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The Studies of Sepharose-Immobilized Lipases: Combining Techniques for the Enhancement of Activity and Thermal Stability

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Abstract: Thermal stability is one of the essential parameters characterizing biocatalysts with potential applications in the chemical and pharmaceutical industries. Therefore, it is extremely important to develop standardized procedures for enzyme stability studies. The paper attempts to assess the thermal stability of immobilized lipases in aqueous buffers: lipase B from *Candida antarctica* (CALB) and lipase from *Candida rugosa* (CRL-OF) immobilized on the Octyl-Sepharose CL-4B carrier. As part of the optimization conditions of the immobilization, the influence of time on the catalytic activity and lipase loading, as well as the effect of temperature on lipase activity (optimal incubation—14 h at 4 °C), was determined. The thermal stability test procedure was carried out for 7 days using a climatic chamber (65 °C) and a refrigerator (4 °C). The studies of immobilized lipases included the assessment of the impact of various solvents (water, citrate buffer, 1,2-dichloropropane—DCP), temperature, light in the visible spectral range (400–800 nm), and additions of calcium ions. The highest value of residual activity (564.5 ± 21.6%) was received by storing the immobilized CALB in citrate buffer (pH 4.0, 500 mM) with the addition of calcium ions (Ca^{2+}). On the other hand, residual activity values for immobilized CRL-OF after storage in the climatic chamber were lower than 5%. A combining of techniques: immobilization onto the support in high ionic strength and low pH, with a technique of extremally high-temperature applied in a climatic chamber, with the addition of Ca^{2+} allowed to achieve of excellent thermal stability of the immobilized CALB, with increasing of catalytic activity more than five-fold. Additionally, performing studies on the thermal stability of the tested lipases using a climatic chamber seems to be particularly promising in the context of unifying and standardizing storage guidelines, enabling the comparison of results between different laboratories, as well as enhancing catalytic activity.

Keywords: lipase from *Candida rugosa*; lipase B from *Candida antarctica*; Octyl-Sepharose CL-4B; immobilization; thermal stability; incubation time; climatic chamber; aqueous buffers; combining techniques

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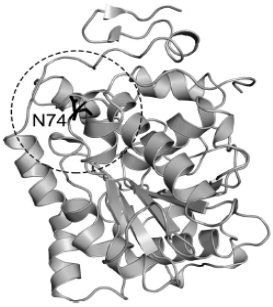
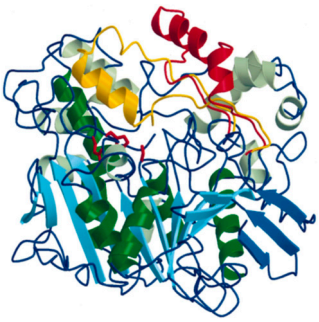
1. Introduction

The modern trends in achieving new drugs and precursors are often related to enzymes, which are the basis of biocatalysis reactions [1]. Despite the established position of chemical synthesis, alternative reactions with the use of enzymatic catalysts are earning more interest from researchers. Due to the milder reaction conditions, the possibility of reuse, and environmental friendliness, biocatalysis has advantages over chemical reactions [2]. It should be noted that the efficiency of reactions catalyzed by enzymes differs

and requires many improvements and modifications. Thus, it is a substantial challenge for numerous research groups.

Lipases, belonging to hydrolases, are a meaningful group of enzymes used in reactions of pharmaceutical importance [3]. These enzymes are most often characterized by the presence of a lid that covers the enzyme's active site [4] and demonstrate high catalytic activity in both hydrophilic and lipophilic media [5]. The mentioned proteins can adjust to the reaction medium and alter their activity, excluding extreme conditions in which the protein structure is deactivated [6]. One of the best-known and most widely applied lipases are lipase B from *Candida antarctica* (CALB) and lipase from *Candida rugosa* (CRL) [7,8]. In Table 1, the comparison of the structure and properties of these lipases has been shown.

Table 1. The comparison of the structure and properties of CALB and CRL.

	Lipase B from *Candida antarctica*	**References**	**Lipase from *Candida rugosa***	**References**
Molecular structure		[8]		[9]
Active site	Serine-105, Histidine-224, Aspartate-187	[10]	Serine-209, Histidine-449, Glutamate-341	[9,11]
The presence of a lid	Debated	[12]	Presence	[9,11,13]
Isoelectric point or points range	pH 6.0	[14]	pH 4.0–5.5	[15]
Optimum temperature	10–60 °C	[16]	30–50 °C	[17]
Optimum pH	6.0–9.0	[16]	7.0	[17]
Isoforms	1	[18]	5	[19]
Enantiopreference	*R*-enantiomers, *S*-enantiomers	[20]	*S*-enantiomers	[21]
Carbon chain length preference	Short-length	[6]	From short to long	[22]

In biocatalysis reactions, lipases are commonly used in the free (native) form [23]. It should be noted that methods with the aim of modifying the catalytic activity are constantly developed. One of the techniques enabling to increase in the lipase's enzymatic activity and enhancing its stability is immobilization onto support (carrier) [24,25]. After the immobilization process, lipase could be more resistant to a wide range of reaction conditions (e.g., temperature, pH, ionic strength). An essential aspect of the application of this technique is the possibility of immobilized lipase recovery from the reaction medium and reuse in subsequent reaction cycles. Lipase immobilization can be carried out via various interactions. One of the most commonly used is adsorption, ionic or covalent interactions, cross-linking, encapsulation, or entrapment [26]. It should be taken into account that each of these techniques has its certain limitations, such as enzyme leakage from the support (adsorption, ionic bond), change in the enzyme structure (covalent bond, cross-linking), or diffusion barrier (encapsulation, entrapment) [6]. Therefore, one

of the directions for this study is an improvement of the immobilization methods by the application of multipoint interaction/bonding as well as attempts at limiting the disadvantages associated with the use of each method separately [27]. Another technique to influence enzyme activity is to control the temperature and, also, the application of metal ions during the storage of biocatalysts [28]. Our paper describes studies on the use of a higher-than-optimal temperature (stress conditions) and the addition of calcium ions in order to increase the catalytic activity.

A support material, which is constantly increasing in importance, is agarose [29,30]. Its derivatives (e.g., octyl-agarose) are characterized by well-stability in a broad range of temperatures (up to 75 °C) [31] and pH [32]. The mechanism of the lipase immobilization onto agarose beads is based on physical adsorption via interfacial activation. In the presence of a hydrophobic surface (drops of the natural substrates), the lid movement takes place, exposing the hydrophobic pocket of the enzyme (lipase open form) to the medium [33]. As mentioned, agarose support undergoes various modifications; among others is the addition of functional groups to increase their hydrophobicity (possibly making them more accessible to lipase) and/or for stabilization of the formed catalytic system [34–36]. Octyl Sepharose CL-4B is commercial agarose beads composed of a cross-linked 4% agarose matrix with an octyl group (data gained from GE Healthcare, Uppsala, Sweden). This support allows adsorption through interfacial activation and contributes to the stabilization of immobilized lipase [37].

One of the most valuable features in the search for novel lipases is thermal stability. The lipases characterized by thermostability are extremely important enzymes used in the pharmaceutical, food, and biotechnological industries. The increased stability of lipases enables the maintenance of catalytic activity in various reaction media [38]. It is worth citing that the essential factors for protein lipase structure stabilization are ion interactions and hydrogen bonds [39]. The stability (especially thermal and solvent/medium) in lipase structure can be increased by improving the hydrophobic core and the change in the side chain isoelectric point value [40].

Numerous authors have presented stability studies in the literature [41–43]. It should be noted that there is a lack of precise unified stability test procedures. These standardized tests could be valuable in the study of lipases for potential application in the pharmaceutical and chemical industries. In our current paper, we propose the application of a climatic chamber to study the thermal stability of immobilized onto Octyl-Sepharose CL-4B support lipases (CALB and CRL-OF) stored in aqueous buffer solutions. These lipases have been chosen due to differences in structure, in particular, regarding the lid. As a part of the study, the influence of the various solvents (water, buffer, 1,2-dichloropropane), temperature, light in the visible spectral range (400–800 nm), and the addition of calcium ions on the storage stability of biocatalysts was investigated. The influence of storing the systems in buffers with different ionic strengths on the activity parameters was also tested. In addition, the immobilization procedures were optimized by studying the effect of time and temperature on the catalytic activity as well as on lipase loading. The analysis was conducted with the use of spectrophotometric and titration methods. For potential enhancement of thermal stability, combining techniques were performed. Two methods were combined: (a) immobilization of lipases onto the support in high ionic strength and low pH, with (b) storage of lipases in a climatic chamber at an extremely high temperature in a buffer in high ionic strength and low pH, with the addition of calcium ions.

2. Results and Discussion

2.1. Effect of Immobilization Time on CALB Catalytic Activity and Lipase Loading

CALB was immobilized in phosphate buffer (pH 7.0) and ionic strength (100 mM) using various incubation times. Based on the achieved results of enzymatic activity (U) and the amount of immobilized lipase, the immobilization efficiency (I_e) has been calculated. The obtained parameters are shown in Figure 1.

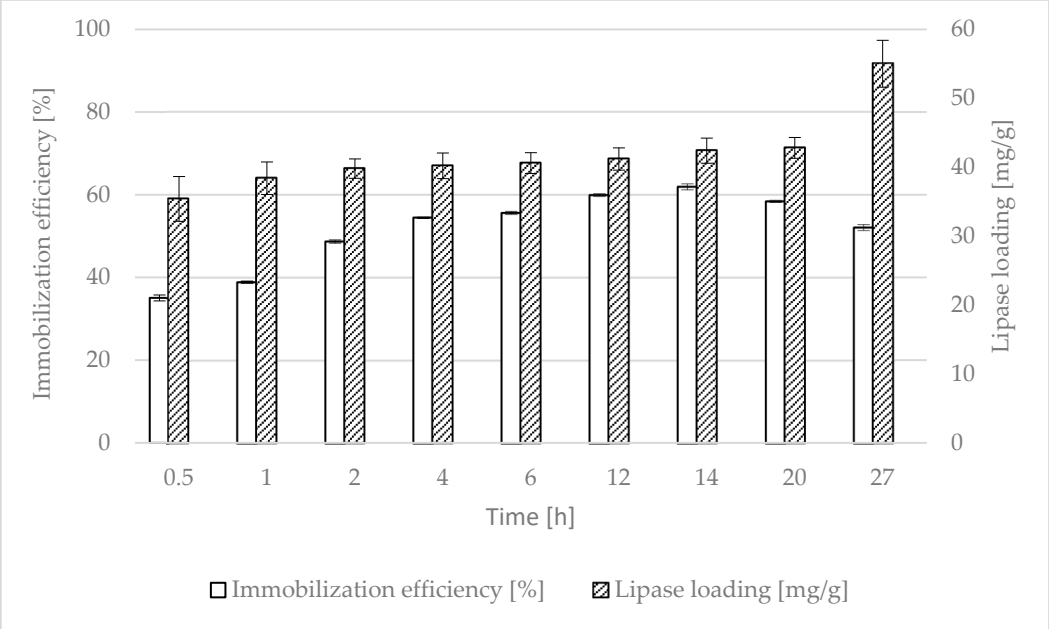


Figure 1. The effect of incubation time on immobilization efficiency (I_e) and the lipase loading (L_L) (mg/g support) of immobilized CALB. Data are presented as means ± standard deviations of three analyses (n = 3). The error bars represent the standard deviations of the mean.

The received results showed an increase (to 14 h of incubation time) of the I_e parameter, along with an increase in the incubation time. Nevertheless, above the 14 h of incubation time, the decrease in I_e was observed. Simultaneously, the lipase loading values increased with incubation time, without decline, as in the case of I_e (14 h—L_L = 42.40 ± 1.81 mg/g). In our study, above 20 h of incubation, the increase in immobilized lipase amount has been noticed. Regarding I_e, it is assumed that the cause of this phenomenon can be support overloading and related to its interactions of immobilized enzymes and diffusion limitations. It is supposed that the decrease in enzymatic activity can also be induced by the denaturation of the enzyme structure. Moreover, different incubation times in order to achieve maximal enzymatic activity may result from enzyme support [44]. The decrease in activity could also be the effect of the steric hindrance of lipase molecules and the limitation of mass transfer [45], as well as the leakage of lipase molecules [46]. On the other hand, it is believed that prolonged immobilization time can cause an increase in enzyme activity due to the change in protein conformation [47]. Studies on the impact of time on CALB activity have been described in the literature [44,47,48].

The presented data in our paper suggest the substantial effect of immobilization time on the catalytic activity of immobilized CALB. Optimizing this parameter seems to be a crucial step in developing novel catalytic systems. Peirce et al. [29] carried out the immobilization process of CALB onto Octyl-Sepharose CL-4B with the application of phosphate buffer at pH 7.0 with ionic strength of 5 mM. The enzyme was completely immobilized within 30 min with a low effect on enzyme activity. It should be mentioned that the ionic strength of the phosphate buffer in the cited paper was 20 times less than the buffer used in our studies, which could affect the increase in incubation time. Analyzing the received data, it was decided that in the next stages, the incubation time would be 14 h.

2.2. Effect of Immobilization Temperature on CALB Activity

The CALB was immobilized onto Octyl-Sepharose CL-4B support using various temperatures. The lipolytic activity of lipase (U) has been assessed. Based on obtained results (U), the relative activity (A_{rel}) of immobilized preparations and immobilization efficiency (I_e) were calculated (Table 2).

Table 2. The effect of various temperature conditions on CALB activity.

First Step		Second Step				Results	
Stirring (5 min) in Ice Bath	**Stirring (5 min) at Room Temperature**	**Incubation 14 h at 4 °C**	**Incubation 14 h at Room Temperature (22 °C)**	**Incubation 14 h at 37 °C**	**Stirring during 14 h of Incubation**	**Relative Activity (%)**	**I_e (%)**
+		+				93.20 ± 3.02	55.20 ± 1.00
	+	+				100.00 ± 2.25	61.90 ± 0.74
	+		+			90.70 ± 2.55	52.30 ± 0.84
	+		+		+	90.70 ± 1.22	51.50 ± 0.40
	+			+		95.40 ± 2.41	57.70 ± 0.79
	+			+	+	93.20 ± 0.75	54.90 ± 0.25
Alternative protocol: Incubation time: 30 min, 22 °C, constant stirring						38.70 ± 2.53	21.15 ± 0.83
Alternative protocol: Incubation time: 1 h, 22 °C, constant stirring						56.70 ± 2.11	37.41 ± 0.69

Reaction conditions: immobilized CALB onto Octyl-Sepharose CL-4B (50 mg), phosphate buffer (100 mM, pH 7.4), the emulsion of gum Arabic and olive oil, temperature 37 °C, incubation 30 min. Data are presented as means ± standard deviations of three analyses (n = 3).

The immobilization was performed at three different temperatures—4 °C, 22 °C, and 37 °C. In each tested temperature, the relative activity value was above 90%, except for alternative protocols. Hence, it can be stated that the immobilization was optimal in low, room, and elevated temperatures. As suggested above, the lowest values of A_{rel} were noted when immobilization was carried out according to alternative protocols, i.e., within 30 and 60 min at 22 °C, with constant mixing (38.70 ± 2.53% and 56.70 ± 2.11%, respectively).

Based on the literature data, enzymes are immobilized usually at temperatures range 4 to 60 °C, and one of the most commonly used temperatures in the immobilization process of CALB is room temperature [49]. Coskun et al. [44] carried out the CALB immobilization in the temperature range of 20–60 °C; Li et al. [48] immobilized the CALB onto ECR1030 resin at 30 °C and Gokalp et al. [49] immobilized the CALB onto amorphous silica support at 25 °C. Cunha et al. [50], on the other hand, immobilized the CALB onto various polymer supports, and the process was conducted at 4 °C.

It should be noted that the higher immobilization temperature could result in an increase in the immobilized enzyme loading [51]. At the same time, the high amount of immobilized enzyme does not always influence the increase in enzyme activity [6]. The overloading of the support with the enzyme could generate a steric hindrance and, thus, inhibit the access of the enzyme to the substrate. Moreover, it should be pointed out that the origin of the applied enzyme has a reflection in its activity. Our studies indicate that there is no meaningful effect of temperature (in the range of 4 to 37 °C) on the immobilized biocatalysts activity. However, due to slightly higher activity values gained at 4 °C, for the further project step, this temperature was chosen.

2.3. Effect of Immobilization Conditions on CRL-OF Enzymatic Activity

Lipase from *Candida rugosa* (CRL-OF) was immobilized onto Octyl Sepharose CL-4B under similar conditions as in the case of CALB. The incubation time was 14 h; the temperature was 4 °C, and the pH and ionic strength of buffer—pH 7.0 and 100 mM. The application of the developed procedure allowed us to obtain the activity of immobilized CRL-OF 37.65 ± 2.84 U. Immobilization efficiency values were also determined for the examined time points: 0.5 h—10.2 ± 0.1%; 1 h—13.5 ± 0.1%; 2 h—16.7 ± 0.2%; 4 h—20.5 ± 0.4%; 6 h—22.3 ± 0.4%; 12 h—22.1 ± 0.2%; 14 h—22.4 ± 0.2%; 20 h—18.5 ± 0.3%; 27 h—15.7 ± 0.1%. Considering the results, the immobilization time of 14 h was selected. It should be noted that in the case of this lipase, the highest I_e values were recorded in the immobilization time range of 6 h to 14 h. The reached values of U (for immobilized

form) were remarkably higher than the activity parameters achieved for CALB (published in previous studies) [12]. Nevertheless, a comparison of the results is not entirely possible due to different immobilization protocols and support types. The significant changes in activity are most likely to the molecular structure of the lipase. As was presented in Table 1, the presence of a lid in the CRL-OF structure has been proved. On the other hand, as described in the literature [12,50], the oligopeptide helix in CALB may not sufficiently cover the enzyme's active site. Therefore, as it was shown in Table 1, the presence of a lid in the CALB structure is debated. These differences in structure can be of great importance to lipase activity. It should be emphasized that the lid movement in the presence of a hydrophobic surface permits alter in lipase conformation from closed to open form.

The higher activity values of CRL immobilized onto agarose beads in comparison with CALB were shown in a paper by Da Rocha et al. [52]. This trend was seen only when the lipase activity was measured with the use of *p*-NPB as a substrate. According to literature data, the immobilization times of CRL were 1–5 h (the optimal incubation time was 2 h) [53], 12 h [54], 5 h [55], 1–6 h (the optimal incubation time was 3.5 h) [56]. Regarding the effect of temperature, it is believed that the increased temperature enhances the accessibility and mobility of enzymes. However, at temperatures beyond the optimal range, thermal denaturation of biocatalysts may occur [56].

2.4. Climatic Chamber Thermal Stability Tests of CALB and CRL-OF

Immobilized lipases were placed in a climatic chamber and in a refrigerator for 7 days to determine their thermal stability. The samples were exposed to citrate buffer (pH 4.0, 500 mM), water, calcium ions, 1,2-dichloropropane (DCP), visible light, and high temperature in the climatic chamber (Figure 2). In contrast, the lipases placed in the refrigerator were stored in citrate buffer (pH 4.0, 500 mM) and protected from light. Citrate buffer (pH 4.0 and ionic strength of 500 mM) was selected based on the results of our previous paper [57]. The obtained data are presented in Table 3.

Figure 2. The photo of the climatic chamber KBP P240. The temperature was 65 °C, and the visible spectral range was 400–800 nm.

Analyzing the obtained results of the lipolytic activity of immobilized CALB stored in solutions in a climatic chamber and in a refrigerator, it was shown that the tested lipase is characterized by excellent thermal stability. It should be emphasized that CALB stored in citrate buffer exhibited higher lipolytic activity than the lipase analyzed immediately after immobilization. In addition, it was noted that calcium ions added to the citrate buffer positively affected the thermal stability of the immobilized CALB. The addition of calcium ions to the buffer allowed it to receive over five times higher catalytic activity (residual

activity values of 564.5 ± 21.6%), compared to the lipase tested directly after immobilization, and over 60% higher values of residual activity, in comparison with the lipase sample in the buffer without the addition of calcium ions (564.5 ± 21.6% to 340.9 ± 30.2%). It is assumed that increased lipase activity can be connected with the binding of Ca^{2+} to the internal structure of the enzyme, thereby influencing the solubility and behavior of the ionized fatty acids at interfaces [58]. It is pointed out that the addition of Ca^{2+} could influence the changes in lipase conformation [59] and also that Ca^{2+} ions may create complexes with ionized fatty acids, which could facilitate their removal in reactions at the water–oil interface [60]. The residual activity values for lipase stored in water with calcium ions was 82.8 ± 17.9%, which proves relatively good stability. However, a significant increase in activity was not recorded, as was in the case with lipase suspended in citrate buffer. A positive effect of high temperature on the tested lipase is noticeable regarding the lipolytic activity. Higher values of the residual activity of the samples stored in the buffer at a temperature of 65 °C were found compared to the samples at the temperature of 4 °C. It is suggested that conformational changes generated by the enzyme inactivation could affect in various ways on the lipase activity relative to different substrates. The higher activity could be connected, among others, to reducing steric hindrances or enzyme conformational changes [52]. Furthermore, no negative impact of visible light on CALB stability was observed. Moreover, the addition of an organic solvent—1,2-dichloropropane (DCP)—to the citrate buffer did not greatly reduce the system's stability. However, there was an evident decrease in the residual activity values compared to the system stored without this solvent (340.9 ± 30.2% to 237.6 ± 30.2%).

The received data on the thermal stability of the immobilized CALB allows concluding that the storage of the immobilized system in citrate buffer (pH 4.0, 500 mM) with the addition of calcium ions significantly contributes to the increase in thermal stability and activity of the enzyme. The stability tests demonstrate that the created system is stable, and, what is extremely important, storing the optimized catalytic system in a climatic chamber increased its activity. Therefore, it should be considered to include the stage of lipase storage (after immobilization) in a climatic chamber in the procedure of optimizing CALB immobilization on the tested support. The technique of immobilization in high ionic strength and at low pH allowed to obtain higher enzyme activity than when buffers with lower ionic strength were used. Thereby, a citrate buffer with pH 4.0 and ionic strength of 500 mM was applied to store the immobilized lipases. To our knowledge, combining techniques of immobilization in high ionic strength and storage in the climatic chamber in extremely high temperatures in citrate buffer with the addition of calcium ions is, to a slight extent, described in the literature. Preliminary stability test results for CALB in dry form were described in the previous paper [57]. It should be emphasized that studies on catalytic systems in climatic chambers are rarely shown in the biocatalysis literature [6]. Thus, it is our hope that the data presented in this paper may contribute to the opening of discussions on the influence of conditions in climatic chambers to enhance the activity of immobilized protein catalysts. By comparing the results obtained during storage in the refrigerator (4 °C) to those from the climatic chamber, it was found to be more optimal to store the developed systems in the climatic chamber (in our study, 65 °C—stress conditions).

The results of the thermal stability study of the second lipase (CRL-OF) stored in the climatic chamber indicate low stability of the formed enzyme system. After 7 days of storage in the climatic chamber, a significant decrease in activity was noted—residual activity did not exceed 5%. Assumably the denaturation of catalytic proteins under the impact of temperature occurred [44]. It is worth noting that the storage of the immobilized enzyme at 4 °C (in the refrigerator) provided maintenance of the stability of the lipase (residual activity 89.9 ± 3.4%)—a decrease in the activity of about 10% compared to the lipase tested immediately after immobilization. That means that the temperature can be an influential factor in reducing the system's stability and decreasing lipase activity. The results suggest that the obtained catalytic system is very sensitive to high temperatures. It is assumed that visible light likely has a minor effect on stability. However, it is difficult to

state clearly, because, in each analyzed sample, after 7 days of CRL-OF storage in a climatic chamber, very low values of residual activity were received. In our study, it was established that the optimal storage temperature for this immobilized lipase was 4 °C.

Table 3. Effect of storage conditions in the climatic chamber and refrigerator on the thermal stability of the immobilized CALB (a) and CRL-OF (b) in an aqueous or aqueous–organic medium.

(a)

Storage Conditions							Immobilized CALB		
Citrate Buffer (pH 4.0, 500 mM)	**Water**	**Ca^{2+}**	**Vis (400–800 nm)**	**T [65 °C]**	**T [4 °C]**	**DCP**	**CALB Residual Activity [%]**	**Activity CALB [U]**	**Activity CALB [U/g Support]**
+				+			340.9 ± 30.2	3.30 ± 0.29	66.04 ± 5.85
+		+		+			564.5 ± 21.6	5.47 ± 0.21	109.38 ± 4.19
	+	+		+			82.8 ± 17.9	0.80 ± 0.17	16.04 ± 3.47
+					+		220.4 ± 26.3	2.14 ± 0.25	42.71 ± 5.09
+			+	+			409.7 ± 13.1	3.97 ± 0.13	79.38 ± 2.55
+				+		+	237.6 ± 30.2	2.30 ± 0.29	46.04 ± 5.85

(b)

Storage Conditions							Immobilized CRL-OF		
Citrate Buffer (pH 4.0, 500 mM)	**Water**	**Ca^{2+}**	**Vis (400–800 nm)**	**T [65 °C]**	**T [4 °C]**	**DCP**	**CRL-OF Residual Activity [%]**	**Activity CRL-OF [U]**	**Activity CRL-OF [U/g Support]**
+				+			4.2 ± 0.5	1.47 ± 0.19	29.38 ± 3.85
+		+		+			3.8 ± 0.9	1.30 ± 0.33	26.04 ± 6.67
	+	+		+			0.4 ± 0.3	0.14 ± 0.10	2.71 ± 1.92
+					+		89.9 ± 3.4	31.14 ± 1.83	622.71 ± 36.67
+			+	+			2.3 ± 0.7	0.80 ± 0.25	16.04 ± 5.09
+				+		+	2.3 ± 0.3	0.80 ± 0.10	16.04 ± 1.92

Reaction conditions: immobilized CALB or CRL-OF onto Octyl-Sepharose CL-4B (50 mg); phosphate buffer (100 mM, pH 7.4); the emulsion of gum Arabic and olive oil; temperature 37 °C; incubation 30 min. Refrigerated storage conditions: temperature (4 °C); closed tube; no light. Data are presented as means ± standard deviations of three analyzes (n = 3).

It should be remarked that there is an apparent relationship between the results of the stability of both lipases and the effect of temperature. It is believed that this may be related to the structure of the lipases—the presence or absence/small size of a lid [4]. This part of the lipase structure is considered to contribute significantly to the stability of the tested enzymes. An immobilized CALB characterized by the absence or small size of a lid was more stable than CRL with the well-documented presence of a lid. The developed immobilization conditions promote the stability of the immobilized CALB.

Free lipases (CALB and CRL-OF) were also stored in a climatic chamber and a refrigerator. It was demonstrated that the lipolytic activities of free lipases after a week of storage in a climatic chamber (in the same conditions as for immobilized lipases) were lower than 0.1 U (CALB below 0.1 U; CRL-OF below 0.05 U), which may suggest the protein denaturation process. However, after storage in the refrigerator (4 °C, in citrate buffer at pH 4.0 and ionic strength of 500 mM), the activity of free CALB was 0.66 ± 0.08 U, and immobilized CALB—2.14 ± 0.25 U. On the other hand, the activity of free CRL-OF after storage in the refrigerator was 120.24 ± 3.22 U, and the immobilized CRL-OF—31.14 ± 1.83 U.

The I_e values, after 7 days of storage in the refrigerator, for the immobilized CALB was 324.24 ± 5.57%, while for the immobilized CRL-OF, the I_e value was 25.89 ± 1.16%.

As part of our project, the possibility of reusing immobilized lipases was also investigated (the immobilized lipases were not stored in the climatic chamber). It was shown that the relative activity of the tested lipases after the third reaction cycle was: 63.0 ± 0.8% for CALB and 52.0 ± 1.1% for CRL-OF. The decrease in activity probably resulted from the desorption of the enzyme, which was favored by the reaction mixture containing fatty acid triglycerides and Arabic gum.

Studies on the effect of temperature on CALB activity and stability have been reported in the literature. Da Rocha et al. [52] observed that immobilized lipases exhibited a significant increase in activity at 55 °C when they were earlier incubated at 70 °C or 75 °C. In the beginning, the activity of the immobilized CALB was observed to increase (almost five-fold after 15 min, triacetin) and then to drop (after 4 h, three-fold initial activity remained). In relation to Octyl-CRL, it was attended to a four-fold rise in the activity after 15 min (triacetin), and after 4 h, a 50% decline in activity was noticed. Cunha et al. [50] showed no decrease in CALB activity over a period of at least 2 months when stored at 4–20 °C. Chiaradia et al. [61] performed a storage stability assessment of immobilized CALB by incubating the lipase for 30 days at 4 °C in sodium phosphate buffer (0.1 M, pH 7.6). The authors demonstrated that immobilization could affect conformational changes of lipase and, via it, the final properties of the enzymes—the residual activity of 100% at 60 °C and 80 °C, compared to 95% at 40 °C.

Stability studies of immobilized lipase from *Candida rugosa* (CRL) were also reported in the literature. Vetrano et al. [62] carried out thermal stability tests CRL entrapped in alginate beads. After 7 days of study, at 25 °C, there was no reduction in activity, whereas, at 50 °C, after one day, the activity was 70% and remained unchanged till the end of the study (7 days). Aghaei et al. [54] noticed that very high temperatures could deactivate the enzyme by denaturing its structure. The authors determined the storage stability (30 days at 4 °C) of immobilized CRL on epoxy-activated cloisite 30B. After 30 days, the relative activity of the immobilized biocatalyst was 87.3%. Aghabeigi et al. [53] studied the thermal stability of immobilized CRL on the graphene oxides magnetized with $NiFe_2O_4$ nanoparticles. The three-dimensional structure of the lipase changed with increasing temperature, which resulted in a decrease in enzyme activity. It was also mentioned that immobilization provides a more robust structure for the enzyme. The immobilized enzyme lost 22% of its activity (relative to the maximum obtained at 30 °C) after 1 h at 60 °C. Yu et al. [63] stated that immobilization limits the conformational migration capability of lipase and increases the structure rigidity, as well as enhancing the resistance of lipase to inactivating agents. At 70 °C (1 h), immobilized CRL retained 54.75% of relative enzyme activity.

In the literature, we can also find publications describing the effect of the addition of calcium ions on activity and stability. Arana-Pena et al. [31] tested the lipase from *Pseudomonas fluorescens* (PFL) immobilized on octyl-agarose beads and stated that the presence of $CaCl_2$ during immobilization (if high loading of the PFL biocatalyst occurred) created much more stable biocatalysts than the enzyme immobilized in the absence of this additive, whereas, in the case of lowly loaded PFL biocatalysts, the addition of $CaCl_2$ during the immobilization increased the enzyme activity. Ameri et al. [64] presented data on significantly improved immobilized *Bacillus atrophaeus* lipase (BaL) activity when Ca^{2+} was applied. Pazol et al. [65] indicated that fatty acid liberation from lipopolysaccharides (LPS) in the reaction catalyzed by CALB, was enhanced by the addition of Ca^{2+}. However, in the literature, there are also documents describing the ambiguous influence of the tested ions. Yu et al. [66] noticed no clear effect of Ca^{2+} on the activity of used model enzymes (among other CALB). Similarly, Arana-Pena et al. [67] presented that the addition of 10 mM $CaCl_2$ had no evident effect on the octyl-CALB stability. The influence of calcium ions has also been presented in other papers [68,69].

2.5. Effect of Lipase Storage in Buffers of Different Ionic Strength—24 h Stability Test

The influence of citrate buffer with pH 4.0 and different ionic strengths (50, 100, 300, 500, and 700 mM) on the stability of immobilized lipases (CALB and CRL-OF) was investigated. After immobilization under optimized conditions, the immobilized lipase was transferred to buffers with different ionic strengths and placed in a refrigerator (4 °C) for 24 h. Citrate buffer (pH 4.0) was selected based on the results of our previous paper [57]. Then, the lipolytic activity was examined, and the relative activity was calculated (Figure 3).

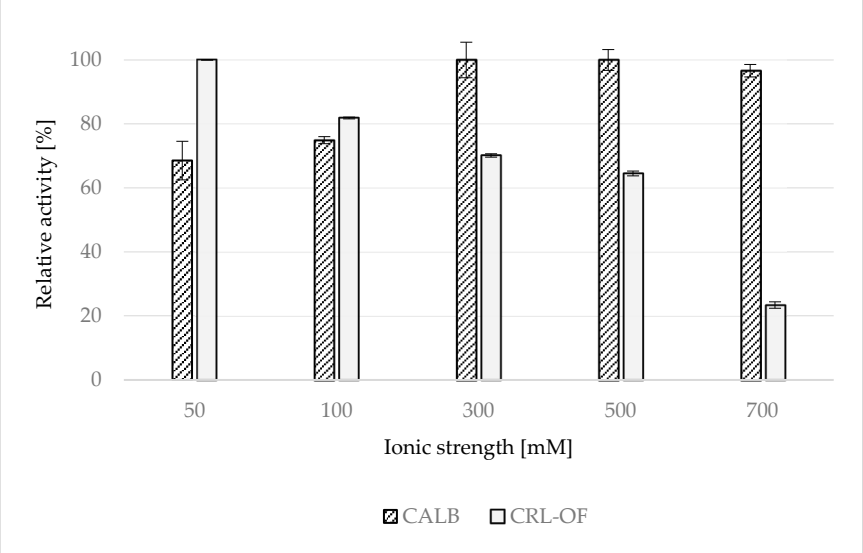


Figure 3. Effect of lipase storage in buffers of different ionic strengths on stability. Reaction conditions: immobilized CRL-OF or CALB onto Octyl-Sepharose CL-4B (50 mg); phosphate buffer (100 mM, pH 7.4); the emulsion of gum Arabic and olive oil; temperature 37 °C; incubation 30 min. Data are presented as means ± standard deviations of three analyzes (n = 3). The error bars represent the standard deviations of the mean.

The relative activity results determined for the CALB and CRL-OF indicate a significant effect of the buffer ionic strength on the stability of the catalytic systems during 24 h storage at 4 °C. For CRL-OF; the optimal ionic strength value of the citrate buffer was 50 mM (relative activity 100.0 ± 0.0%), while for CALB, the optimal range of ionic strength during storage was 300 to 700 mM (300 mM—100.0 ± 5.5%, 500 mM—100.0 ± 3.3%, 700 mM—96.6 ± 1.9%). The received data may suggest a relationship with the structure of the lipases, i.e., the presence of a lid (small size or its absence). For the immobilized CRL-OF, an inverse relationship can be observed: with an increase in the ionic strength of the buffer, there was a decrease in lipolytic activity. The results exhibit a better medium for storing this lipase (immobilized onto the tested support) being a buffer with a lower ionic strength—50 mM. On the other hand, in the case of a CALB characterized by no or small lid, for maintaining stability, a buffer with higher ionic strength is preferable. The tests performed show that, regarding CRL-OF, not only the temperature during storage in the climatic chamber was a factor affecting the decrease in stability, but probably, also the storage in a buffer at high ionic strength (500 mM). It should be emphasized that CRL-OF is more sensitive under the storage in high ionic strength buffers than CALB, which exhibited high stability at higher buffer ionic strength values.

3. Materials and Methods

3.1. Materials

Lipase B from *Candida antarctica* (CALB, produced in yeast) was purchased from Chiral Vision (Leiden, The Netherlands), and lipase from *Candida rugosa* (CRL-OF) was supplied by Meito Sangyo (Tokyo, Japan). Octyl-Sepharose CL-4B (GE Healthcare, Uppsala, Sweden),

olive oil, 1,2-dichloropropane (DCP), and Bradford reagent were bought from Sigma-Aldrich (Steinheim, Germany). Arabic gum, phenolphthalein, citric acid monohydrate, calcium chloride anhydrous, disodium hydrogen phosphate dihydrate, monosodium hydrogen phosphate monohydrate, *o*-phosphoric acid (75%), and analytical weighed amount of sodium hydroxide with a concentration of 0.1 M were purchased from POCH (Gliwice, Poland). Trisodium citrate was achieved from Chempur (Piekary Śląskie, Poland). Methanol and acetone were gained from StanLab (Lublin, Poland). The water used in this study was filtrated by Milli-Q Water Purification System (Millipore, Bedford, MA, USA). The thermal stability of immobilized lipases was studied in climatic chamber KBF P240 (Tuttlingen, Germany). Preparation of Octyl-Sepharose CL-4B was performed by centrifuge Eppendorf Spin Mini Plus (AG, Hamburg, Germany) and mixer vortex Velp Scientifica ZX4 (Usmate, Italy). The buffers were prepared by SevenMulti pH-meter (Mettler-Toledo, Schwerzenbach, Switzerland). The other used sorts of equipment were UV-Vis U-1800 spectrophotometer (Hitachi, Tokyo, Japan), Unimax 1010 incubator (Heidolph, Schwabach, Germany), and burettes (Simax, Sazava, Czech Republic).

3.2. Octyl Sepharose CL-4B Preparation Protocol

The Octyl Sepharose CL-4B suspension (110 µL) was placed into an Eppendorf tube. Afterward, 1 mL of filtered water was inserted into the tube with support suspension, and the mixture was mixed using a vortex for 3 min, followed by centrifugation for 15 min at 9000 rpm. Then, the supernatant was removed from the Eppendorf tube, and the beads were weighed (50 mg).

3.3. Immobilization of CALB onto Octyl-Sepharose CL-4B

The immobilization method was carried out in our laboratory based on literature data [5,12], with slight changes. An amount of 10.0 mg of CALB was placed in an Eppendorf tube (2.0 mL) with 1.0 mL of phosphate buffer—pH 7.0, 100 mM (for thermal stability studies immobilization in citrate buffer—pH 4.0, 500 mM and mixed for 5 min at room temperature was applied). The sample stayed for 15 min at room temperature. After this time, the CALB suspension was pipetted and then inserted into the Eppendorf tube (2.0 mL) containing 50 mg of a prepared Octyl-Sepharose CL-4B support. The samples were mixed for 5 min and then kept for the appropriate time and at an adequate temperature. After immobilization, the CALB concentration in the supernatant was determined with the use of the Bradford method. The procedures were carried out in triplicate.

The process was repeated under various immobilization conditions:

(a) immobilization time—the samples were mixed for 5 min at room temperature and then stayed at a temperature of 4 °C for 0.5, 1, 2, 4, 6, 12, 14, 20, and 27 h;
(b) temperature—the samples were mixed for 5 min at room temperature or in an ice bath, and then CALB was incubated for 14 h in the following temperature conditions: 4 °C (refrigerator); 22 °C (room temperature); and 37 °C; additionally selected samples were stirred during 14 h of incubation (at room temperature and 37 °C).

The alternative method was an incubation time of 30 min and 1 h at room temperature (22 °C) with constant stirring.

3.4. Immobilization of CRL-OF onto Octyl-Sepharose CL-4B

The immobilization method was studied in our laboratory based on the literature data [6,12], with some modifications. An amount of 10.0 mg of CRL-OF was placed in an Eppendorf tube (2.0 mL) with 1.0 mL of phosphate buffer—pH 7.0, 100 mM (for thermal stability studies immobilization in citrate buffer—pH 4.0, 500 mM and mixed for 5 min at room temperature). The sample was kept for 15 min at room temperature. After this time, the lipase suspension was pipetted and then inserted into the Eppendorf tube (2.0 mL) containing 50 mg of a prepared Octyl-Sepharose CL-4B support. The sample was mixed for 5 min at room temperature and allowed to stay at a temperature of 4 °C for the appropriate

time. After immobilization, the CRL-OF concentration in the supernatant was determined with the use of the Bradford method. The procedures were carried out in triplicate.

The process was repeated under various immobilization conditions:

(a) immobilization time—the samples were mixed for 5 min at room temperature and then stayed at a temperature of 4 °C for 0.5, 1, 2, 4, 6, 12, 14, 20, and 27 h.

3.5. Determination of the Amount of Immobilized CALB and CRL-OF by Bradford Method

The amount of enzymatic protein immobilized on the Octyl-Sepharose CL-4B was established by a modified Bradford method [5,6,12]. The study was carried out using the UV-Vis spectrophotometric method (λ = 595.0 nm), measuring the absorbance of the free lipase remaining in the suspension after the immobilization process (concentration range: 1.0–10.0 mg/mL). The measurement was made in triplicate. The amount of CALB and CRL-OF immobilized onto the Octyl-Sepharose CL-4B was calculated with a calibration curve equation (R^2 = 0.999 ± 0.003 for CALB and R^2 = 0.999 ± 0.009 for CRL-OF). The result was the three-sample mean. The lipase loading (L_L) (mg/g support) was determined based on the obtained data.

3.6. Lipolytic Activity of CALB and CRL-OF

The lipolytic activity of immobilized and free CALB and CRL-OF was determined by the titrimetric method, as described in previous studies and the literature data [70]. The olive oil was applied as the substrate. The reaction mixture contained the lipase (CALB or CRL-OF) immobilized onto Octyl Sepharose CL-4B or free lipase, 3.0 mL phosphate buffer (pH 7.4), and the 5.0 mL of emulsion composed from an equal volume of olive oil and water suspension of Arabic gum (7% w/v). The mixtures were incubated at 37 °C for 30 min with rotation at 600 rpm. Then, the reaction was interrupted by adding 5.0 mL of methanol and 5.0 mL of acetone. The titration was performed using 0.05 M NaOH standard solution at room temperature, with phenolphthalein as the indicator. The control was carried out without lipase—as a blank. The enzymatic activity (U and U/g support), immobilization efficiency (I_e), relative activity, and residual activity were calculated. Immobilization efficiency I_e (%) was defined as the ratio between the activity of immobilized lipase and the activity of the same amount of free protein in the solution that had been immobilized onto the support. This parameter was determined using the following equation:

$$I_e = \frac{U_I}{U_B} \times 100\%$$

where I_e—immobilization efficiency; U_I—the activity of the lipase immobilized onto the support; U_B—the activity of the same amount of free protein in the solution that had been immobilized onto the support.

Relative activity (A_{rel}) (%) was determined as the ratio between the activity of every sample and the maximum activity of the sample under the test conditions. Residual activity (%) was calculated as the ratio between the activity of every sample (from a climatic chamber or refrigerator) and the activity of a sample that had not been subjected to a thermal stability test in a climatic chamber or refrigerator. Analyses were performed in triplicate. In addition, A_{rel} was also calculated in reusability studies—after 30 min of incubation; the supports were separated from the reaction mixture and used in another reaction cycle.

3.7. Climatic Chamber Thermal Stability Tests of CALB and CRL-OF in Aqueous Buffers

The method for the thermal stability studies of immobilized CALB and CRL-OF was prepared in our laboratory. After immobilization in citrate buffer (pH 4.0, 500 mM), the supernatant was collected, and then, one of the following solutions was added to the immobilized lipase: 1 mL of citrate buffer (pH 4.0, 500 mM); solution of $CaCl_2$ (10 mM) in citrate buffer (pH 4.0, 500 mM)—1 mL; solution of $CaCl_2$ (10 mM) in water—1 mL; 1 mL of a solution composed of 0.5 mL of citrate buffer (pH 4.0, 500 mM) and 0.5 mL of DCP.

Then, the Octyl-Sepharose CL-4B beads with enzymes were stored in closed glass vials in a climatic chamber KBF P240. The temperature was maintained at 65 °C; the visible spectral range was 400–800 nm. The studies were conducted for 7 days. The immobilized lipase beads (in 1 mL of citrate buffer—pH 4.0, 500 mM) were also stored in closed glass vials in a refrigerator. After 7 days, the enzymatic activity (U and U/g support) and residual activity (%) of immobilized lipases were evaluated by the method described in Section 3.6. The storage conditions of immobilized lipases in an aqueous medium are shown in Table 3. Free lipases (CALB and CRL-OF) were also stored for 7 days in a climatic chamber and a refrigerator.

3.8. Effect of Lipase Storage in Buffers of Different Ionic Strength—24 h Stability Test

To Eppendorf tubes containing immobilized lipases (CALB or CRL-OF) 1 mL of buffer at pH 4.0 and appropriate ionic strength (50, 100, 300, 500, 700 mM) was added. Then, the samples were incubated for 24 h at 4 °C (refrigerator). After this time, the lipase lipolytic activity was determined according to the method described in Section 3.6. The enzymatic activity (U) and the relative activity (%) have been calculated.

4. Conclusions

The thermal stability studies of immobilized lipases in aqueous buffers: lipase B from *Candida antarctica* and lipase from *Candida rugosa* carried out in a climatic chamber (stress conditions—65 °C) indicate excellent stability of CALB, in contrast to CRL-OF. Storing the immobilized CALB in a climatic chamber in citrate buffer (pH 4.0, 500 mM) resulted in an increase in lipolytic activity (hydrolysis of olive oil). The addition of calcium ions (Ca^{2+}) to the citrate buffer made it possible to obtain more than five times higher CALB activity compared to samples not stored in the chamber. The 24-h test demonstrated that the optimal ionic strengths of the storage buffer for CALB were in the range of 300 to 700 mM, while for CRL-OF, the lower ionic strength of 50 mM was more advantageous. The use of a combining of such techniques as immobilization onto the support in high ionic strength buffer and then storing immobilized lipases in a climatic chamber at stress conditions (65 °C) in high ionic strength aqueous buffer with calcium ions is a strategy to a very limited extent described in the literature. Additionally, the application of climatic chambers potentially enables the unification of results and effective and reliable interlaboratory analysis in accordance with the International Conference on Harmonization (ICH) guidelines and the requirements of the pharmaceutical industry. What is worth emphasizing is that thermal stability studies could also be potentially applied to other hydrolases.

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