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The effect of spacer arm on hydrolytic and synthetic activity of *Candida rugosa* lipase immobilized on silica gel

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ABSTRACT

Candida rugosa lipase was covalently immobilized onto silica gel in two different ways: via glutaraldehyde (L_{GAL}) and via hydrophobic spacer arm (1,6 diamino hexane) (L_{SA}). Free lipase, L_{GAL} and L_{SA} were used to investigate the hydrolysis of two different substrates, namely *p*-nitrophenyl palmytate (*p*NPP) and *p*-nitrophenyl acetate (*p*NPA), both in aqueous medium. In addition, these lipase samples were used to synthesize the *p*NPP from *p*-nitrophenol (*p*NP) and palmytic acid (PA) and *p*NPA from *p*NP and acetic acid (AA), both in hexane medium. Hydrolytic and synthetic activities of L_{SA} were higher than those of free lipase and L_{GAL}. Synthetic activities of free lipase, L_{GAL} and L_{SA} for *p*NPA in the presence of *p*NP and AA within hexane medium were higher than those of hydrolytic activities for *p*NPA in aqueous medium. The same tendency was also observed with *p*NPP. The effects of pH and temperature on hydrolytic and synthetic activities were investigated for all lipase preparations. Operational stability was the highest for L_{GAL} and L_{SA} when these enzymes were used for *p*NPP synthesis and in hexane medium, after 100 repeated uses, 68% and 51% of initial activities remained, respectively, at the end of 100 repeated cycles. Free lipase lost all of its activity within 15 and 20 days when stored at 25 °C and 5 °C, respectively. However, L_{GAL} showed 54% and 70% of initial activity at the end of 60 storage days at 25 °C and 5 °C, respectively, while these values were observed as 36% and 60% for L_{SA}.

1. Introduction

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is a very interesting enzyme due to its ability to catalyze both hydrolysis and formation of the ester bond depending on the nature of reaction medium. Lipase hydrolyzes ester bond to give carboxylic acid and alcohol in aqueous medium, while it constitutes ester bond from carboxylic acid and alcohol in nonaqueous medium. Therefore, on account of its hydrolytic and synthetic activities, it is becoming increasingly useful for chemical and pharmaceutical industries involving hydrolysis of oil and fats, esters [1]; synthesis of fatty acid esters as cosmetic ingredients [2,3], flavor esters [4–6], surfactants [7], amino acid esters [8], biodiesel [9–12], and so on.

Lipases immobilized onto various carriers were investigated in terms of their either hydrolytic [1,13–16] or synthetic activity [4,8,9,11,12,17–20] or both hydrolytic and synthetic activities [10,21–28]. It was reported that the hydrophilicity or hydrophobicity of the carriers used in immobilization of lipase affected the catalytic behavior [21,22,24]. The effect of the chain length of substrate on the lipase activity was also investigated [17,29–32].

One of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially due to the steric hindrance between enzyme and substrate molecules.

Spacer-arm incorporation through enzyme and support is a common method to reduce undesirable interactions between the functional groups on the carrier surface and the large enzyme molecule and to lower steric hindrance [33]. Spacer arms with low-molecular weight usually consist of linear hydrocarbon chains with functionalities on both ends for any coupling to the support and enzyme protein [34]. There are several studies on using the various spacer arms for different enzyme immobilization [13,22,24,33,35–39].

Changes in the kinetic behavior of an enzyme resulting from its attachment to an insoluble matrix have been the subject of a number of investigations and have been attributed to conformational changes in the enzyme, alterations in the microenvironment generated by the matrix and other limitations created by the solid phase [40].

This study was mainly aimed to investigate both hydrolysis and synthesis of the carboxylic acid ester of *p*NP by catalyzing *Candida rugosa* lipase. To this end, lipase preparations were used not only for

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Nomenclature

APTES	3-aminopropyltriethoxysilane
DAH	1,6-diaminohexane
GAL	glutaraldehyde
L_{GAL}	lipase immobilized via GAL
L_{SA}	lipase immobilized via spacer arm (1,6-diaminohexane)
*p*NP	*para*-nitrophenol
PA	palmytic acid
AA	acetic acid
*p*NPA	*para*-nitrophenylacetate
*p*NPP	*para*-nitrophenylpalmytate

hydrolysis of *p*NPA in aqueous medium, but also for synthesis of this ester product from *p*NP and AA in hexane medium. Similar studies were also conducted for *p*NPP hydrolysis and synthesis using the same way with appropriate substrates. There are many studies about synthetic activity of lipase using aliphatic alcohol and fatty acid in the literature [4,17,19,21–23,25]. Instrumental techniques, such as GC or HPLC, or titrimetric methods were used to determine the synthetic activity in these studies. In the present study *p*NP which is an aromatic alcohol was used as alcohol and synthetic activity was determined by simple spectrophotometric method. It was thought that this method was novel and it would be the first in its area.

Another major aim of this research was to investigate the effect of the hydrophobic spacer arm of the support used in lipase immobilization on the catalytic efficiency and stability. Three different lipase preparations were used; free lipase, lipase immobilized on silica gel via glutaraldehyde (L_{GAL}) and lipase immobilized on silica gel via hydrophobic spacer arm, 1,6-diaminohexane (L_{SA}). Reaction conditions were optimized in terms of optimum pH and temperatures, and kinetic parameters were determined. Operational stabilities for all reactions and storage stabilities using synthesis of *p*NPA were also investigated.

2. Materials and methods

2.1. Materials

Lipase from *C. rugosa* (1170 U mg^{-1}), *p*-nitrophenol (*p*NP), palmitic acid (PA), *p*-nitrophenyl palmitate (*p*NPP), *p*-nitrophenyl acetate (*p*NPA), acetic acid (AA), 1,6-diamino hexane (DAH), glutaraldehyde (GAL), 3-aminopropiltrietoxysilane (APTES) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Silica gel was purchased from Merck A. G (Darmstadt, Germany). All other chemicals used were analytical grade.

2.2. Methods

2.2.1. Preparation of carriers

Silica gel was activated by method described in detail in earlier study [41] including three steps: cleaning with 5% HNO_3 solution, formation of alkyl amine derivative with APTES and activation with GAL solution, respectively. At the end of this procedure Carrier I was obtained. This carrier was used not only for lipase immobilization to get L_{GAL}, but also for preparing the Carrier II which had spacer arm. To this end, Carrier I was incubated in (DAH) solution at pH 7, 50 °C for 5 h to insert spacer arm [13]. After excessive washing and drying, carrier bound DAH was again activated by GAL. Carrier II was then ready for lipase immobilization to get L_{SA}.

2.2.2. Ninhydrine method for amino group

The amount of free amino groups of Carriers after certain step was determined by Ninhydrine method [42]. Three-fold diluted Ninhydrine reagent (Sigma) was added onto 0.1 g of carrier and reaction mixture was left for 20 min at 90 °C in a covered boiling water bath. The reactants were then cooled in a cold-water bath below 30 °C and the contents were diluted with 5 ml of 50% (v/v) ethanol/water solution. The absorbance level at 570 nm was measured. First the amount of the amino groups on APTES treated carrier was found at and following GAL activation the decrease in the amino group, which determines the amount of aldehyde on the Carrier I, was calculated. The same measurements were carried out for DAH treated support and then GAL treated support to determine the amount of aldehyde group on the Carrier II. The concentration of amino group was determined by standard curve obtained using alanine amino acid at the same procedure.

2.2.3. Lipase immobilization

One gram of carrier (Carrier I or II) was incubated with 10 ml of lipase solution at 2 mg ml^{-1} for 2 h at 15 °C. After immobilization period, unbound protein was removed by excessive washing. Protein content of washing solution was determined by Lowry [43]. The amount of unbound enzyme protein was subtracted from the total amount of enzyme protein used for immobilization and the amount of bound enzyme protein was calculated as mg protein g carrier^{-1}. Fig. 1 shows the lipase bound covalently on Carrier I (L_{GAL}) and Carrier II (L_{SA}).

2.2.4. Hydrolytic activity assay

The hydrolytic activities of free and immobilized lipase preparations in aqueous medium were determined according to the method reported by Ye et al. with only minor modification [16]. The reaction mixture was composed of 1 ml ethanol containing 0.5% *p*NPA or *p*NPP and 1 ml buffer solution in a tube. The reaction commenced on addition of 100 μl (1 mg ml^{-1}) free or 10 mg immobilized lipase preparations and it was allowed to continue for 15 min in a shaker. To stop reaction for *p*NPP hydrolysis, 5 ml of 0.5N Na_2CO_3 solution was added. Due to low solubility of *p*NPP

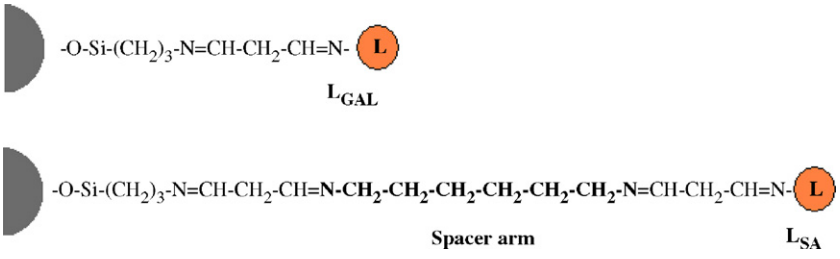

Fig. 1. Immobilized lipases via GAL (L_{GAL}) and via 1,6-diaminohexane (L_{SA}).

in aqueous medium, reaction mixture at emulsion form was centrifugated and absorbance value resulting from the release of *p*NP of homogen supernatant was measured at 410 nm. In the case of *p*NPA hydrolysis, 5 ml buffer at pH 7.0 was added instead of Na_2CO_3 solution because *p*NPA is instable at pH higher than pH 7.0 [44] and absorbance value was measured immediately within 10 s. The same experiments were repeated for non-enzymatic medium by using 100 μl buffer solution instead of free lipase or 10 mg Carrier I or Carrier II instead of immobilized lipase. Standard curves of *p*NP were prepared as in activity assay of *p*NPP and *p*NPA in Na_2CO_3 or pH 7 buffer solution, respectively. Concentration of *p*NP released by enzymatic reaction was determined and the activity was expressed as μmol *p*NP min^{-1} mg $protein^{-1}$.

2.2.5. Synthetic activity assay

Synthetic activity of lipase was investigated in organic medium with 2 ml of hexane containing 1 mM *p*NP and 50 mM PA (or AA). Reaction commenced on addition of 100 μl (1 mg ml^{-1}) free or 10 mg immobilized lipase and it continued for 15 min in a shaker. On completion of reaction time, 1 ml solution was removed from the reaction mixture and dropped into 3.5 ml of 25 mM NaOH solution to recover unreacted *p*NP by vortexing. The absorbance value was measured at 410 nm. The same experiment was carried out for non-catalyzed medium by using the same material as in hydrolytic activity assay. Concentration of *p*NP was determined by standard curve of *p*NP at different concentration prepared in hexane and extracted with 3.5 ml of 25 mM NaOH solution. Concentration difference of *p*NP between non-catalyzed and catalyzed medium was used to calculate the amount of *p*NP participated in esterification reaction. Activity was expressed as μmol *p*NP min^{-1} mg $protein^{-1}$.

2.2.6. Characterization of free and immobilized lipases

It was possible to study optimum pH of lipase preparations (ranging from 5 to 7.5) for only hydrolytic activity due to its occurrence in aqueous medium. However, the effect of temperature on lipase activity was investigated for both hydrolytic activity and synthetic activity at different temperatures between 20 and 55 °C.

The effect of substrate concentration on the lipase activity of free lipase, L_{GAL} and L_{SA} was studied at predetermined optimal conditions through hydrolysis and synthesis of the *p*NPP or *p*NPA. The maximum reaction rate (V_{max}) and Michaelis–Menten coefficient (K_M) values were determined from the Lineweaver–Burk plot for all enzyme preparations and reaction systems.

Operational stabilities of L_{GAL} and L_{SA} were studied for hydrolytic and synthetic activities using a packed bed column reactor. 100 mg of immobilized enzyme was loaded into column and then 2 ml of substrate was added. After 15 min reaction time, reaction mixture was removed from the column and the released *p*NP (for hydrolytic activity) or remaining *p*NP (for synthetic activity) was measured as in activity assay. The same types of measurements were repeated 100 times by the same enzyme reactor. In order to prevent the influence of storage time on the enzyme activity there was just 20 s between two subsequent cycles and all measurements were carried on unintermittently.

Storage stabilities of enzyme preparations of free lipase, L_{GAL} and L_{SA} were investigated in terms of synthesis of the *p*NPA in hexane from *p*NP and AA. Free enzyme at 10 mg ml^{-1} in pH 7 buffer solutions and L_{GAL} and L_{SA} as solid forms were incubated at 5 and 25 °C for 2 months and the remaining activities were measured periodically for 60 days storage periods.

All experiments were repeated three times and standard deviations of results were less than 6%.

3. Results and discussions

3.1. The effect of spacer arm on enzyme immobilization

The amount of amino group bound on silica gel obtained from after treatment with APTES was determined as 210.2 μmol g $carrier^{-1}$. After treatment of this carrier with GAL, it was determined that there was a decrease of 92.6% in the amount of amino groups (as 15.5 μmol g $carrier^{-1}$) due to participation in imine bond formation. Henceforth, It was calculated that Carrier I had 194.6 μmol aldehyde of GAL per gram of carrier. L_{GAL} was obtained by immobilization of lipase on Carrier I.

Carrier I was also used for incorporation of spacer arm which caused increasing at the amino group of silica gel from 15.5 to 84.7 μmol g $carrier^{-1}$. Finally, this carrier was activated again with GAL and then Carrier II whose GAL content was determined as 69.4 μmol aldehyde g $carrier^{-1}$ was obtained. Carrier II was used to get L_{SA}. The amounts of bound lipase protein were determined as 9.0 and 9.8 mg g $carrier^{-1}$ for L_{GAL} and L_{SA}, respectively. Although aldehyde content of Carrier I was 2.8 times as much as that of Carrier II, it was observed that the amount of bound enzyme protein was not proportional to with the aldehyde content of the carriers. This may be explained by steric hindrance between enzyme molecules which were very close to the support surface when Carrier I was used. However, in the case of Carrier II, apart from the attachment of an eight atom-hydrophobic arm the addition of five carbon atoms from GAL may have prevented any undesirable side interactions between large enzyme molecules and the support. In this way, protein to protein and protein to support interactions decreased and this caused more bindings. Similar results were reported about various enzyme immobilization on various supports earlier by several researchers. When Bayramoğlu et al. who immobilized *C. rugosa* lipase onto poly(GMA-HEMA-EGDMA) microspheres via GAL and via DAH spacer arm, they found the amount of bound lipase as 16.1 and 28.3 mg g^{-1}, respectively [13]. Bulmuş et al. who immobilized glucose oxidase on PMMA via spacer arms at different length, reported that the amount of bound enzyme increased by the length of the spacer arm [35]. Bayramoğlu et al. immobilized urease enzyme on plain and spacer arm (DAH) attached poly (HEMA-

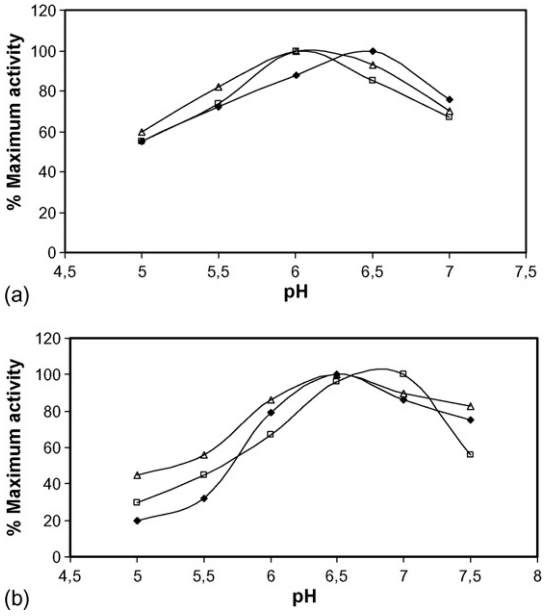

Fig. 2. (a) The effect of reaction pH on the *p*NPA hydrolyzing (♦: free, □: L_{GAL}, △: L_{SA}). (b) The effect of reaction pH on the p-NPP hydrolyzing (♦: free, □: L_{GAL}, △: L_{SA}).

GMA) and they found the amount of bound enzyme as 102.7 and 120.9 μg cm^{-2}, respectively [38].

3.2. The effect of pH on hydrolytic activity

The effect of pH on *p*NPA and *p*NPP hydrolysis is given in Fig. 2a and b, respectively. Free lipase revealed maximum activity at pH 6.5 for both *p*NPA and *p*NPP hydrolysis. The highest activities of *p*NPA and *p*NPP hydrolysis were observed at pH 6.0 and 7.0 for L_{GAL} and at pH 6.0 and pH 6.5 for L_{SA}, respectively. It was seen that immobilized lipase preparations showed higher activity in slightly more acidic medium for *p*NPA hydrolysis. *p*NPA hydrolysis was not studied at alkaline pH values due to its instability at pH values exceeding 7.0 [44].

3.3. The effect of temperature on hydrolytic and synthetic activity

Free lipase, L_{GAL} and L_{SA} samples were used to study the effect of reaction temperature on the catalytic activities of hydrolysis of *p*NPA and *p*NPP in aqueous medium and the synthesis of *p*NPA and *p*NPP in hexane medium.

As seen in Fig. 3a, maximum activity of free lipase for *p*NPA synthesis was observed at 30 °C, whereas for *p*NPP synthesis, and *p*NPA and *p*NPP hydrolysis at 35 °C. It was also found out that *p*NPP hydrolysis was the most affected reaction by the temperature.

The effect of temperature on the activity of L_{GAL} was investigated and results are given in Fig. 3b where L_{GAL} exhibited maximum activity at higher temperatures when compared with free lipase. Maximum activities of L_{GAL} were observed at 35 °C for *p*NPA synthesis, at 40 °C for *p*NPA hydrolysis and at 45 °C for both hydrolysis and synthesis of *p*NPP.

The effect of temperature on the activity of L_{SA} (Fig. 3a) was more like free enzyme rather than L_{GAL}. While maximum activities of L_{SA} were observed at 30 °C for *p*NPA synthesis, at 35 °C for both *p*NPP synthesis and *p*NPA hydrolysis and it was observed at 40 °C for *p*NPP hydrolysis. Higher optimum temperature values were caused by the changing physical and chemical properties of the enzyme. The covalent bond formation, via amino groups of the immobilized lipase, might as well have reduced the conformational flexibility and resulted in higher activation energy for the molecule to reorganize the proper conformation for bonding to its substrate [13].

3.4. Kinetic parameters

Kinetic parameters of free lipase, L_{GAL} and L_{SA} for hydrolytic activity were assayed at substrate concentration from 0.02 to 0.3 mM for *p*NPP and from 0.012 to 0.12 mM for *p*NPA in aqueous medium. In the case of synthetic activity, 0.1–1 mM *p*NP and 50 mM AA or PA were used in hexane solution. Kinetic parameters were obtained from Line viewer–Burk plots and results are given in Table 1.

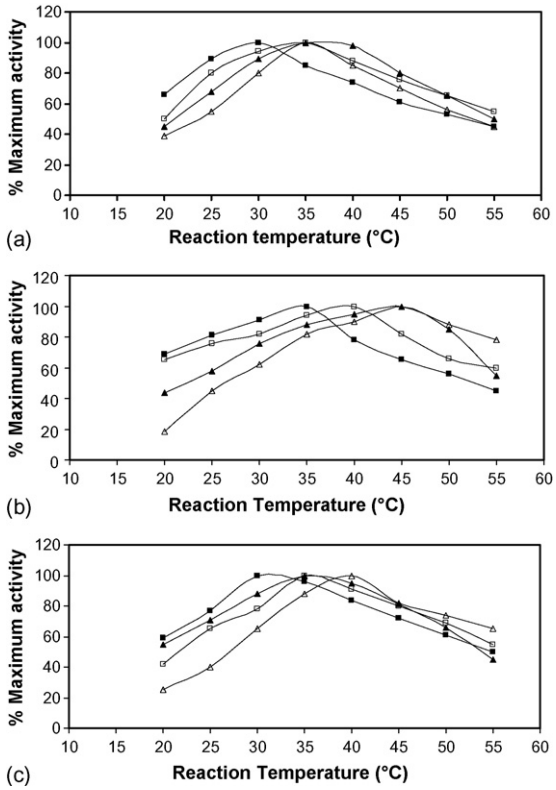

Fig. 3. (a) The effect of reaction temperature on the activity of free lipase (△: *p*NPP hydrolyzing, □: *p*NPA hydrolyzing, ▲: *p*NPP synthesis, ■: *p*NPA synthesis). (b) The effect of reaction temperature on the activity of L_{GAL} (△: *p*NPP hydrolyzing, □: *p*NPA hydrolyzing, ▲: *p*NPP synthesis, ■: *p*NPA synthesis). (c) The effect of reaction temperature on the activity of L_{SA} (△: *p*NPP hydrolyzing, □: *p*NPA hydrolyzing, ▲: *p*NPP synthesis, ■: *p*NPA synthesis).

As seen in Table 1, all lipase samples showed higher activities for synthesis of the *p*NPP in the presence of PA and *p*NP in hexane solution. The highest activities were observed for L_{SA}, whereas the minimum activities were found for L_{GAL}. Both hydrolytic and synthetic activities were higher for longer chain substrate, which is PA here. Similar tendency was emphasized in literature [22,30]. It was also seen that synthetic activities of L_{GAL} and L_{SA} were higher than those of free lipase. Stark and Holmberg [24] immobilized lipase on tresyl activated silica directly and via PEG as hydrophilic spacer arm. They investigated the effect of spacer arm on transesterification reactions in nonaqueous medium, yet they did not observe any difference. Then they suggested that the ideal spacer for lipase use in nonaqueous medium would probably be a noncharged hydrophobic molecule. In the present study, DAH a hydrophobic molecule, was used as spacer arm; indeed this sample (L_{SA}) showed the highest synthetic activity in hexane as nonaqueous medium. L_{SA}, also showed the highest hydrolytic activity when compared with free

Table 1
Kinetic parameters of free lipase, L_{GAL} and L_{SA}

	Free lipase			L_{GAL}			L_{SA}		
Substrate	V_{max}[a]	K_M[b]	V_{max}/K_M	V_{max}	K_M	V_{max}/K_M	V_{max}	K_M	V_{max}/K_M
*p*NPA	1.46	7.6	0.19	1.38	11.3	0.12	1.76	8.15	0.22
*p*NPP	2.43	0.9	2.7	1.93	1.6	1.2	2.8	1.2	2.3
*p*NP + AA	1.65	1.1	1.5	1.79	0.5	3.6	1.85	0.8	2.3
*p*NP + PA	2.75	1.3	2.1	2.97	0.4	7.4	3.55	0.3	11.8

[a] U/mg lipase.
[b] mM.

lipase and L_{GAL}. This result can be explained by the positive effect of the spacer in terms of reduced interaction between enzyme and the particle of silica [24]. Without spacer arm, the lipase, in an attempt to maximize its contact with the surface, might have lost its active conformation, and thus, low activity results may have occurred [13]. In addition to spacer arm constituted by aliphatic chains of 7 atoms, GAL of 5-atoms has been used to move the immobilized lipase away from the support. In this way, more areas of the L_{SA} might have been accessible to its substrate(s) [39]. As seen in Table 1, K_M values of free lipase at hydrolytic activities were lower than those of L_{GAL} and L_{SA}. Yet, in the case of synthetic activities, the highest K_M values were observed for free lipase. It is known that there is a negative relation between K_M value of the enzyme and affinity to its substrate. So, it can be said that substrate affinity of free lipase was higher for the hydrolytic activity than synthetic activity considering the K_M values of L_{GAL} and L_{SA}. This can be explained by homogeneity of lipase in reaction medium. Free lipase aggregated because it was insoluble in the organic medium, while immobilized lipase scattered on a large carrier surface area may have easily contacted with substrates [16]. Immobilization might have improved the catalytic activity of the lipase, most probably by acting on its conformation and dispersing it more as on the support than direct use in the hexane solution as the reaction medium.

3.5. Operational stability

The operational stability of an immobilized enzyme without appreciable loss of enzyme activity is important for the economic viability of a biosynthetic process. Immobilized lipases were used repeatedly and similar tendency was observed for both L_{GAL} and L_{SA} (Fig. 4a and b); operational stabilities were higher for synthetic activities than those of hydrolytic activities. It was clearly seen that the maximum stability was shown by the *p*NPP synthesis, whereas the minimum was shown by the hydrolysis of the *p*NPA. Operational stability was the highest for L_{GAL} and L_{SA} when these enzymes were used for *p*NPP synthesis and in hexane medium; 68% and 51% of initial activities remained, respectively, at the end of 100 repeated cycles. In aqueous medium carboxylic acid is released during reaction, in present case PA or AA. Therefore, microenvironment of enzyme may be more acidic and this probably may have caused the partial denaturation of the enzyme.

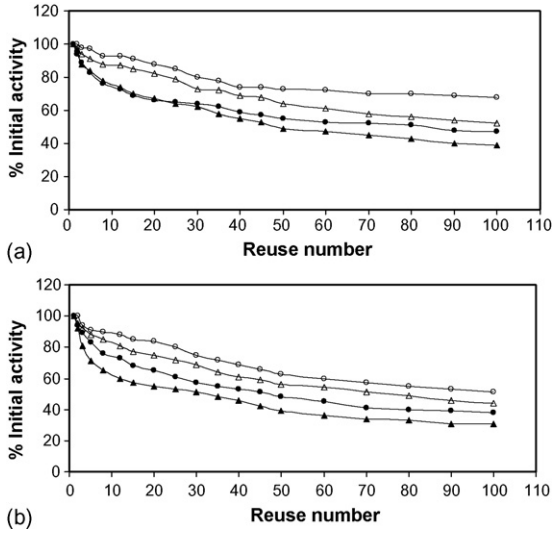

Fig. 4. (a) Operational stabilities of L_{GAL}: (○) *p*NPP synthesis, (△) *p*NPA synthesis, (●) *p*NPP hydrolyzing, (▲) *p*NPA hydrolyzing. (b) Operational stabilities of L_{SA}:(○) *p*NPP synthesis, (△) *p*NPA synthesis, (●):*p*NPP Hydrolyzing, (▲) *p*NPA hydrolyzing.

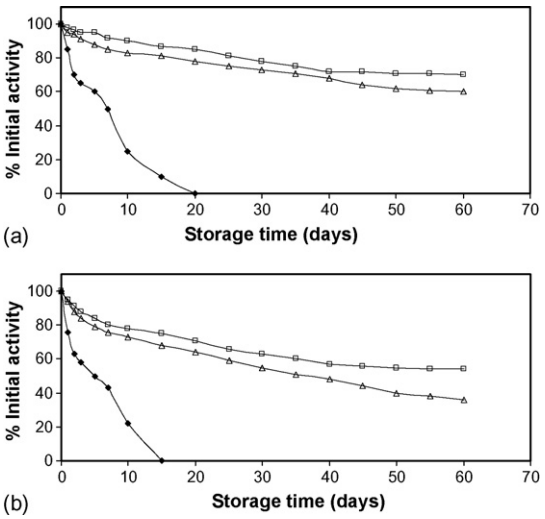

Fig. 5. (a) Storage stabilities of free lipase: (♦), L_{GAL} (□) and L_{SA} (△) at 5 °C. (b) Storage stabilities of free lipase: (♦), L_{GAL} (□) and L_{SA} (△) at 25 °C.

It was also found that, L_{GAL} was more stable than L_{SA} for all reaction types.

3.6. Storage stability

The free lipase, L_{GAL} and L_{SA} were stored at 5 and 25 °C under the same conditions, at both temperatures, activities of L_{GAL} and L_{SA} during storage time decreased more slowly than those of free lipase (Fig. 5a and b). The free lipase lost all its activities within 20 and 15 days at 5 and 25 °C, respectively. It was clearly observed that, L_{GAL} had high storage stability. At the end of the 60 days storage time, L_{GAL} and L_{SA} preserved about 70% and 60% of their initial activities at 5 °C, respectively. However, L_{GAL} and L_{SA} showed about 54% and 36% of their initial activities at 25 °C, respectively.

4. Conclusion

Three different *C. rugosa* lipase preparations, free lipase, L_{GAL} and L_{SA} were used in two different reaction systems: hydrolysis of *p*NPP and *p*NPA in aqueous medium and synthesis of *p*NPP (from *p*NP and PA) and *p*NPA (from *p*NP and AA) in hexane medium. In this study, a novel simple spectrophotometric method was used for determination of synthetic activity of lipase in nonaqueous medium with aromatic alcohol used as *p*NP.

The results indicated that:

- The most active form was L_{SA} for both synthetic and hydrolytic activities. Meanwhile, the positive effect on the catalytic activity was obtained by using hydrophobic noncharged spacer arm at immobilization.
- It was L_{GAL} that had the most stable form for operation and storage.
- Synthetic activities were higher than hydrolytic activities. That is to say, lipase preparations were more active for synthesis *p*NPP and *p*NPA than for their hydrolysis.
- Maximum activities were observed for esterification of *p*NP and PA,
- Hydrolytic and synthetic activities were higher when substrate included PA.

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