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Immobilization of *Candida rugosa* lipase onto spacer-arm attached poly(GMA-HEMA-EGDMA) microspheres

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Abstract

Epoxy group-containing poly(GMA-HEMA-EGDMA) microspheres were prepared by suspension polymerisation. The epoxy groups of the poly(GMA-HEMA-EGDMA) microspheres were used for the covalent attachment of *Candida rugosa* lipase and 1,6-diaminohexane (i.e., spacer-arm). *C. rugosa* lipase was also covalently immobilised onto the spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres using glutaric dialdehyde as a coupling agent. The maximum lipase immobilization capacities of the poly(GMA-HEMA-EGDMA) and poly(GMA-HEMA-EGDMA)-spacer-arm attached microspheres were 16.1 and 28.3 mg g^{-1}, respectively. The attachment of the spacer-arm resulted in an increase in the apparent activity of the immobilised lipase with respect to the enzyme immobilised via the epoxy groups of the microspheres. The activity yield of the lipase immobilised on the spacer-arm attached microspheres was up to 45%, and this was 9% for the enzyme immobilized through epoxy groups. Therefore, the rest of the immobilization study was carried out using only spacer-arm attached microspheres. The optimum temperature for lipase immobilised on the spacer-arm attached microspheres was 5 °C higher than that of the free enzyme and was also significantly broader. The immobilised lipase had better resistance to temperature inactivation than did the free form.

Keywords: Lipase; Immobilised enzyme; Spacer-arm; Microspheres; Hydrogels

1. Introduction

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is an important enzyme in biological systems, where it catalyses the hydrolysis of triacylglycerol to glycerol and fatty acids. The enzyme is distributed among higher animals, plants and microorganisms in which it plays a key role in lipid metabolism. Lipase has been widely used for enzymatic organic synthesis and clinical analysis. In previous works, lipases from several microbial and animal sources were immobilized by different methods, including covalent attachment to activated supports (Gomes, Pereira, & de Castro, 2004; Huang, Liao, & Chen, 2003) entrapment in gel (Reetz, Zonta, & Simpelkapt, 1996) and adsorption on materials (such as zirconium) surfaces (Bellezza, Cipiciani, & Costantino, 2003), ion exchange resins (Gandhi, Vijayalakshmi, Sawant, & Joshi, 1996) acrylic polymers (Bryjak et al., 1997), emulsion membrane reactors (Giomo & Drioli, 2003), nylon fibres (Braun, Klein, & Lopez, 1996) and silica (Soares, de Castro, de Mareas, & Zanin, 2000).After immobilization of lipase, changes were observed in enzyme activity, optimum pH, affinity for substrate and stability (Balcao, Paiva, & Malcata, 1996; Garcia, Garcia,

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Martinez, & Aracil, 2000; Ivanov & Schneider, 1997; Krishna, Manohar, Divakar, Prapulla, & Karanth, 2000; Moreno et al., 1997). The extent of these changes depended on the source of enzyme, the type of support and the method of immobilization. It is, thus, important that the choice of proper support material and immobilization method over the free bioactive agent should be well justified (Bajpai & Bhanu, 2003; Bayramoğlu, Denizli, & Arıca, 2002a; Cetinus & Oztop, 2003; Huang et al., 2003).

The availability of a large number support materials and methods of enzyme immobilisation leave virtually no bioactive species without a feasible route of immobilisation. It is, thus, important that the choice of support material and immobilisation method over the free bioactive agent should be well justified (Arıca, 2004a; Bayramoğlu, Denizli, & Arıca, 2002b; Bayramoğlu, Yılmaz, & Arıca, 2004; Jia et al., 2002; Sharma, Kumar, & Sawhney, 2003; Yodoya et al., 2003; Zhou & Chen, 2001; Zhou, Chen, & Xuemeli, 2003). Acrylic copolymers are especially versatile as a family of carrier materials for enzyme immobilisation that can be prepared with a wide variety of properties. Among them, epoxy group-carrying acrylic copolymers exhibited some significant advantages as a potential carrier matrix (i.e., easy and stable covalent linkages with different groups, such as amino, thiol and phenolic ones under mild experimental conditions (Bayramoğlu, Altınok, Bulut, Denizli, & Arıca, 2003; Xu, Li, & He, 1995)). Some of the methods employed to prepare functional support material are: (1) derivatisation of support surface with various activating agents (such as cyanogen bromide, epichlorohydrin or carbodiimide); (2) preparation of support by co-polymerisation of different monomers containing reactive groups, such as glycidylmethacrylate, 2-hydroxyethylmethacrylate and ethyleneglycoldimethacrylate. The latter, of course, reduces the number of steps involved in the functional support preparation, an important advantage in enzyme immobilization, because required amounts of functional groups can be incorporated into the support by adjusting the mole ratio of functional group carrying monomer in the polymerisation mixture (Arıca, 2004b).

In this study, an acrylic copolymer support containing functional epoxy groups was synthesised in the microsphere form from the monomers glycidylmethacrylate, 2-hydroxyethylmethacrylate and ethyleneglycoldimethacrylate. The epoxy groups of poly(GMA-HEMA-EGDMA) microspheres were used for lipase immobilization and attachment of 1,6-diaminohexane (i.e., spacer-arm). Lipase was also immobilised onto spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres using glutaric dialdehyde as coupling agent. The optimum pH and temperature for the free and immobilised enzymes were investigated. Thermal deactivation of the free and immobilised enzymes at various temperatures was studied.

2. Materials and methods

2.1. Materials

Lipase (from *Candida rugosa*, lyophilised powder) was supplied by the Sigma Chemical Co. (St. Louis, MO, USA) and used as received. 2-Hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (methacrylic acid 2,3 epoxypropyl isopropyl ether; GMA) and ethyleneglycoldimethacrylate (EGDMA) and α-α′-azo-isobisbutyronitrile (AIBN) were obtained from Fluka AG (Switzerland), and the monomers distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until used. 1,6 Diaminohexane, polyvinyl alcohol (PVA), gum arabic, bovine serum albumin (BSA) and glutaric dialdehyde were obtained from Sigma Chemical Co. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of poly(GMA-HEMA-MMA) microspheres

Poly(GMA-HEMA-EGDMA) microspheres were prepared via suspension polymerisation. The aqueous continuous phase was comprised of 0.2 M NaCl. The organic phase contained GMA (7.5 ml), HEMA (10 ml), EGDMA (7.5 ml; cross-linker) and isopropyl alcohol (15 ml, containing 5.0% polyvinyl alcohol as stabilizer) were mixed together with 0.5 g of AIBN in 30 ml of toluene. The polymerisation reactor was placed in a water bath and heated to 65 °C. The reactor was then equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The polymerisation mixture was placed into a dropping funnel and was introduced drop-wise into the reactor over about 10 min, while stirring at 200 rpm under a nitrogen atmosphere. The polymerisation reaction was maintained at 75 °C for 2.0 h and then at 85 °C for 2.0 h. After the reaction, the microspheres were filtered under suction and washed with distilled water and ethanol. The product was dried in a vacuum oven. The microspheres were sieved and the 50–100 μm size fraction was used for further reactions.

2.3. Immobilisation of Candida nugosa lipase onto poly(GMA-HEMA-EGDMA) microspheres

The spacer-arm (i.e., 1,6-diaminohexane) was covalently attached onto the epoxy groups carrying poly-(GMA-HEMA-EGDMA) microspheres. The reaction was carried out with 1.0% 1,6-diaminohexane solution at 65 °C, at pH 10 in a reactor containing 10 g microspheres and the mixture was stirred magnetically for 5 h. After the reaction, the spacer-arm-attached poly-(GMA-HEMA-EGDMA) microspheres were washed with distilled water.

The spacer-arm (i.e., 1,6-diaminohexane)-attached poly(GMA-HEMA-EGDMA) microspheres (5 g) were equilibrated in phosphate buffer (10 ml, 100 mM, pH 7.0) for 6.0 h, and transferred to the same fresh medium containing glutaric dialdehyde (20 ml, 0.5% (v/v)). The activation reaction was carried out at 25 °C for 12 h, while continuously stirring the medium. After the reaction period, the excess glutaric dialdehyde was removed by sequentially washing the microspheres with distilled water, acetic acid solution (100 mM, 100 ml) and phosphate buffer (100 mM, pH 7.0). The resulting modified poly(GMA-HEMA-EGDMA) microspheres were dried in a vacuum oven at 40 °C.

Immobilisation of lipase on the poly(GMA-HEMA-EGDMA) and spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres was carried out at 22 °C in a shaking water bath for 1.0–8.0 h. The microspheres were treated with enzyme solution (5.0 mg ml^{-1} lipase) in phosphate buffer (100 mM, pH 8.0). After a predetermined period, the enzyme-loaded microspheres was immediately transferred to ethylene diamine solution (5.0 mg ml^{-1} ethylene diamine) in same buffer solution to block the reactive groups on the microspheres. Physically bound enzyme was removed, first by washing the supports with saline solution (20 ml, 1.0 M) and then with phosphate buffer (100 mM, pH 7.0) and was stored at 4 °C in the same fresh buffer until used.

The amount of immobilised lipase on both microspheres was determined by measuring the initial and final concentrations of protein within the adsorption medium using Coomassie brilliant blue as described by Bradford(1976). A calibration curve, constructed with BSA solution of known concentration (0.05–0.50 mg ml^{-1}), was used for the calculation of protein in the enzyme and wash solutions.

2.4. Activity assays of free and immobilized lipase

The activities of free and immobilized lipase were determined by olive oil hydrolysis. A 100-ml olive oil emulsion was prepared by mixing olive oil (50 ml) and gum arabic solution (50 ml, 7% w/v). The assay mixture consisted of emulsion (5 ml), phosphate buffer (2.0 ml, 100 mM, pH 7.0) and free enzyme (0.5 ml, 1.0 mg ml^{-1}) or immobilized enzyme (0.5 g microspheres). Oil hydrolysis was carried out at 35 °C for 30 min in a shaking water-bath at 100 rpm. The reaction was stopped by the addition of 10 ml of acetone–ethanol solution (1:1 v/v). The liberated fatty acid in the medium was determined by titration with 25 mM NaOH solution. These activity assays were carried out over the pH range 4.0–9.0 and temperature range 20–60 °C to determine the pH and temperature profiles for the free and the immobilized enzymes. One lipase unit corresponded to release of one μmol fatty acid per min under the assay conditions. The specific activity is the number of lipase units per mg protein.

2.5. Thermal stability measurements of free and immobilised enzymes

Thermal stabilities of the free and immobilised lipase preparations were carried out by measuring the residual activity of the enzyme exposed to two different temperatures (50 and 60 °C) in phosphate buffer (100 mM, pH 7.0) for 120 min. A sample was removed after 15 min time intervals and assayed for enzymatic activity.

The results were converted to relative activities (percentage of the maximum activity obtained in that series). The residual activity was defined as the fraction of total activity recovered after covalent attachment on the poly(GMA-HEMA-EGDMA) microspheres compared with the same quantity of free enzyme.

2.6. Storage stability

This experiment was conducted to determine the stabilities of free and immobilized lipase preparations after storage in phosphate buffer (100 mM, pH 7.0) at 4 °C for 12 weeks. The residual activities were then determined as described above and activity of each preparation was expressed as a percentage of its residual activity compared to the initial activity.

2.7. Characterisation of poly(GMA-HEMA-EGDMA) microspheres

2.7.1. Determination of the water content

The spacer-arm attached poly(GMA-HEMA-EGDMA) microspheres were allowed to soak in distilled water for 24 h, swollen microspheres (~1 g) were weighed after removing the excess water and dried in vacuum oven at 60 °C for 24 h to constant weight.

2.7.2. Scanning electron microscopy

Scanning electron micrographs of the dried poly-(GMA-HEMA-EGDMA) microspheres were obtained using a JEOL, JMS 5600 scanning electron microscope, after coating with gold under reduced pressure.

2.7.3. Determination of the epoxy groups content

The available epoxy group content of the poly(GMA-HEMA-EGDMA) microspheres was determined by the pyridine-HCl method as described previously (Sidney, 1967).

2.7.4. Elemental analysis

The amount of covalently bound 1,6 diaminohexane on the microspheres was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen stoichiometry.

3. Results and discussion

3.1. Properties of poly(GMA-HEMA-EGDMA) microspheres

In the present study, porous epoxy group-containing poly(GMA-HEMA-EGDMA) microspheres were prepared from HEMA, GMA and EGDMA via suspension polymerisation in the presence of an initiator (AIBN). The amount of available epoxy groups was determined by titration of pyridine-HCl solution with 0.1 M NaOH and was found to be 0.8 mmol g^{-1} microspheres.

The spacer-arm was covalently attached through the epoxy groups of the poly(GMA-HEMA-EGDMA). Elemental analyses of the spacer-arm-attached poly-(GMA-HEMA-EGDMA) microspheres were performed, and the amounts of the incorporated amino groups were found to be 1.4 mmol g^{-1} from the nitrogen stoichiometry.

The water content is very important when use of the matrix, in enzyme immobilisation, is contemplated. The water content of the spacer-arm-attached poly-(GMA-HEMA-EGDMA) microspheres was determined to be 48%. It should be noted that this is a moderate swelling degree and suitable for use as column packing material for application of immobilized enzymes in a continuous flow system.

Scanning electron microscopy (SEM) micrographs, presented in Fig. 1, show the surface structures of poly-(GMA-HEMA-EGDMA) microspheres. The SEM micrograph shows that the microspheres have a porous surface structure (Fig. 1). The porous surface properties of the poly(GMA-HEMA-EGDMA) microspheres would favour a higher immobilization capacity for the enzyme, due to increase in the surface area.

3.2. Immobilisation of lipase onto poly(GMA-HEMA-EGDMA) microspheres

The aim was to determine an efficient relationship between enzyme and support by moving the enzyme away from the surface of microspheres, via incorporation of spacer-arms. Lipase was covalently immobilized on both poly(GMA-HEMA-EGDMA) and (after activation) spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres with glutaric dialdehyde. Lipase was immobilized on the poly(GMA-HEMA-EGDMA) microspheres via a coupling reaction between the free amino groups of enzyme and epoxy groups of the support. In the case of activated microspheres, a coupling reaction, between amine groups of the enzyme and aldehyde groups of the supports, could be considered via shift-base formation. Glutaric dialdehyde can readily react with amino groups; therefore, the aldehyde group content should be close to the amino group content on the microspheres. The SH groups of lipase can also react with glutaric dialdehyde after the amine groups have been used. It is important to note that $-NH_2$ groups are more liable than $-SH$ groups to react with glutaric dialdehyde.

The attachment of spacer-arm (i.e., 1,6-diaminohexane) to the poly(GMA-HEMA-EGDMA) microsphere surface could prevent undesirable side interactions between large enzyme molecules and support. In this way, more areas for the immobilized lipase could become accessible to lipid substrate. It should be noted

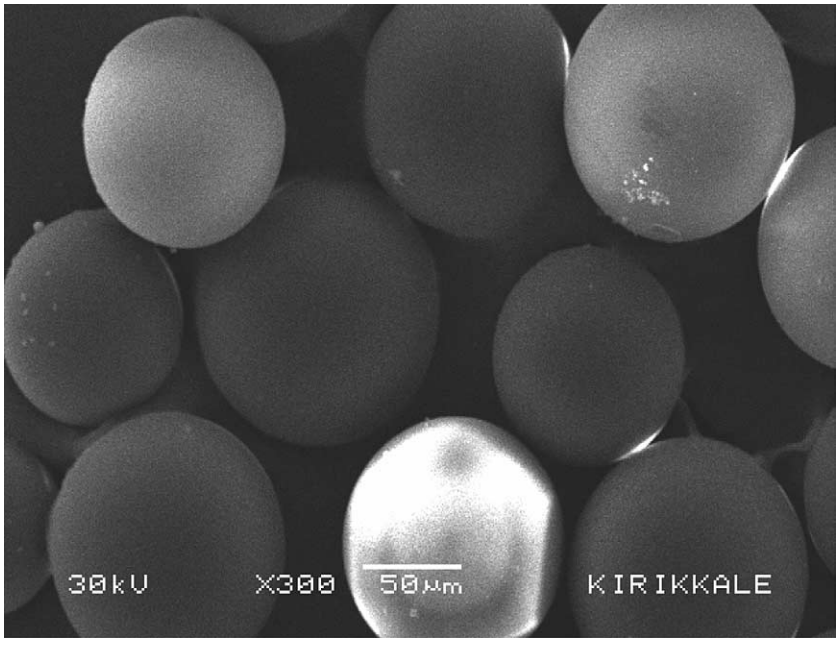

Fig. 1. SEM micrographs of poly(GMA-HEMA-EGDMA) microspheres.

that the coupling agent, glutaric dialdehyde, has a 5-carbon length and it should act as an extra spacer-arm in this immobilization method. The recovered activity yield of the lipase immobilised on the spacer-arm-attached microspheres was up to 45%, and it was 9% for the enzyme immobilized through epoxy groups. The attachment of the spacer-arm resulted an increase in the apparent activity of the immobilised lipase with respect to the enzyme immobilised via the epoxy groups of the microspheres. The maximum lipase immobilization capacities of the poly(GMA-HEMA-EGDMA) and poly(GMA-HEMA-EGDMA)-spacer-arm-attached microspheres were 16.1 and 28.3 mg g^{-1}, respectively. Without spacer-arm, the lipase, in attempting to maximize its contact with the surface, could lose its active conformation, and consequently, low activity (1420 U g^{-1}) results. Therefore, the rest of the immobilization study was carried out using the spacer-arm-attached microspheres. The measured specific activity of the free lipase was 980 U mg $protein^{-1}$.

The effects of enzyme coupling time on the immobilization capacity and on the enzyme activity were studied with spacer-arm attached microspheres and are presented in Fig. 2. An increase in the coupling duration time led to an increase in the immobilization efficiency (from 12.5 to 27.9 mg g^{-1} microspheres) but this relationship levelled off at around 6.0 h. Thus, a maximum enzyme loading of 28.3 mg g^{-1} microspheres was observed after 8.0 h. Further increase in the coupling duration time (up to 8.0 h) did not lead to a significant change in the immobilization capacity. On the other hand, the relation between lipase loading and resultant lipase activity was linear up to 19.8 mg g^{-1} microspheres, then a plateau was observed (Fig. 2). This observation can be explained either by overcrowding of the immobilized lipase in the pore space of the matrix, as a result of which relatively large substrate (olive oil) diffusion limitations occur, or by protein–protein interactions becoming more important and these hinder the substrate conversions. In view of these observations,

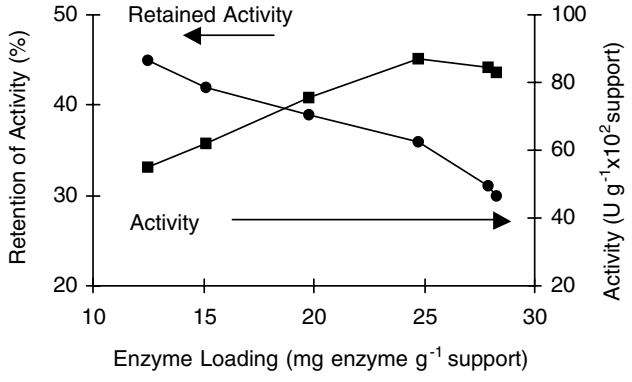

Fig. 2. Effect of enzyme loading on the recovered immobilised enzyme activity.

19.8 mg protein g^{-1} microspheres was used in the rest of the experiments and this was obtained after a 4-h coupling reaction.

Several publications have appeared describing the investigation of new supports for lipase immobilisation via adsorption. For example, the adsorption of *Candida rugosa* lipase on poly(styrene-divinylbenzene) supports was studied and the hydrolytic activity of the immobilised enzyme was between 60 and 500 U g^{-1} support (de Oliveira, Alves, & de Castro, 2000) The same source of lipase was immobilised on chitin particles and the activity yield of the covalently immobilised lipase was 250 U g^{-1} support (Gomes et al., 2004). The adsorption of modified *Candida rugosa* lipase on organic polymer microspheres was studied and the highest immobilised enzyme activity was obtained (2.5 U g^{-1} support) when lipase was adsorbed on to XAD7 support (Basri, Ampon, Yunus, Md. Razak, & Salleh, 1994). The lipase from *Candida cylindracea* was immobilised onto poly (Vac-DVB) magnetic particles and the activity was about 6750 U g^{-1} supports (Guo, Bai, & Sun, 2003). The covalent immobilization of *Candida rugosa* lipase on chitosan beads resulted in an activity of about 19.3 U g^{-1} (Chiou & Wu, 2004). In another study, *Candida rugosa* lipase was immobilised on to chitosan beads by activating its hydroxyl groups with carbodiimide, followed by cross-linking lipase to the amino groups with glutaraldehyde, and this immobilisation method yielded a retained activity of about 74% (Hung, Giridhar, Chiou, & Wu, 2004). Candida rugosa lipase was immobilised onto zirconium phosphate and phosphonate and the recovered activity of the immobilised enzyme was between 32% and 41% (Bellezza et al., 2003). All lipase immobilisation studies reported have been performed under different conditions. Therefore, it is almost impossible to compare the immobilisation results. However, the highest immobilised enzyme activity obtained (7570 U g^{-1}), with the presented novel immobilisation technique, appears to be quite promising for various industrial applications.

3.3. Biochemical properties of free and immobilised lipase

A comparative study between free and immobilised lipase is provided in terms of pH, temperature, and thermal stability. The effect of pH on the activity of free and immobilized lipase on the spacer-arm attached microspheres in olive oil hydrolysis was determined in the pH range 4.0–9.0 and the results are presented in Fig. 3. The optimum pH of the free enzyme was 7.0. The optimum pH value of the immobilized lipase was shifted by 1.0 unit to the alkaline region. This observed displacement toward to the neutral region for the immobilized lipase is because pH conditions in the pore space of the polymeric matrix are different from those in the rest of the solution. In other similar report, the

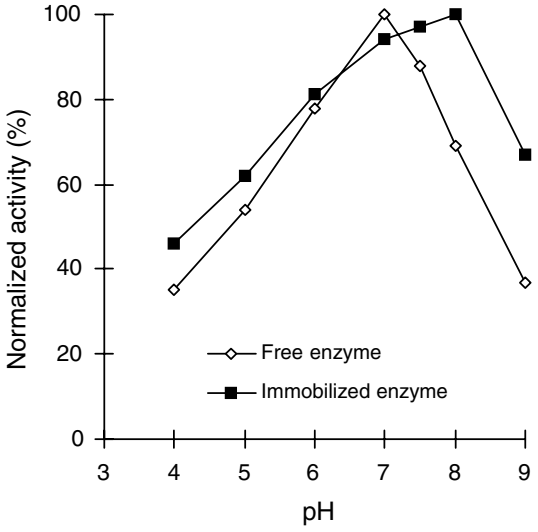

Fig. 3. Effect of pH on the activity of free and lipase immobilised on the spacer-arm attached poly(GMA-HEMA-EGDMA) microspheres.

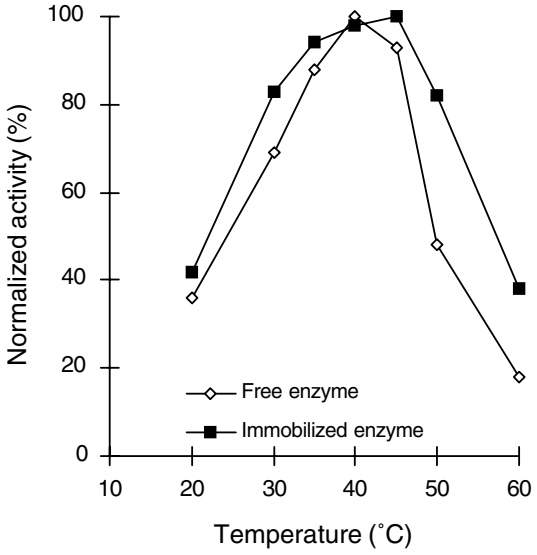

Fig. 4. Effect of temperature on the activities of free lipase and lipase immobilised on the spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres.

optimum pH value of the immobilized lipase was generally slightly shifted toward the alkaline region (Arıca, 2000, 2004a; Bayramoğlu et al., 2004). 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. These results could probably be attributed to the stabilisation of lipase molecules, resulting from multipoint attachment of the enzyme molecules on the surface of the poly(GMA-HEMA-EGDMA) microspheres.

The activities obtained in a temperature range of 20–60 °C were expressed as percentage of the maximum activity (Fig. 4). The apparent temperature optimum for free enzyme was about 40 °C while that for the immobilized enzyme was about 45 °C (Fig. 4). The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation, via amino groups of the immobilised lipase, might also reduce the conformational flexibility and result in a higher activation energy for the molecule to reorganize the proper conformation for binding to substrate. One of the main reasons for enzyme immobilisation is the anticipated increase in stability toward various deactivating forces, due to restricted conformational mobility of the molecules following immobilisation (Arıca, 2000, 2004a; Phadtare, D'Britto, Pundle, Prabhune, & Sastry, 2004).

3.4. Thermal stability

Thermal stability experiments were carried out with free and immobilized enzymes, which were incubated in the absence of substrate at various temperatures

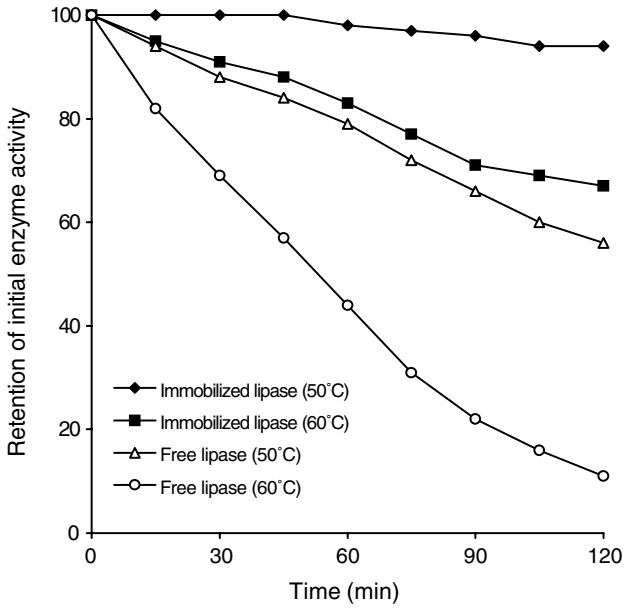

Fig. 5. Thermal stabilities of free lipase and lipase immobilised on the spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres.

(Fig. 5). At 50 °C, the free and immobilised lipases retained their activities at levels of 56% and 94% during a 120 min incubation period. At 60 °C, the free and the immobilised enzymes retained their activities about levels of 11% and 67%, respectively. The immobilised lipase was inactivated at a much slower rate than that of the native form. The increase in the thermal stability of the immobilised lipase may arise from the conformational integrity of the immobilised enzyme structure after covalent binding to the supports through the

amino and/or cysteine residues in the enzyme (Arıca, 2004b; Phadtare, Vinod, Wadgaonkar, Rao, & Sastry, 2004; Phadtare et al., 2004). These results suggest that thermostability of immobilised lipase increased considerably because of multi-point covalent immobilisation of enzyme onto spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres.

3.5. Storage stability

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The free and the immobilised lipase on the spacer-arm-attached microspheres were stored in phosphate buffer solution (50 mM, pH 7.0) at 4 °C for a predetermined period. Under the same storage conditions, the activity of the immobilised lipase decreased more slowly than that of the free lipase. The free enzyme lost all its activity within a 4-week period. The immobilised lipase preserved about 67% of its initial activity during a 12-week storage period (Fig. 6). Thus, the immobilised lipase exhibits a higher stability than does the free counterpart. The higher stability of the immobilised lipase could be attributed to the prevention of autodigestion and thermal denaturation as a result of covalent attachment of the lipase on the surface of spacer-arm-attached poly-(GMA-HEMA-EGDMA) microspheres. In our previous studies, we observed that of hydrogel carrier provides a protective microenvironment for enzymes and yields higher stabilities (Arıca, 2000, 2004b). On the basis of this observation, a modified hydrophilic support should provide a stabilisation effect, minimising possible distortion effects imposed from the aqueous medium on the active site of the immobilized enzyme. The generated covalent bonds between enzyme and support should also convey a higher conformational stability to the immobilized enzyme. Thus, the modified hydrophilic support and the immobilization method provide a longer shelf life than that of the free counterpart.

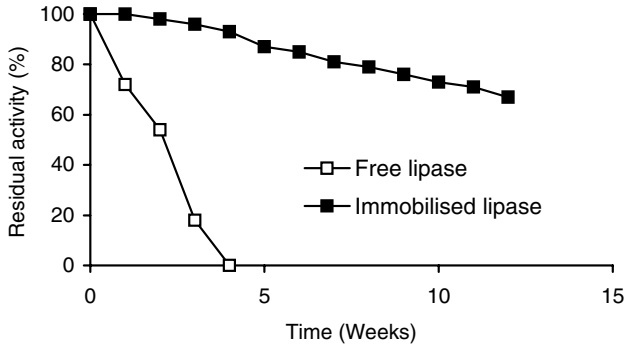

Fig. 6. Storage stabilities of free lipase and lipase immobilised on the spacer-arm-attached poly(GMA-HEMA-EGDMA) microspheres.

4. Conclusion

One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme structure, the immobilization methods, and type of support. In this study, immobilisation of *Candida rugosa* lipase on the poly(GMA-HEMA-EGDMA) microspheres was studied by two different coupling methods. In the first method, the enzyme was directly coupled to the support via epoxy groups. In the second method, the spacer-arm (i.e., 1,6-diaminohexane) was attached through the epoxy groups of the support and the enzyme, lipase, was then covalently immobilised after activation of amino groups of the spacer-arm with glutaric dialdehyde. The attachment of the spacer-arm resulted in an increase in the apparent activity of the immobilised lipase with respect to the enzyme immobilised via the epoxy groups. Lipase, immobilised on the spacer-arm-attached microspheres, retained much activity over wider ranges of temperature and pH than those of the free form. A high operational stability, obtained with the immobilised lipase, indicates that the immobilised lipase could successfully be used in a continuous system for the hydrolysis of lipids. In addition, the poly-(GMA-HEMA-EGDMA) microspheres proposed in this work showed promising potential for applications to enzyme immobilization. Finally, a very promising result of this work was the observation that the activity of the immobilised enzyme becomes less sensitive to reaction conditions than that of the free counterpart.

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