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ORIGINAL PAPER

Immobilization of lipase from *Candida rugosa* on Sepabeads®: the effect of lipase oxidation by periodates

Nevena Ž. Prlainović · Zorica D. Knežević-Jugović · Dušan Ž. Mijin · Dejan I. Bezbradica

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Abstract The objective of this paper was the investigation of a suitable Sepabeads® support and method for immobilization of lipase from *Candida rugosa*. Three different supports were used, two with amino groups, (Sepabeads® EC-EA and Sepabeads® EC-HA), differing in spacer length (two and six carbons, respectively) and one with epoxy group (Sepabeads® EC-EP). Lipase immobilization was carried out by two conventional methods (via epoxy groups and via glutaraldehyde), and with periodate method for modification of lipase. The results of activity assays showed that lipase retained 94.8% or 87.6% of activity after immobilization via epoxy groups or with periodate method, respectively, while glutaraldehyde method was inferior with only 12.7% of retention. The immobilization of lipase, previously modified by periodate oxidation, via amino groups has proven to be more efficient than direct immobilization of lipase via epoxy groups. In such a way immobilized enzyme exhibited higher activity at high reaction temperatures and higher thermal stability.

Keywords Enzyme immobilization · *Candida rugosa* lipase · Sepabeads® · Periodate oxidation

Introduction

Lipases (E.C. 3.1.1.3) are highly stereoselective catalysts of great importance for the modern chemical and pharmaceutical industries. They catalyze cleavage of carboxy ester bonds in tri-, di-, and mono-acylglycerols to glycerol and fatty acids [1]. They also catalyze various other reactions, such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Enzymes, such as lipases, accept a broad range of substrates and require no cofactor for reaction [2]. They are resistant to solvents and are exploited in a broad spectrum of biotechnological applications [3]. Novel biotechnological applications, such as biopolymer synthesis [4], biodiesel production [5–7], treatment of fat-containing waste effluents [8], enantiopure synthesis of pharmaceuticals [9, 10] and nutraceutical agents [11], have been established successfully.

The high cost of lipases, however, makes enzymatic processes economically unattractive. Immobilization of enzymes is the key to expand the applications of these natural catalysts by enabling easy separation and purification of products from reaction mixtures and efficient recovery of enzyme proteins [12]. The use of immobilized lipases is a possible solution to this problem because the enzyme can be recovered from the product and reused [13]. Immobilization of enzyme also enhances its thermal and chemical stability and allows heterogeneous catalysis of enzymatic reactions [14, 15]. The concept of immobilizing enzymes on insoluble supports has been the subject of considerable research for over 30 years and consequently, many different methodologies and a vast range of applications have been suggested. Among the various developed immobilization techniques, immobilization using covalent binding has been most widely studied, because it provides the creation of a strong link between the enzyme and the carrier and does not permit the loss of enzyme by desorption from the support. Enzyme can be immobilized on a large number of different carriers such as inorganic materials, naturals and synthetic polymers [13, 16–18]. In this work, our attention has been focused on the immobilization and

N. Ž. Prlainović · Z. D. Knežević-Jugović · D. Ž. Mijin · D. I. Bezbradica (✉)
Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 3503, 11120 Belgrade, Serbia
e-mail: dbez@tmf.bg.ac.rs

biochemical characteristics of lipase from *Candida rugosa* immobilized on different Sepabeads® EC carriers. These carriers have a particle size distribution of 150–300 μm and a specific gravity of 1.13 g cm^{-3}. They have a highly porous metacrylic polymer matrix with average pore diameter of 30–40 nm, high mechanical stability, high resistance to microbial attack and do not swell in water. The raw materials applied for the production of these supports are included in the EU list of register for the processing of food stuff [19]. Different carriers from this group have been used not only for immobilization of lipase [20, 21], but also for immobilization of other hydrolases such as penicillin G acylase [22], β-galactosidases [23] or phytases [24]. A number of previous investigations point out high retained activity and stability of enzymes immobilized on Sepabeads® supports with epoxy-groups [20, 23–26].

In this paper, lipase from *C. rugosa* was immobilized on three types of Sepabeads® carriers: Sepabeads® EC-EP, Sepabeads® EC-EA Sepabeads® EC-HA. Immobilization on Sepabeads® EC-EP was performed using conventional procedure for direct binding via epoxy groups, while for immobilization on supports with amino groups two strategies were tested: support activation with glutaraldehyde or oxidation of enzyme's carbohydrate portion by sodium-periodate. Immobilized lipases with highest activity yields were then characterized. The effect of temperature and pH on the activity was tested, and also stability profiles of different immobilized enzymes were compared.

Experimental

Materials

Candida rugosa lipase (EC 3.1.1.3), type VII, was obtained from Sigma Chemical Co. (St. Louise, USA). Sepabeads® EC-EP, EC-EA and EC-HA (particle sizes 150–300 μm, average pore diameter 30–40 nm, water retention 55–65%) were kindly donated by Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy). All other chemicals were of analytical grade.

Immobilization of lipase

Three different immobilization techniques were used, as well as three different supports.

Immobilization via epoxy groups

As the Sepabeads® EC-EP has epoxy groups on its surface, the first immobilization technique was direct binding. Immobilization of lipase was performed by direct binding on polymers. 1 g of unmodified carrier was incubated with 35 cm^3 of native lipase solution in 1.25 M potassium phosphate buffer (0.5–4 mg cm^{-3}) at room temperature, for 48 h on a shaker at 150 rpm. After the incubation the unbound enzyme was removed by washing with distilled water (3 × 20 cm^3) and afterwards with potassium phosphate buffer pH 7.0 (3 × 20 cm^3) and stored in it at 4 °C until use.

Immobilization by glutaraldehyde

Sepabeads® EC-EA and EC-HA have amino groups as functional groups, so the first step in immobilization process involves activation of the supports with glutaraldehyde. Activated supports were prepared by suspending 5 g of the supports in 20 cm^3 of 2% (w/v) glutaraldehyde. The suspension was kept under mild stirring at 25 °C for 1 h. After that the supports were filtered and washed several times with phosphate buffer. After the activation was finished the immobilization was performed by incubating 1 g of activated polymer with native lipase solution in 20 mM potassium phosphate buffer (pH 8.0). Concentration of lipase was varied between 0.5 and 4 mg cm^{-3}. The samples were left on a shaker for 20 h at room temperature. When the immobilization was completed, the unbound enzyme was removed by washing three times with 20 mM potassium phosphate buffer, pH 8.0 and stored in this buffer at 4 °C until use.

Immobilization by periodate

Lipase was oxidized by periodate as described previously [27], by incubating 3 g of lipase in 510 cm^3 of acetate buffer (pH 5.0) and then 360 cm^3 of 50 mM $NaIO_4$ solution was added. The mixture was kept in the dark at 4 °C for 6 h with occasional stirring. The reaction was quenched with 0.66 cm^3 of ethylene glycol for 30 min. To remove the by-products, the oxidized lipase solution was then dialyzed against 50 mM acetate buffer (pH 5.0) for 18 h. Polymers with amino groups were incubated with oxidized lipase in sodium acetate buffer (pH 5.0) at 4 °C for 48 h. After that, the polymers were washed three times with 50 mM phosphate buffer (pH 7) and stored in it until use.

Enzyme activity assay

Activity of the immobilized lipase was determined by a method based on measuring the effect of the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) [28]. The substrate solution was prepared by adding solution A (40 mg of *p*-NPP in 12 ml of propane-2-ol) to 9.5 ml of solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 in 90 ml of distilled water) drop wise with intense stirring. The obtained emulsion remained stable for at least 2 h. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of phosphate buffer (50 mM) and 0.1 ml of suitably diluted

enzyme. The assay mixture was incubated at 37 °C for 30 min and the *p*-nitrophenol released was measured at 410 nm on UV spectrophotometer (Shimadzu 1700, Shimadzu Corporation, Kyoto, Japan). One unit of activity was expressed as μmol of *p*-nitrophenol released per minute under the assay conditions.

Determination of lipase concentration

Lipase concentration was determined according to Lowry's method [29] using starting enzyme preparation as a standard. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced into the coupling reaction mixture and the amount of enzyme in the filtrate after immobilization.

Determination of pH optimum of immobilized enzyme

Optimum pH determination of free and immobilized lipase was carried out by changing incubation pH between 3.75 and 9.0. 0.1 g of immobilized enzyme was incubated in 2 ml of certain buffer. After 30 min lipase activity was determined by a method based on measuring the effect of the hydrolysis of p-nitrophenyl palmitate (*p*-NPP), described previously.

Determination of temperature optimum of immobilized enzyme

Optimum temperature was determined by measuring the activity of immobilized enzyme at different temperatures (4, 25, 37, 45, 60 and 80 °C). 0.5 g of biocatalyst was incubated in 2 ml of 20 mM phosphate buffer (pH = 7.0).

Thermal stability assay

The thermal stability assay was performed at 75 °C in a 50 mM phosphate buffer pH 7. 0.5 g of immobilized enzyme was incubated in 2 ml of phosphate buffer in a constant temperature water bath. After a certain period of time (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 h), samples were taken and the residual activities were determined by the *p*-NPP method. The remaining enzyme activity is expressed as a percentage compared with the activity of biocatalysts that was not subjected to a thermal treatment, whose activity is marked as 100%.

Results and discussion

In this paper, we have examined the immobilization of lipase from *C. rugosa* on three polymethacrylate particulate polymers with different functional groups. One of the applied carriers has epoxy groups (Sepabeads® EC-EP) and the other two have amino groups (Sepabeads® EC-EA and Sepabeads® EC-HA) as functional groups. Carriers with amino groups vary in length of hydrocarbons chain that bonds the amino group and polymethacrylate surface. Amino groups of Sepabeads® EC-EA carrier are separated from its surface with two carbon atoms (ethylendiamino spacer) and amino groups of Sepabeads® EC-HA carrier with six carbon atoms (hexamethylenamino spacer).

The immobilization on Sepabeads® EC-EP was performed using direct binding to amino groups of enzyme via epoxy groups. For immobilization on supports with amino groups two immobilization techniques have been applied: immobilization on glutaraldehyde-activated support and immobilization of enzyme modified by periodate method on non-modified support.

Comparison of immobilization methods

The first step was to determine the best immobilization support and immobilization method with respect to enzyme loading, catalytic activity and coupling yield. In order to optimize immobilization conditions and utilization of enzyme activity, the effect of enzyme concentration in the attachment solution on enzyme loading, enzyme coupling yield and the activity of the immobilized enzyme were examined. Fig. 1 shows the enzyme loadings and enzyme coupling yields on Sepabeads® EC-EP, EC-EA and EC-HA, respectively.

It can be seen that loading on Sepabeads® EC carriers increase rapidly in the examined range of enzyme concentrations. The increase of enzyme loading was almost linear with Sepabeads® EC-EA and EC-HA, while in experiment with Sepabeads® EC-EP mild increase was observed at lipase concentrations up to 2 mg cm^{-3} with steep increase of enzyme loading with further increase of offered lipase concentration. Consequently, in a case of carriers with amino groups enzyme coupling yield reaches the maximum value with the lowest concentration of enzyme, while in immobilization on Sepabeads® EC-EP coupling yield continuously increased within examined range. The highest enzyme loading (109.2 mg g^{-1}) was achieved with Sepabeads® EC-EP, while in immobilizations with supports containing amino groups, enzyme loadings were slightly lower, in narrow range between 98.7 and 100.8 mg g^{-1} of support. Higher loading on Sepabeads® EC-EP, in spite of significantly lower density of functional groups than Sepabeads® EC-EA and EC-HA (Table 1), confirms significantly higher reactivity of epoxy groups, in comparison with amino groups. Additionally, continuous increase of coupling yield throughout the observed range of enzyme concentration (Fig. 1a) could indicate that immobilization via epoxy groups is irreversible. On the other hand, it seems

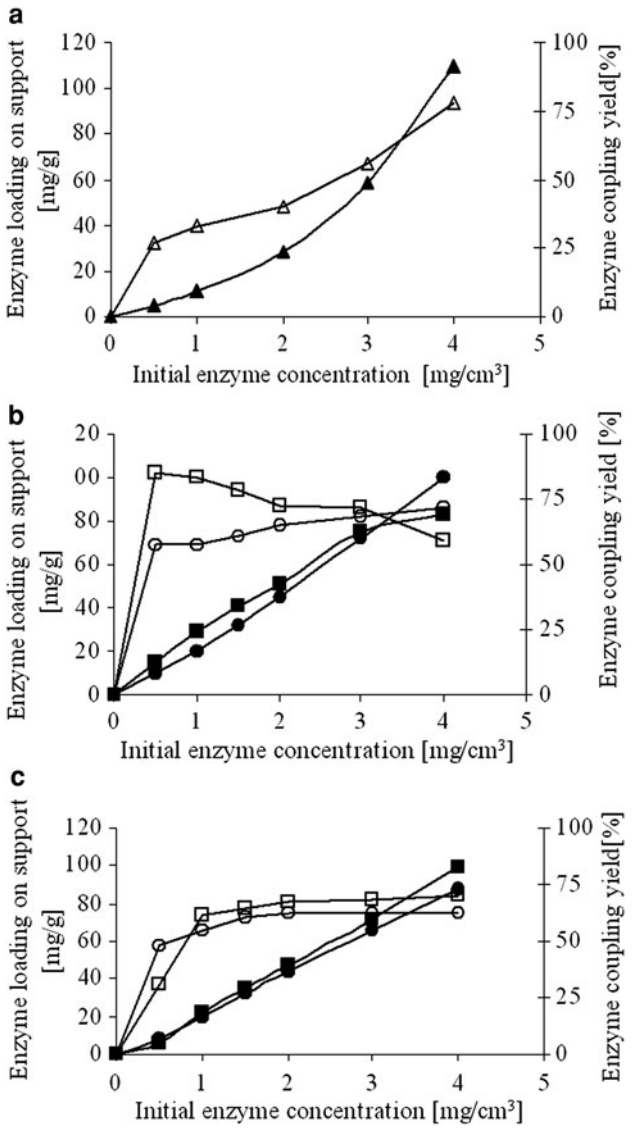

Fig. 1 The influence of the initial enzyme concentration on the enzyme loading on (**a**) Sepabeads® EC-EP (*filled triangle*), (**b**) Sepabeads® EC-EA–GA method (*filled circle*) and Sepabeads® EC-EA–PJ method (*filled square*), (**c**) Sepabeads® EC-HA–GA method (*filled circle*) and Sepabeads® EC-HA–PJ method (*filled square*) and the enzyme coupling yield for (**a**) Sepabeads® EC-EP (*open triangle*), (**b**) Sepabeads® EC-EA–GA method (*open circle*) and Sepabeads® EC-EA–PJ method (*open square*), (**c**) Sepabeads® EC-HA–GA method (*open circle*) and Sepabeads® EC-HA–PJ method (*open square*)

Table 1 Concentration of functional groups on Sepabeads® EC carriers [30]

Carrier type	Concentration of functional groups (mmol/g wet)
Sepabeads® EC-EP	0.11
Sepabeads® EC-EA	0.6
Sepabeads® EC-HA	0.7

that immobilization on supports with amino groups is reversible since ratio between immobilized and non-bounded enzyme is almost constant throughout observed enzyme concentration range (Fig. 1b, c). The maximum observed enzyme coupling yields were 78% for EC-EP, 84.7% for EC-EA and 70.5% for EC-HA.

Comparing the results for enzyme immobilization by the glutaraldehyde and periodate methods, it can be observed that slightly higher amounts of the enzyme are loaded and higher immobilization yields are achieved using periodate method. Amount of the immobilized enzyme also depends on the length of the carbon chain that connects functional group with the polymer surface. The amount of the immobilized lipase on support with a shorter spacer length, Sepabeads® EC-EA, (99.9 mg g^{-1} support for GA method and 100.8 mg g^{-1} support for PJ method) is slightly higher than on the carrier with six carbon spacer length, Sepabeads® EC-HA, (90.9 mg g^{-1} support GA method and 98.7 mg g^{-1} for PJ method).

From the practical point of view, the activity of the enzyme preparations has a greater significance. Effects of enzyme loading on the activity of the biocatalysts and the activity coupling yield are given in Fig. 2.

The immobilization on glutaraldehyde-activated supports resulted with immobilized derivative of low activity, since lipase retained only 12.75 and 9.37% of the initial activity after immobilization on Sepabeads® EC-HA and EC-EA, respectively. Nevertheless, approach involving chemical modification of carbohydrate portion of enzyme with periodate was more efficient, leading to 87.6% retention of free lipase activity for Sepabeads® EC-HA and 40.95% for Sepabeads® EC-EA. This is probably due to the targeted binding through the carbohydrate moiety that is not essential for the enzyme activity. The increase of the spacer length to 7 and 11 carbon atoms for Sepabeads® EC-EA and EC-HA, respectively, by adding five glutaraldehyde carbons drastically reduced activity of the immobilized preparations. These results could indicate favorable interactions between support surface and the enzyme. It seems that polymer surface provides a suitable microenvironment for stabilization of active conformation of lipase and/or allows easier access of substrate. This is in agreement with previous studies reporting that internal geometry of Sepabeads® EC-EP offers large plane surfaces where the enzyme may undergo intense interactions with the support [26]. On the other hand, glutaraldehyde is a bifunctional reactive agent capable of reacting with all surface amino groups of the enzyme. That can lead to a conformational changes, and therefore to altered activity of the enzyme if these groups are in vicinity of active site. Also, it has been previously reported that glutaraldehyde had caused the denaturation of immobilized enzyme [13, 31, 32]. Additionally, it can be seen (Fig. 2b, c) that discrepancies

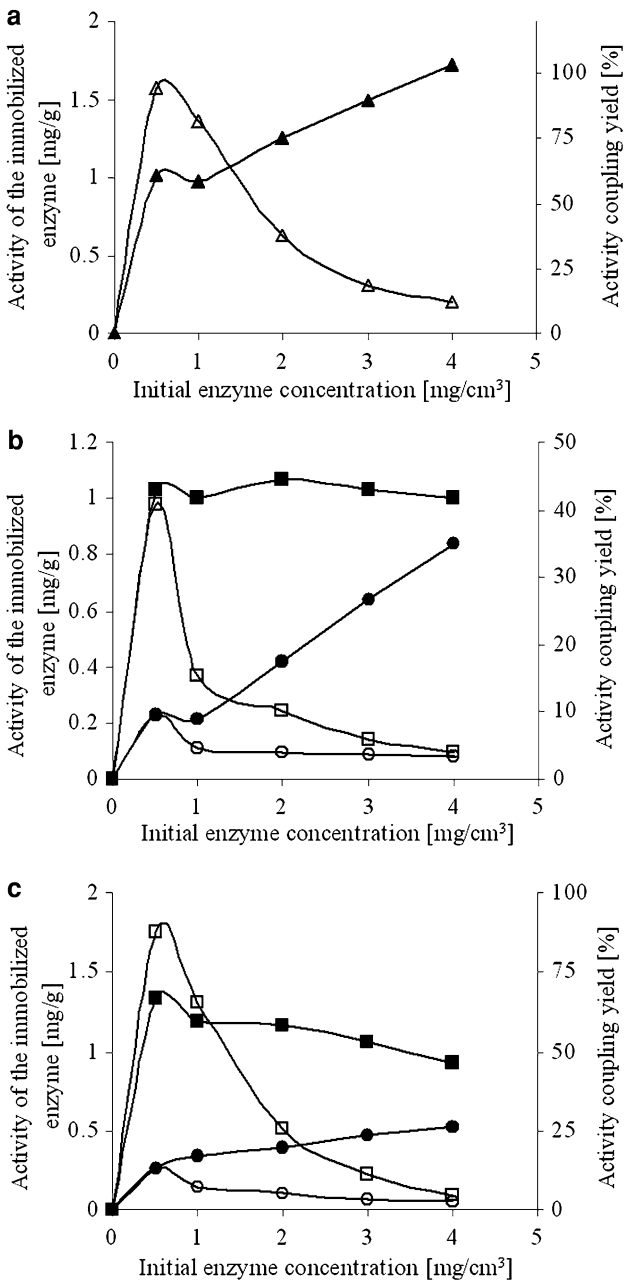

Fig. 2 The influence of the initial enzyme concentration on the activity of the enzyme immobilized on (**a**) Sepabeads® EC-EP (*filled triangle*), (**b**) Sepabeads® EC-EA–GA method (*filled circle*), Sepabeads® EC-EA–PJ method (*filled square*) and (**c**) Sepabeads® EC-HA–GA method (*filled circle*), Sepabeads® EC-HA–PJ method (*filled square*) and on activity coupling yield (**a**) Sepabeads ® EC-EP (*open triangle*), (**b**) Sepabeads® EC-EA–GA method (*open circle*) and Sepabeads® EC-EA–PJ method (*open square*), (**c**) Sepabeads® EC-HA–GA method (*open circle*) and Sepabeads® EC-HA–PJ method (*open square*)

between Sepabeads® EC-HA and Sepabeads® EC-EA are more pronounced and forthright than with respect to coupling yield (Fig. 1b, c). It is clear that Sepabeads® EC-HA provides significantly higher activity yields with both immobilization methods. Similar trends with supports containing longer spacer arm on surface have been previously observed, and it was usually ascribed to better flexibility of enzyme conformation and reduced risk of substrate diffusion limitations when enzyme is on larger distance from surface of support [33, 34].

The highest activity yield was achieved using Sepabeads® EC-EP, since after immobilization on this support lipase retained 94.8% of native activity. Also, overall immobilized activity was significantly higher when this support was used -1.73 IU g^{-1} support compared with 1.33 and 1.03 IU g^{-1} support, obtained using Sepabeads® EC-HA and Sepabeads® EC-EA, respectively. This high value of retained activity is a very good result in regard to previous researches [35–37]. When the residual activities of the immobilized preparations, for all immobilization techniques and all applied carriers, were compared it was concluded that maximum activity yields were achieved at the lowest concentration of the enzyme. Further increase of offered enzyme concentration resulted with steep decrease of activity coupling yield. It is plausible that the enzyme binds first via the best oriented functional groups, which does not lead to conformational changes that reduce its activity. Increasing the enzyme concentration leads to unfavorable binding and to a conformational changes that reduce the activity of lipase. Therefore, to achieve the optimum utilization of lipase activity it is necessary to work with the lowest concentrations of enzyme. For supports with amino groups immobilization at low lipase concentration is more efficient with respect to utilization of support, since activity per g of support did not change significantly with increase of offered lipase concentration (Fig. 2b, c). On the other hand, when immobilization was performed with Sepabeads® EC-EP continuous increase of activity was achieved indicating that for complete utilization of support binding potential higher concentrations of enzyme should be applied, especially if the enzyme is not too expensive.

Biochemical properties of free and immobilized lipase

In order to evaluate the effect of immobilization on catalytic properties of lipase from *C. rugosa,* a comparative study between free and immobilized lipase is provided in terms of pH, temperature, and thermal stability. Since results of previous experimental series indicate that glutaraldehyde-activation of supports with amino groups leads to very low activity coupling yields, in this part of study these immobilized derivatives were not characterized.

pH optimum of immobilized enzyme

The effect of pH on the activity of free and immobilized lipase was determined in the pH range 3.75–9.00, and the

results are presented in Fig. 3. The pH optimum of the lipase immobilized on the carriers with amino groups was shifted to the alkaline region relative to the free enzyme, while the pH optimum for carrier with epoxy groups remains the same as for free enzyme. The shift of pH optimum can be ascribed to the fact that binding of lipase to a carrier via its free amino groups results in an increase in the amount of the acidic groups and enzyme gains more polyanionic character [38]. The pH profile of free lipase is broader than those of the immobilized lipase in alkaline region, which indicates that the immobilization did not preserve the enzyme activity. Only in acidic range slight broadening was observed with lipase immobilized via epoxy-support.

Temperature optimum of immobilized enzyme

Activity of free and immobilized lipase was assayed at different temperatures in the range 4–80 °C. The results are presented in Fig. 4. The apparent temperature optimum for free lipase was about 37 °C. It can bee seen that the temperature optimum varies depending on the type of carrier. Immobilization on Sepabeads® EC-EA did not affect the temperature optimum, while the immobilization on Sepabeads® EC-EP affected adversely and shifted the optimum to a 25 °C. Only in the case of carrier with amino groups, with a six carbon spacer (Sepabeads® EC-HA) immobilization had a positive effect and shifted the optimum temperature to a higher temperature −45 °C. It can also be observed that immobilization on supports with amino groups resulted in significant broadening of temperature profile; hence immobilized lipase retained around 80% of their activity at 80 °C. Such a result indicates that chemical modification of carbohydrate portion of lipase molecule by periodate and subsequent covalent immobilization of lipase via introduced carbonyl groups can significantly broaden area of application of lipase from *C. rugosa* to reactions that need to be performed at higher temperatures because of low solubility of substrates at temperature optimum of free lipase [39]. It is plausible that the increase in temperature optimum occurred because covalent bond formation, via amino groups of the immobilized lipase, might also reduce the conformational flexibility and result in higher activation energy for disturbing lipase catalytic conformation.

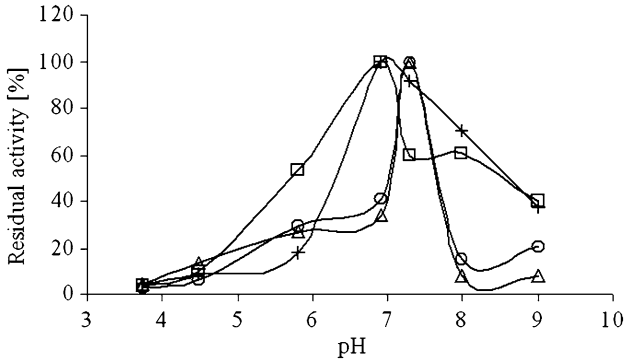

Fig. 3 pH profiles of the free lipase (+), lipase immobilized on Sepabeads® EC-EP (*open square*), on Sepabeads® EC-EA (*open triangle*) and on Sepabeads® EC-HA (*open circle*)

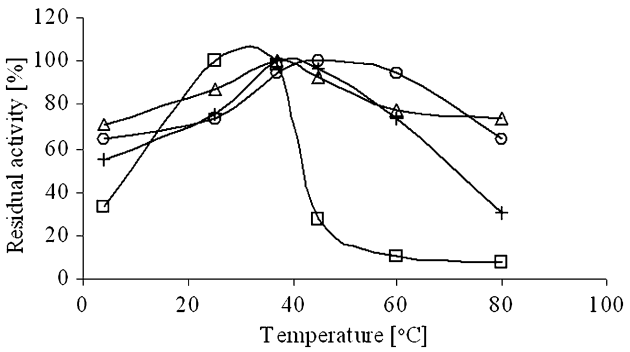

Fig. 4 Temperature profiles of the free lipase (+), lipase immobilized on Sepabeads® EC-EP (*open square*), on Sepabeads® EC-EA (*open triangle*) and on Sepabeads® EC-HA (*open circle*)

Thermal stability of immobilized enzyme

For complete characterization of immobilized enzymes and better evaluation of prospect of their application, it is necessary to compare stability of different enzyme derivatives. In our study, thermal stability assay was performed by incubation of immobilized and free lipase at 75 °C for 5 h. Obtained results are illustrated in Fig. 5. For easier comparison of stabilities half-lives of different derivatives were determined, and obtained results are presented in Table 2. Immobilization of lipase increased lipase stability, as it was expected. It was clearly demonstrated (Fig. 5) that immobilization of periodate-modified lipase on supports containing amino groups resulted in immobilized lipases with significantly increased stability. Half-lives of these

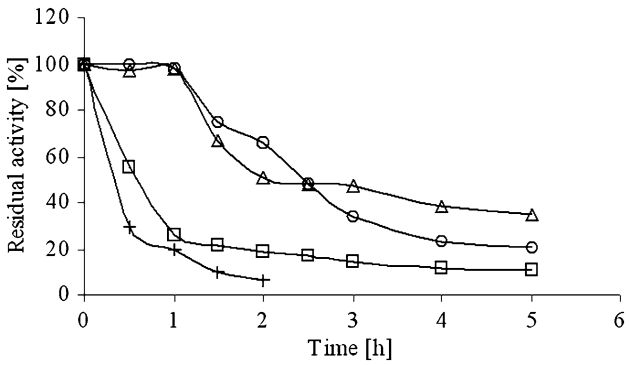

Fig. 5 Thermal stabilities of the free lipase (+), lipase immobilized on Sepabeads® EC-EP (*open square*), on Sepabeads® EC-EA (*open triangle*) and on Sepabeads® EC-HA (*open circle*) at 75 °C

Table 2 Half-lives of free and immobilized lipase

Derivatives	Half-lives (h)
Sepabeads® EC-EP	0.6
Sepabeads® EC-EA	2.2
Sepabeads® EC-HA	2.4
Free lipase	0.3

derivatives were around five times higher in comparison with non-modified lipase immobilized via epoxy groups.

Also, the method of immobilization had significant impact on the shape of stability curves. In experiments with lipase immobilized via amino groups of supports two or three stages of enzyme inactivation are distinct, while inactivation of lipase immobilized on Sepabeads® EC-EP occurred within only one stage resembling first order kinetics. Multiphasic inactivation of immobilized lipase modified with periodate could be consequence of multipoint covalent attachment of enzyme leading to different activation energies for bond breaking of different subpopulations of immobilized enzyme, which resulted in large discrepancies between degradation rates [40, 41]. It is plausible that higher stability of these derivatives could be due to previously reported specific immobilization behavior of carbonyl groups created by periodate oxidation. To be precise, Mateo et al. reported that rates of immobilization on glyoxyl supports strongly depend on the surface density of reactive amino groups on enzyme surface [42]. It was suggested that even first step of immobilization process involved multipoint covalent attachment, not just reaction between one amino group and one carbonyl group. Hence, it seems that, based on previous findings and our results, periodate oxidation of carbohydrate portion of lipase caused formation of carbonyl groups that promote multipoint covalent attachment resulting with highly stabilized immobilized lipases.

Conclusions

In this study was it was proved that various Sepabeads® EC supports which contain different functional groups can be used for *C. rugosa* lipase immobilization with enzyme loads exceeding 100 mg per g of dry support and high retention of lipase activity after immobilization. Significant improvement in application of supports with amino groups (Sepabeads® EC-HA and Sepabeads® EC-EA) was achieved when immobilization was performed with lipase previously oxidized with periodate. These immobilized lipases have shown drastically greater activity in comparison with non-modified lipase immobilized on same supports previously activated with glutaraldehyde. Also, the temperature profile of these immobilized enzymes was significantly "broader" than profile of free lipase, indicating the prospect of application of such immobilization method for preparation of enzymes that catalyze reactions at higher temperatures. Finally, periodate-modified lipase immobilized via amino groups of support has exhibited higher thermal stability than lipase immobilized via epoxy groups of support (Sepabeads® EC-EP), indicating that after proper modification of enzyme supports with amino groups can be even more attractive for enzyme immobilization than supports with epoxy groups.

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