Journal of Molecular Catalysis B: Enzymatic 57 (2009) 109–114

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Synthesis and characterization of cyclodextrin-based polymers as a support for immobilization of *Candida rugosa* lipase

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ARTICLE INFO

*Article history:*
Received 3 May 2008
Received in revised form 17 July 2008
Accepted 31 July 2008
Available online 14 August 2008

*Keywords:*
Enzyme immobilization
*Candida rugosa* lipase
β-Cyclodextrin
Covalent bonding

ABSTRACT

The objective of this study was to prepare cross-linked β-cyclodextrin polymers for immobilization of *Candida rugosa* lipase. The structures of synthesized macrocyclic compounds were characterized by Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA) and scanning electron microscope (SEM) techniques. Properties of the immobilized systems were assessed and their performance on hydrolytic reaction were evaluated and compared with the free enzyme. The influence of activation agents (glutaraldehyde (GA) and hexamethylene diisocyanate (HMDI)) and thermal and pH stabilities of the biocatalyst was evaluated. After the optimization of immobilization process, the physical and chemical characterization of immobilized lipase was performed. Obtained data showed that the immobilized enzyme seemed better and offered some advantages in comparison with free enzyme. It can be observed that the free lipase loses its initial activity within around 80 min at 60 °C, while the immobilized lipases retain their initial activities of about 56% by HMDI and 82% by GA after 120 min of heat treatment at 60 °C.

Results showed that the specific activity of the immobilized lipase with glutaraldehyde was 62.75 U/mg protein, which is 28.13 times higher than that of the immobilized lipase with HMDI.



## 1. Introduction

Enzymes are often immobilized onto solid supports to increase their thermal and operational stability, and recoverability [1,2]. Various methods available for enzyme immobilization can be divided into two general classes: chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weak interactions between support and enzyme exist [3–6]. Protocols for covalent enzyme immobilization often begin with a surface modification or activation step. The native amino groups or the active groups resulting from surface modification can be bound to aldehyde groups using glutaraldehyde [7,8].

Aldehyde groups in the support and amine groups in the enzyme are a good choice to make the multipoint attachment and, therefore, to obtain highly stable enzyme derivatives. In literature [9–14] when multipoint immobilization occurs, although the first binding is fast, the multi-interaction process requires longer times to achieve the correct alignment of the reactive groups of enzyme and support. Therefore, the fact that a higher stabilization was achieved after longer incubation times indicates that *Candida antarctica* lipase is attached to the support by more than one bond. Amine groups (terminal and in lysine residues) are very reactive, abundant on the enzyme surface and form Schiff bases with the aldehyde groups of the support. The number of covalent bonds between the support and the enzyme depends on the degree of activation of the support (concentration of aldehyde groups in the support surface) and on the concentration of amine groups in the enzyme molecule [11,14].

Moreover, for enzyme immobilization, it is very important to choose supports from different nature [15] which can be classified into three general types: inorganic particles, synthetic polymers and natural macromolecules. Natural macromolecules, including chitosan [16–18], cellulose [19,20], agarose [21] and carrageenan [22] with excellent biocompatibility and hydrophilicity, are nontoxic, biodegradable and inexpensive.

Cyclodextrins (CDs), another class of compounds with a macrocyclic structure, have been successfully used to improve enzymes activity and to increase the reaction rate and *E* in enzyme-catalyzed reactions in organic solvents [23,24]. They are a family of chiral cyclic α-(1–4)-linked D-glucose oligomers with six, seven, or eight glucose units, corresponding to α-, β-, and γ-homologues, possessing toroidal conformation in the solid state and in solution. The internal hydrophobic cavity and the external hydrophilic rim of chemically modified cyclodextrins render them ideal for modeling enzyme substrate binding [25]. Owing to the above orientation

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doi:10.1016/j.molcatb.2008.07.014

of the hydroxyl groups on both sides of the toric cavity, the CD periphery is hydrophilic, which favors the hydration and solubility in aqueous solutions. On the other hand, the interior of the CD cavity may be more hydrophobic because nonbonding electron pairs of the glycosidic oxygen are present along with a high electron density and Lewis-base properties. The physicochemical properties and inclusion capacity of CDs derive directly from this special topology of the OH groups. One of the most remarkable characteristics of CDs is their ability to form inclusion complexes with a wide range of hydrophobic molecules [26–30].

The immobilization of enzymes is carried out by the formation of inter- and intra-molecular cross-linkages between the enzyme molecules by means of bifunctional reagents. Glutaraldehyde and isocyanate derivatives have been used as a cross-linker for the immobilization of enzymes in which the amino groups of a protein are expected to form a Schiff base with the glutaraldehyde [31–38]. The glutaraldehyde technique is very versatile and may be used in very different fashions [39,40]. However, in terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed on supports bearing primary amino groups offers very good results in many cases, because it permits the cross-link between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support.

To our best knowledge, there exists no report on the use of β-cyclodextrin polymer as support and hexamethylene diisocyanate (HMDI) for activating agents for immobilization of lipases. The aim of this work was to study the influence of two different activating agents (hexamethylene diisocyanate and glutaraldehyde) on *Candida rugosa* lipase (CRL) activity and stability after immobilization on β-cyclodextrin polymer.

## 2. Materials and methods

### 2.1. Materials

Lipase (from *C. rugosa*, lyophilized powder) was supplied by the Sigma Chemical Co. (St. Louis, MO) and used as received. Bradford reagent, bovine serum albumin 99% (BSA), *p*-nitrophenylpalmitate (*p*-NPP), and hexamethylene diisocyanate were purchased from Sigma-chemical Co. (St. Louis, MO). β-Cyclodextrin, *N*-dimethyl formamide (DMF), acetone and ethanol were provided from Merck (Darmstadt, Germany). All aqueous solutions were prepared with de-ionized water that had been passed through a Millipore Milli-Q Plus water purification system. All other chemicals were of analytical grade and used without further purification Merck (Darmstadt, Germany).

### 2.2. Characterization of support

Thermogravimetric analysis (TGA) was carried out with Seteram thermogravimetric analyzer. The sample weight was 15–17 mg. Analysis was performed from room temperature to 800 °C at a heating rate of 10 °C/min in an argon atmosphere with a gas flow rate of 20 mL/min. The surface morphology of samples was examined by scanning electron microscope (SEM, Jeol, JSM 5310, Japan). FTIR analysis was carried out using a PerkinElmer 1605 single channel Fourier transform spectrophotometer. Infrared spectra of the samples pressed in KBr pellets were obtained at a resolution of 2 $cm^{-1}$ between 4000 and 400 $cm^{-1}$. UV–vis spectra were obtained with a Shimadzu 160 A UV–vis recording spectrophotometer.

### 2.3. Preparation of β-CD-based polymers

Two grams of β-CD (1.76 mmol) was dissolved in 15 mL of dry DMF in a 100 mL round bottom flask at room temperature. Then 17.6 mmol of hexamethylene diisocyanate in 5 mL of dry DMF for polymer 1 was added dropwise. The mixture of polymer was stirred at 70 °C for 3 h. The precipitate was filtered and washed with acetone several times. The support 1 (β-CD–HMDI) was dried in vacuum for 24 h.

A 5-mL of ethylenediamine in acetonitrile (5 mL) was added dropwise to the solution of support 1 in 10 mL acetonitrile. The mixture was stirred at room temperature for 45 min. The product (β-CD-$NH_2$) was filtered and washed with water several times and dried in vacuum for 12 h.

Glutaraldehyde activation was made by contacting β-CD based polymer containing amino groups (β-CD-$NH_2$) with glutaraldehyde (5 mL, 12.5%) for 24 h at room temperature. Afterwards, the activated polymer (β-CD–GA) was washed with phosphate buffer solution (pH 7.0) to remove the excess of the activating agent. Then the support 2 (β-CD–GA) was used for the immobilization of *C. rugosa* lipase.

### 2.4. Immobilization procedure

#### 2.4.1. Method A

One gram of the support 1 (β-CD–HMDI) was suspended by dissolving 0.25 g of lipase powder in 10 mL buffer solution. Flask was incubated at 30 °C at 180 rpm. After 6 h, the support was washed thoroughly with 50 mM phosphate buffer (pH 7.0). Immobilized enzyme was analyzed for the expression of bound lipase activity. The preparation was then lyophilized and stored at 4 °C until use.

#### 2.4.2. Method B

Two grams of the support 2 (β-CD–GA) was suspended by dissolving 0.5 g lipase powder in 15 mL of buffer solution. Flask was incubated at 30 °C at 180 rpm. After 6 h, the support was washed thoroughly with 50 mM phosphate buffer (pH 7.0). Immobilized enzyme was analyzed for the expression of bound lipase activity. The preparation was then lyophilized and stored at 4 °C until use.

### 2.5. Determination of enzyme activity

Activity of the free and immobilized lipase was assayed using 14.4 mM *p*-nitrophenyl palmitate in 2-propanol as substrate. The reaction mixture consisting of 1 mL of 50 mM phosphate buffer (pH 7.0 for immobilized lipase) containing 25 mg of immobilized lipase (or 0.1 mL free lipase) was initiated by adding 1 mL of substrate and mixed for 5 min at 30 °C. The reaction was terminated by adding 2 mL of 0.5N $Na_2CO_3$ followed by centrifuging at 4000 rpm for 10 min. The increase in the absorbance at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Shimadzu UV-160A (Japan) spectrophotometer. A molar extinction coefficient ($\varepsilon$410) of 15,000 $M^{-1}$ $cm^{-1}$ for *p*-nitrophenol was used [41]. One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol/min of *p*-NPP under the conditions of assay.

### 2.6. Immobilization yield

Immobilization yield (IY) was calculated by measuring the difference between protein concentration on the supernatant before ($Ct_0$) and after ($Ct_t$) immobilization [12], according to the following equation:

$$IY(\%) = \frac{Ct_0 - Ct_t}{Ct_0} \quad (1)$$

### 2.7. Protein assay

Protein content was estimated by the method of Bradford [42] using Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as the standard.

### 2.8. pH and temperature stability

The pH stabilities of the free and immobilized lipases were assayed by immersing them in PBS (50 mM) in the pH range of 4–10 for 1 h at 30 °C and then determining their activities.

The thermal stabilities of the free and immobilized lipases were assayed by immersing them in PBS (50 mM, pH 7.0) for 2 h at 60 °C and periodically determining their activities.

## 3. Results and discussion

### 3.1. Characterization of β-CD based polymer (β-CD–HMDI)

In this work a cross-linked β-CD based polymer (β-CD–HMDI) was prepared by the reaction of β-CD with HMDI. The polymer precipitated in a short time when the molar ratio of reactants was 1:10 (β-CD–HMDI). The cross-linked polymer was insoluble in water, as well as in organic solvents. Characterization of the polymer was confirmed by combination of FTIR and TGA. The FTIR spectrum of the polymer shows the appearance of two strong absorption band at 2278 and 1733 $cm^{-1}$ which are the characteristic of the –N=C=O groups and the carbonyl group (N–C=O), respectively (Fig. 1a). The wide absorption band at 3350 $cm^{-1}$ indicates the presence of the OH and free NH stretching absorption for the urethane groups.

The thermal stability of the polymer was evaluated by thermal gravimetric analysis . It was found that the polymer undergoes two-step thermal degradation. The first step arises from decomposition of β-CD, while the second is due to the polymer. According to the literature [43], CD starts to decompose at temperatures ranging from 290 to 300 °C. The onset of degradation for CD polymer was found to occur at 350 °C. This is quite high as compared to conventional polyurethanes, which generally begin to decompose around 200–220 °C. The enhanced thermal stability of β-CD based polymer is attributed to its cross-linked nature, since it is well-known that cross-linking leads to increased thermal stability.

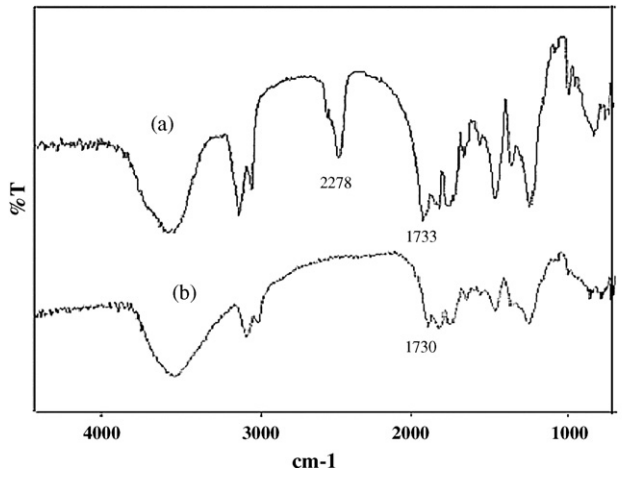
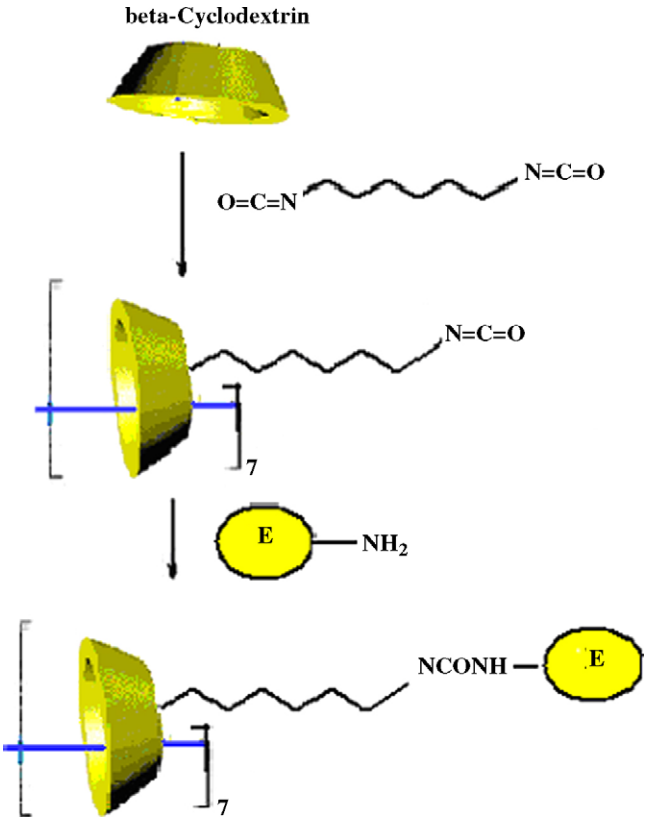



**Fig. 1.** FTIR spectra of (a) β-CD–HMDI and after (b) immobilization.




**Scheme 1.** Synthesis of β-CD based polymer and lipase immobilization (method A).

### 3.2. Immobilization of lipase

An illustrative scheme of the support 1 (β-CD–HMDI)–lipase complex formed by the immobilization of lipase to the isocyanate groups of support 1 by cross-linking with HMDI is shown in Scheme 1. During activation, the consumption of isocyanate was followed by measuring the absorbance of the –N=C=O antisymmetric stretching band at 2278 $cm^{-1}$ (corresponding of isocyanate group) in the FTIR spectrum of β-CD–HMDI (Fig. 1a). By the completion of reaction, the isocyanate group of support 1 was reacted with the amino group of the enzyme to form urethane (–N–C=O) which was confirmed by the appearance of a band at 1730 $cm^{-1}$ in the IR spectra of this compound (Fig. 1b).

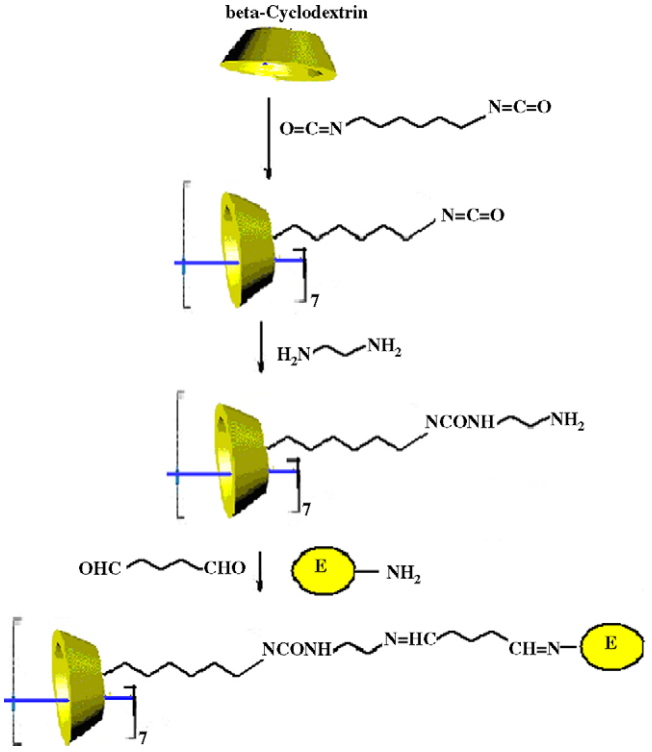
According to the method B, support 1 (β-CD–HMDI) was reacted with ethylendiamine in acetonirile to give β-CD-$NH_2$ (Scheme 2) which was confirmed by the appearance of an amine band at 1626 $cm^{-1}$ and the disappearance of isocyanate band at 2278 $cm^{-1}$ in the IR spectra of this compound (Fig. 2b). Thus, the compound (β-CD-$NH_2$) was treated with glutaraldehyde to give β-CD–GA which was present as seen from the 1715 $cm^{-1}$ carbonyl band (Fig. 2c). The activated polymer (β-CD–GA) was reacted with the amino group of the enzyme to form imino group (–CH=N–).

The immobilization of lipase on the activated supports (β-CD–HMDI or β-CD–GA) was performed at 30 °C for 6 h with low stirring in the standard buffer (50 mM phosphate buffer pH 7.0). Protein loading, activity and specific activity of the lipase immobilized by the two different immobilization protocols are shown in Table 1. The support 2 (β-CD–GA) was found to be more efficient compared to the support 1 (β-CD–HMDI) with respect to expression of immobilized lipase activity. The β-CD–GA was found to give 16.16 U/g of support with 176.7% activity yield, while β-CD–HMDI was found to give 8.82 U/g of support with 6.28% activity yield. The

**Table 1**
Activity of the free and immobilized lipase under optimum reactions conditions

| | Bound protein (μg/g) | Bound protein yield (%) | Immobilization yield (%) | Lipase activity (U/g support) | Specific activity (U/mg protein) | Activity yield (%) |
|---|---|---|---|---|---|---|
| Free lipase | – | – | – | 2.49[a] | 35.60 | 100 |
| Method A | 3.94 | 45 | 55 | 8.82 | 2.23 | 6.28 |
| Method B | 257.5 | 9.8 | 90 | 16.16 | 62.75 | 176.70 |

[a] Dimension is U/mL.




**Scheme 2.** Synthesis of β-CD based polymer and lipase immobilization (method B).

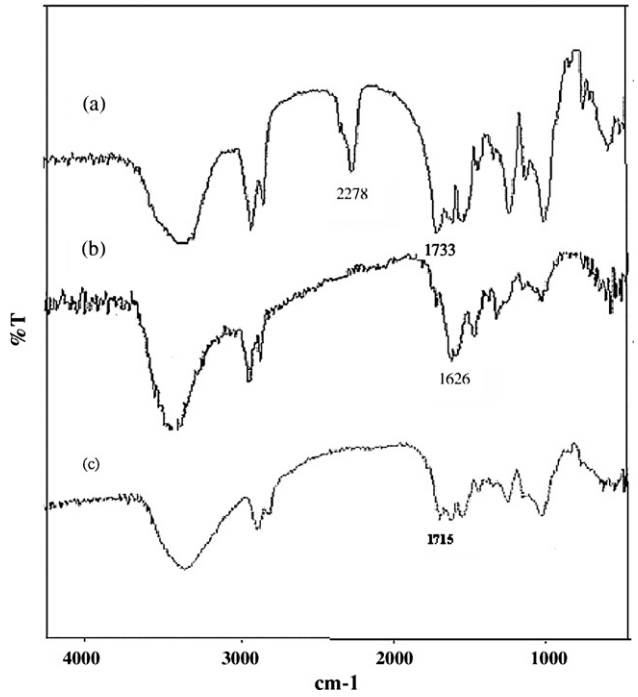



**Fig. 2.** FTIR spectra of (a) β-CD–HMDI polymer, (b) β-CD-$NH_2$ polymer, and (c) β-CD–GA polymer.

best results of immobilization yield and hydrolytic activities of the immobilized enzyme were reached when glutaraldehyde was used as activating agent. This result was not surprising because in the literature glutaraldehyde is well-known as the best activating agent to immobilize enzymes. It has given some good stabilization factors in many instances [44–46].

To verify changes in the immobilized enzymatic preparation during this reaction, an SEM image obtained after GA treated support was compared with image obtained for the immobilized lipase. After immobilization, the surface cavity of the GA treated polymer (Fig. 3) was filled by the rounded structure, which is presumably protein aggregate.

### 3.3. Effect of pH and temperature on the activity of immobilized enzyme

Fig. 4 illustrates the effect of pH on the activity of the free and the immobilized lipases. pH is one of the most influential parameters altering enzymatic activity in an aqueous medium. Immobilization of enzyme is likely to result in conformational changes of enzyme resulting in a variation of optimum pH. Fig. 4 shows that maximum enzyme activity is exhibited at pH 7.0 by free as well as immobilized enzyme (β-CD–HMDI), indicating the absence of conformational changes in the enzyme during covalent binding. Furthermore, the pH profiles of the immobilized lipases are broader than that of the free enzyme, which means that the immobilization methods preserved the enzyme activity over a wider pH range. However, the optimum pH value of the immobilized enzyme (β-CD–GA) was slightly shifted toward alkaline region. The shift depends on the method of immobilization as well as the structure and charge of the matrix. It might be a result of the basic of the imino functionalized surface of the support, the imino groups on the support surface can prevent the uniform distribution of hydrogen ions between the surface and the bulk solution [47–49]. In another research, investigated by de Castro and co-workers [50], optimum pH for immobilized lipase on polyvinyl alcohol activated with glutaraldehyde was found to be 8.8. In a recent study, lipase (*C. rugosa)* when was immobilized on chitosan beads with glutaraldehyde, was stable in the pH range of 5.0–8.0 with optimum pH 9.0 [51].

In addition, an improved stability upon covalent immobilization may explain this shift in the optimal pH. These results could probably be attributed to the stabilization of lipase molecules, resulting from multipoint attachment of the enzyme molecules on the surface of the β-cyclodextrin-based polymer [52,53]. Such a phenomenon had also been observed by other researchers [54,55]. It was attributed to the formation of covalent bonds, which limited the transition of enzyme conformation, against the change of pH. On the other hand, the results suggested that the immobilized lipases possessed better pH stability than the free one. The effect of temperature on free and immobilized lipases are given in Fig. 5. The effect of temperature on the activity of free and immobilized lipases for *p*-NPP hydrolysis at pH 7.0 in the temperature range of 25–60 °C is shown in Fig. 5. It was found that the optimum temperature for the free enzyme was approximately 35 °C, while it shifted nearly to 40 °C for immobilized lipase (β-CD–HMDI) and 45 °C for immobilized lipase (β-CD–GA). One of the main reasons

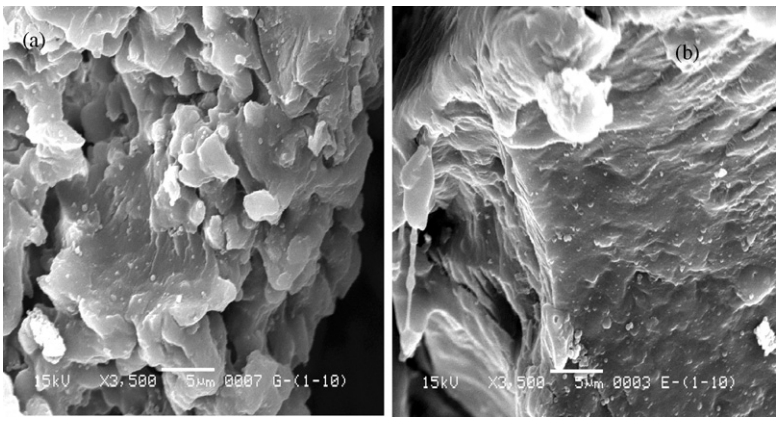



**Fig. 3.** SEM micrographs of GA treated support (a) and lipase immobilized support (b).

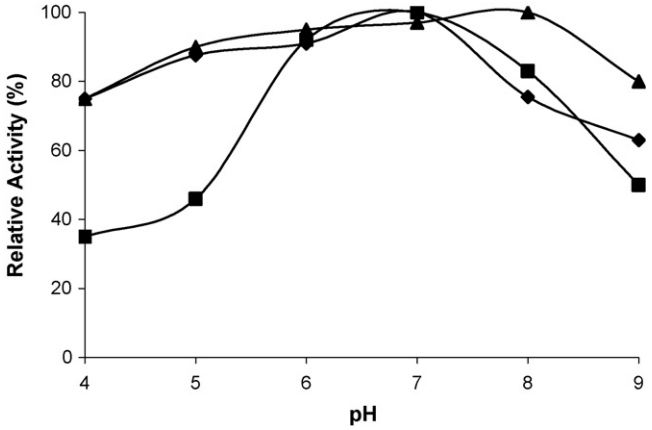



**Fig. 4.** Effect of substrate pH on residual activity of free (■), immobilized lipase (β-CD–HMDI) (♦) and immobilized lipase (β-CD–GA) (▲).

for enzyme immobilization is the anticipated increase in stability toward various deactivating forces, due to restricted conformational mobility of the molecules following immobilization [56–58]. This was either due to the creation of conformational limitation on the enzyme movement as a result of covalent bond formation between the enzyme and the supports or a low restriction in the diffusion of the substrate at high temperature. Thus, the immobilized enzymes showed their catalytic activities at a higher reaction temperature [59]. At higher temperature, free lipase could easily undergone denaturation while immobilized lipase was protected probably in terms of rigid conformation and therefore was able to retain its catalytic activity.

### 3.4. Stability of the immobilized enzyme

The variation of the residual activity of the free and the immobilized lipase with pH is shown in Fig. 6. The immobilized lipases were stable in the pH range from 7 to 9. Free lipase was stable in the pH range from 6 to 7. This indicated that immobilization appreciably improved the stability of lipase in the alkaline region. The immobilized enzymes were more stable than their free forms in the pH (4–9) range.

Fig. 7 shows the thermal stabilities of the free and immobilized lipases. Free and immobilized enzymes were incubated for 2 h at 60 °C and the enzyme activity was measured at various time intervals after cooling the enzyme to 30 °C and following the procedure described earlier. It can be observed that the free lipase loses its initial activity within around 80 min at 60 °C, while the immobilized lipases retain their initial activities of about 82% by HMDI and 56% by GA after 120 min of heat treatment at 60 °C. Lipase immobilized by HMDI showed better thermal stability at same temperature and time periods. These results indicate that the thermal stability of the immobilized lipases is much better than that of the free one since the interaction between the enzyme and the support, which

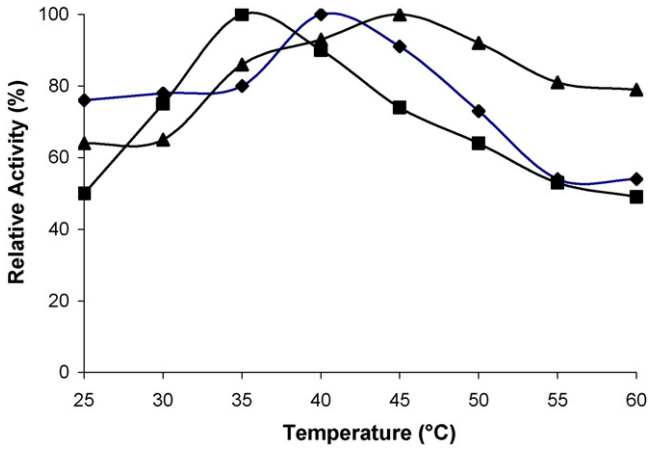



**Fig. 5.** Effect of reaction temperature on the residual activity of free (■), immobilized lipase (β-CD–HMDI) (♦) and immobilized lipase (β-CD–GA) (▲).

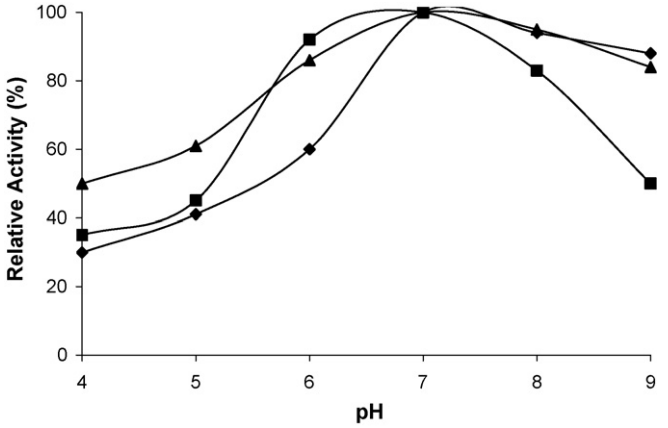



**Fig. 6.** pH stability of free (■), immobilized lipase (β-CD–HMDI) (♦) and immobilized lipase (β-CD–GA) (▲).

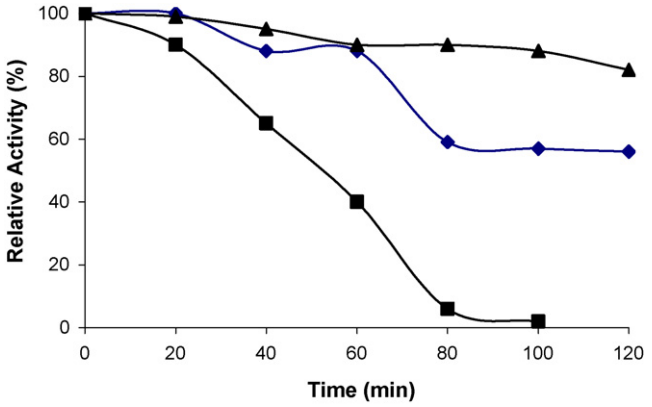



**Fig. 7.** Thermal stability of free (■), immobilized lipase (β-CD–HMDI) (♦) and immobilized lipase (β-CD–GA) (▲).

could prevent the conformation transition of the enzyme at high temperature.

## 4. Conclusions

*C. rugosa* lipase was immobilized by covalent binding on β-cyclodextrin-based polymer chemically modified with different activating agents as glutaraldehyde, and hexamethylene diisocyanate. The use of different activation agents allowed the immobilization of CRL with different properties. A new enzyme support was prepared from β-cyclodextrin-based polymer and glutaraldehyde, on which CRL was immobilized with a high activity recovery. It was observed that the specific activity of the immobilized lipase with glutaraldehyde was 62.75 U/mg protein, which is 28.13 times higher than that of the immobilized lipase with HMDI. The immobilization procedure is simple and easy to carry out. The immobilized enzyme also showed good properties and stabilities, which are important factors when selecting an appropriate enzymic system for different biotechnological applications.

## Acknowledgments

I would like to thank The Research Foundation of Selcuk University (BAP) and The Scientific and Technical Research Council of Turkey (TUBITAK-Grant Number 106T435) for financial support of this work

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