical point for many enzymes, as well as lipases, in relation to their further applications [5-7]. Pancreatic porcine lipase (PPL) [8-10], as well as the other lipases of human and animal pancreas are very sensitive to the environment and substrate [11-13]. It is important to mention here the so-called "interfacial activation" effect [10, 14]). The substrates (various triacylglycerols) must be emulsified in microdroplets with bile acids, phospholipids, etc. On the other side, the small protein-helper (called colipase [8-10]) is the key hinge (hook) to bind to the non-catalytic site of the enzyme and interact with the peptidic "lid" at the catalytic site. The joint action of the microdroplets and colipase opens the "lid" and loops to enable the substrate molecule to enter the hydrophobic channel of the enzyme active site (lipase "open" conformation) [10, 14]. This scheme was modified in our research [15, 16] on lipase activation by polyelectrolytes (instead of colipase) and emulsified substrate that convert the enzyme from "closed" to "open" conformation.

Chitosan, which is obtained from chitin, is the most common marine polysaccharide [17]. Chitosan is a cationic linear heteropolyamino saccharide consisting of N-acetyl-2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucose, linked by β-(1,4)-glucoside bonds [17-19]. Chitosan and chitin have numerous advantages include availability, biocompatibility, biodegradability, nontoxicity, antimicrobial properties, heavy metal ions chelation, gel forming properties, ease of chemical modification, and high affinity to proteins. Owing to these characteristics, these biopolymers, thus far underutilized, are predicted to be widely exploited in the near future in environmentally benign applications, notably in systems working in biological environments [19-21]. Chitosan and its derivatives are recognized as universal biomaterials because of their non-toxicity, low allergenicity,

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biocompatibility, and biodegradability, despite the fact that chitosan is not naturally present in human or animal tissue [18-21]. The cationic nature of chitosan is very specific and allows the formation of electrostatic complexes or multilayer structures with other negatively charged synthetic or natural polymers, as well as with lipases [5, 19-21]. Physical or chemical adsorption of lipase on the chitosan molecules are the most reliable and relatively fast methods used in biotechnology [22-27]. These methods allow to maintain a high catalytic activity and reusability of the lipase, without drastic changes in the PPL conformation [22-27].

The aim of this study is to compare the activity of pancreatic porcine lipase with and without chitosan in order to determine the possibility for further applications.

MATERIALS AND METHODS

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 $CaCl_2$ were chosen; whereas KCl was used to fill the electrode (all obtained from Sigma-Aldrich).

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.

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. The same procedure was applied for PSS and PL solution preparations. 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 bevor the experiment.

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 $CaCl_2$). The resulting solution was stirred using a magnetic stirrer for 10 minutes at room temperature. The pH of the resulting solution was ~ 6.1.

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 ºC, 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.

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. 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%).

RESULTS AND DISCUSSION

The measurements of the catalytic activity of porcine pancreatic lipase (PPL) for 1 hour was performed for reference purposes concerning the further experiments of chitosan-lipase complexes. The following PPL activity values (data given in % to the activity of free lipase) vs. time (in 10 min. intervals) were obtained: 100%, 97.4%, 89.9%, 82.9%, 77.9% and 75.9% after 10, 20, 30, 40, 50 and 60 min., respectively. Thus, the gradual decrease in the PPL activity was observed, but after 1 hour there is almost ¼ of the initial activity was lost.

If the prepared solutions were stored in the refrigerator after each measurement, the following PPL activity values vs. time (of storage) were obtained: 100%, 62.7%, 50.1%, 46.0% and 45.4% after 1st day, 7th day, 14th day, 1 and 2 months, respectively. There was a sharp decrease in the activity of free PPL (by 37.3%) observed during the first 7 days. Further, a relative decrease in activity from 7 to 14 days was another 12.7%, but from 14 days to 1 month - only

about 4.1%. Data on the measurement of the PPL catalytic activity after 1 month and 2 months of storage presented the PPL activity decrease only by 0.6%. Thus, the catalytic activity of PPL after 1-2 months of storage decreased by 54-55% in relation to the values on the first day of measurement and stabilized at this level.

Dynamics of the Catalytic Activity of Free PPL for 1 Hour in Relation to the First 10 Min. of Measurements

The complete study was fulfilled during 2 months (after 7 and 14 days, or 1 and 2 months of storage of the same lipase solutions). In order to characterize the PPL activity in relative values (Table **1**) the obtained data were measured after 7 and 14 days, 1 and 2 months of storage (of the same lipase solutions) and recalculated to the first 10 minutes of measurements (was taken as 100%).

As can be seen from the data comparison the PPL activity dynamics after the same days (months) of storage had some differences. For example, after 7 days, 1 and 2 months of storage, a sharp decrease in PPL catalytic activity was observed by the first 20 minutes of measurements as compared to those values at the first day (Table **1**). After 7 days of storage, some decrease in PPL catalytic activity was observed by the first 30 minutes of measurements as compared to those values at the first day (Table **1**). A small increase in the PPL activity (1.6%) was observed from 30 to 40 minutes (Table **1**). After 7 days of storage the total PPL activity per hour decreased by 20.7% (Table **1**). After 14 days of storage, a sharp drop in lipase activity was observed from 30 to 50 minutes (Table **1**). At the same time, the first 30 minutes of activity remained at the same level. A small increase in the PPL activity (1.9%) was observed from 50 to 60 minutes (Table **1**). After 14 days of storage the total PPL activity per hour decreased by 24.9%. After 1 months of storage, a gradual decrease in PPL catalytic activity was observed all the time and the total PPL activity per hour decreased by 33.8% as compared to those values at the first day (Table **1**). After 2 months storage a complete stabilization of PPL activity was observed from 50 to 60 minutes at the level of about 74.1% (Table **1**). Moreover, the absolute decrease of PPL activity after 2 months storage was almost the same as after 14 days of storage –25.9% and 24.9%, respectively (Table **1**). Thus, the PPL catalytic activity decreased about the same after 7 days, 14 days and 2 months storage, although there were differences in the decline dynamics.

Dynamics of the Catalytic Activity of Free PPL for 1 Hour in Relation to Each 10 Min. of the First 60 Min. of Measurements

The other approach to characterize the same PPL activity is the following: to measure a PPL activity during these 2 months (of the same lipase solutions) and recalculated to the first 60 minutes of measurements. These 60 minutes will be considered as 100% lipase activity (Table **2**).

As can be seen from the data in the Table **2**, the PPL activity dynamics (in relation to the first 60 min. of measurements) had less deviations from the gradual dependences, as compared to those presented in the Table **1**. For example, after 7 and 14 days, 1 and 2 months of storage, a sharp decrease in PPL catalytic activity was observed by the first 10 minutes of measurements as compared to those values at the first day (Table **1**). It is interesting that in the case of 14 days and 1 month storage, the final deviations (after an hour of measurements) were almost the same (as for the first 10 min. of measurements). In contrast, the final deviations (after an hour of measurements) were low or higher as those for the first 10 min. of measurements in

Table 1: Dynamics of the Catalytic Activity of Free PPL for 1 Hour (%) vs. Time of Storage (in Relation to the First 10 Min. of Measurements), pH = 7.0, T = 40ºC

Time, min.	PPL activity for 1 hour (%) in relation to the first 10 min. of measurements				
	1 day	7 days	14 days	1 month	2 months
10	100	100	100	100	100
20	97.4	89.9	98.6	88.3	88.4
30	89.9	83,6	98.4	80.5	85.1
40	82.9	85,2	89.3	73.0	81.3
50	77.9	82.0	73.9	71.2	74.1
60	75,9	79.3	75.1	66.2	74.1

Table 2: Relative Catalytic Activity of Free PPL for 1 Hour vs. Time (%) vs. Time of Storage (in Relation to Each 10 Min. of the First 60 Min. of Measurements), pH = 7.0, T = 40ºC

Time, min.	PPL activity for 1 hour vs. time (%) in relation to the first 60 min. of measurements				
	1 day	7 days	14 days	1 month	2 months
10	100	80.0	57.8	62.2	46.7
20	100	74.4	58.1	55.8	44.2
30	100	71.8	59.0	59.0	46.2
40	100	75.0	63.9	58.3	50.0
50	100	78.8	54.5	63.6	48.5
60	100	77.4	58.1	61.3	51.6

the case of 7 days or 2 months storage, respectively (Table **2**). In general, the gradual decrease in the PPL activity was observed at about ¾ or ½ of the current PPL activity.

Dynamics of Catalytic Activity of PPL-Chitosan Complexes

The study of the dynamics of the catalytic activity of chitosan-lipase complexes for 1 hour was performed for the first time. The hourly dynamics of the studied lipase-chitosan complexes (100: 1, 50: 1, 25: 1, 10: 1 and 5: 1) differ significantly from each other and on the dynamics of free lipase (Tables **3** and **4**).

It is important to consider the features of the dynamics of each complex and further compare them with the free lipase.

Complex PPL:Chit=100:1

This ratio of lipase and chitosan very weakly influenced the catalytic activity of lipase (by only 1.7%), but it affected the hourly dynamics of the changes in lipase activity. In such a complex, lipase was more quickly losing its activity than free lipase. The catalytic activity of the complex PPL:Chit=100:1 was reduced in 1 hour by 34.9%, while for the free lipase - was only 24.1%. Thus, the difference in the final activity was

Table 3: Dynamics of Catalytic Activity of PPL (as Reference) and PPL-Chitosan Complexes (100: 1, 50: 1, 25: 1, 10: 1, 5: 1) for 1 Hour (in Relation to the First 10 Minutes of Measurements, Given as 100%), pH = 7, T = 40ºC

Time, min.	PPL activity for 1 hour vs. time (%) in relation to the first 10 min. of measurements					
	PPL	100:1	50:1	25:1	10:1	5:1
10	100	100	100	100	100	100
20	97.4	90.5	101.6	97.4	93.1	95.0
30	89.9	79.4	96.1	97.2	82.8	95.0
40	82.9	76.2	97,4	91.7	75.9	95.0
50	77.9	68.3	95.8	93.1	72.4	95.0
60	75.9	65.1	99.4	89.1	72.4	85.0

Table 4: Dynamics of Catalytic Activity of PPL (as Reference) and PPL-Chitosan Complexes (100: 1, 50: 1, 25: 1, 10: 1, 5: 1) for 1 Hour (in Relation to the First 60 Minutes of Measurements, Given as 100%), pH = 7, T = 40ºC

Time, min.	PPL activity for 1 hour vs. time (%) in relation to the first 60 min. of measurements					
	PPL	100:1	50:1	25:1	10:1	5:1
10	100	105	98.6	111.4	74.4	51.3
20	100	98.3	94.4	97.4	71.1	50.0
30	100	90.9	91.2	99.3	75.0	59.4
40	100	96.0	92.0	93.2	81.5	70,4
50	100	97.7	93.4	97.7	84.0	76.0
60	100	100	100.8	97.3	87.5	70.8

10.8%. Thus, although it was necessary to spend very little carrier to form this complex, this ratio had no positive effect on lipase stabilization. Therefore, such a ratio of lipase and chitosan in the complex cannot be considered as promising for practical application in biotechnology.

Complex PPL:Chit=50:1

In such a molar ratio of lipase and chitosan, there was also no significant inhibition of lipase - the activity of PPL in the complex is reduced by 3.3% compared with free PPL. At the same time, the study of the hourly dynamics of the catalytic activity of this complex showed that the lipase activity remained at the same level for the whole hour, while the activity of the free PPL dropped after an hour by 24.1%. This indicates the relatively high stabilization of the PPL catalytic activity in this complex (Tables **3**, **4**). To our opinion, the complex PPL:Chit=50:1 can be used stabilize the enzyme in the biotechnological processes. Thus, the advantages of this complex were the following: weak inhibition of the catalytic activity of PPL in such a complex and a small amount of carrier - chitosan, necessary for the formation of this complex, compared with lipase.

Complex PPL:Chit=25:1

The nature of the hour dynamics of this complex is very similar to the PPL:Chit=50:1 complex. Lipase also tends to stabilization as a part of this complex for entire hour, but less effectively. For example, there was a decrease in the catalytic activity of PPL by 10.9% in 1 hour as compared with the activity in the first 10 minutes of measurement (Table **3**). The catalytic activity of the PPL in such a complex decreased by 6.5% compared with the free PPL. This complex was also applicable for biotechnological process, however it had no advantages over the PPL:Chit=50:1 complex. The complex PPL:Chit=25:1 inhibits lipase more strongly than the PPL:Chit=50:1, and the carrier costs for the formation of this complex are needed more than in the complex PPL:Chit=50:1.

Complex PPL:Chit=10:1

A significant inhibition of lipase occured (already by 26.6%) in the case of PPL:Chit=10:1 complex (Tables **3**, **4**). At the same time, the study of the hour dynamics of the PPL activity for this complex showed that the lipase was losing its activity more quickly as compared to the free lipase. In one hour, the catalytic activity of the PPL:Chit=10:1complex decreased by 27.6% (Tables **3**, **4**). Thus, in the case of PPL:Chit=10:1 complex, stabilization of the lipase was not observed, but only significant inhibition was noted. On the other side, inhibition of lipase can be important in the treatment of obesity and other digestive disorders. Data on the peculiarities of lipase inhibition by chitosan of different concentrations may be useful in the development of food additives for the treatment and prevention of such diseases.

Complex PPL:Chit=5:1

In this complex, even more significant inhibition of the catalytic activity of lipase occured - by 46.8% as compared with the activity of free PPL. Despite the high level of inhibition of catalytic activity, its stabilization was observed in hourly dynamics up to 50 minutes (but after 50 minutes there was a sharp drop in lipase activity by 15%) as compared to the level of activity in the first 10 minutes of measurement (Table **4**). This complex, despite the ability to stabilize lipase, is not well suited for biotechnological processes due to the high level of enzyme inhibition, as well as the need for high carrier costs for the formation of this complex. However, these data can also be used for design of food additives, as in the case of the PPL:Chit=10:1 complex. At the same time, the PPL:Chit=5:1 complex has a significant advantage - the ability to stabilize the activity of lipase at the same level, as compare to the PPL:Chit=10:1 complex.

CONCLUSIONS

Thus, the complex PPL:Chit=50:1 is the most promising for application in biotechnology. This complex has better stabilizing properties compared to other complexes, it does not strongly inhibit lipase and requires a small amount of carrier - chitosan for its formation. Less effective, but also applicable in industrial processes, is the PPL:Chit=25:1 complex.

In order to use chitosan as a lipase inhibitor (for example, in the design of food additives), one should pay attention to the properties of the PPL:Chit=10:1 and PPL:Chit=5:1 complexes. An such molar ratios, lipase is inhibited most effectively.

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