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ORIGINAL PAPER

Efficient immobilization of lipase from *Candida rugosa* by entrapment into poly(*N*-isopropylacrylamide-co-itaconic acid) hydrogels under mild conditions

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Abstract Temperature and pH-sensitive hydrogels, based on *N*-isopropylacrylamide and itaconic acid, with varying comonomer ratios and crosslinking agent content, were prepared by free radical crosslinking copolymerization. The immobilization of lipase from *Candida rugosa* was carried out by post-loading entrapment method at different temperatures until equilibrium swelling was achieved. The effects of the hydrogel composition and the immobilization temperature on the hydrogel-binding capacity, immobilized lipase specific activity, as well as the enzyme leakage were studied. It was found that the NiPAAm/IA ratio, crosslinking agent concentration and the temperature at which the entrapment was performed significantly affected the hydrogel-binding capacity. The biocatalysts obtained by entrapment into hydrogel with the highest itaconic acid content at 5 °C exhibited both highest binding capacity and the highest specific activity, but appeared to be less suitable for repeated uses than those obtained at 25 °C.

Keywords Lipase from *Candida rugosa* · Immobilization temperature · Hydrogel · *N*-isopropylacrylamide · Itaconic acid

Introduction

The potential application of lipases as catalysts in industrial organic synthesis has got great attention due to several advantages of these biocatalysts over inorganic,

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metal-derived, or chemical ones. Lipases catalyze many reactions selectively under mild conditions, giving the colorless product of good quality and in high yield percentage. The use of enzymes generally decreases the side reactions and simplifies post-reaction separation problems. Moreover, lipases catalyze reactions resemble closely the pathways designed by nature for the metabolism of living organisms. Thus, the reaction mechanisms and processes using lipases as catalysts are more environmental friendly. Also, products with high purity and high added value are observed aid by the substrate specificity and stereospecific properties of lipases [1].

Until the advent of immobilized enzymes, catalytic reactions were usually performed by adding the enzyme to a solution of the reactants, causing numerous problems such as their one-time application, enzyme sensitivity to changes in temperature and pH, and the contamination of the enzyme after completion of the reaction, removing the enzyme from the reaction mixture [2]. Enzyme immobilization offers economical lipase industrial application enabling enzyme reuse and facilitation of the continuous processes [2, 3].

The immobilization process, which offers great economical advantages, can be achieved by methods of various efficiency [4] and using different supports [5, 6]. Among materials frequently used as supports for lipase immobilization are many different crosslinked natural and synthetic hydrogels [4, 7, 8]. Mild conditions of enzyme immobilization within hydrogels provide its protection from harmful environmental influences. For successful immobilization, the hydrogel must have the mesh size large enough to allow molecules of the substrate and of the reaction product to diffuse in and out and to keep the enzyme entrapped in the hydrogel support. The network parameters and hydrogel morphological and mechanical properties can be easily controlled by appropriate selection of reaction mixture composition and reaction conditions [9–11].

In our previous works, the design and synthesis of temperature- and pH-sensitive hydrogels based on *N*-isopropylacrylamide (NiPAAm) and itaconic acid (IA) and their potential to be applied as supports for immobilization of lipase from *Candida rugosa* (CRL) was investigated and reported [12, 13]. The fact that IA is obtained from non-petrochemical resources [14–16], as well as hydrogel temperature and pH-sensitive behavior, offer their advantageous application. The effectiveness of the immobilized CRL within hydrogels in the olive oil hydrolysis has been demonstrated. However, the hydrogels were obtained by free radical crosslinking copolymerization in the presence of lipase (in situ immobilization) which could cause the enzyme denaturation during chemical radical procedures resulting in lower activities of the immobilized enzyme. To achieve higher activities of the resulting immobilized CRL, an attempt has been made in this article to immobilize CRL into the previously synthesized hydrogels (ready-made support) by so-called post-loading entrapment. This immobilization principle appears very gently, because it is based only on molecular diffusion and steric hindrance and not on direct entrapment during synthesis of the hydrogels [17]. This would minimize deactivation of the enzymes during immobilization process and, in fact, more active and stable biocatalysts could be obtained. Although this method has shown to be a relatively easy, rapid, and safe technique in the case of immobilization of glycerol

dehydratase and aminoacetyl-tRNA synthetase in polyacrylamide or Sephadex leading to the production of highly stable and active biocatalyst, in the last decades there have been few examples based on this technique [18, 19].

However, for this purposes, hydrogels structure and entrapment conditions should be carefully designed and optimized. The mesh width must be narrow enough to prevent leakage of the lipase molecules out of the polymer network, but large enough to allow free diffusion of enzyme during immobilization as well as substrates and products. In addition, the content of IA should be optimized to provide good biocatalytic properties of the biocatalyst and its additional stabilization as a result of enzyme-support ionic interactions.

This article reports on pH and temperature sensitive synthesis of P(NiPAAm/IA) copolymer hydrogels designed for enzyme immobilization. The properties of these polymeric supports were controlled by changing concentration of the anionic component and of the crosslinking agent. The immobilization of CRL was performed by soaking the dried gels in lipase solution at different temperatures (5, 25, and 37 °C) to the equilibrium swelling. The influence of hydrogel composition and the immobilization conditions on the mass of immobilized enzyme and its lipolytic activity were studied.

Experimental

Materials

The reactants used in the study, IA and NiPAAm, were obtained from Acros Organics (Belgium). The crosslinking agent *N*,*N*′-methylenebisacrylamide (MBA) was obtained from Serva Feinbiochemica (Germany). The initiator and the accelerator, potassium persulphate (PPS) and potassium pyrosulphate (PPyroS), were obtained from Merck & Co Inc (Germany) and Acros, respectively. CRL (with nominal specific lipolytic activity of 1468 IU mg^{-1} solid) was obtained from Sigma-Aldrich Chemie GmbH (Germany). NiPAAm was recrystallized from benzene/*n*-hexane mixture (35/75) before use. Other materials were used as received, without purification. For all copolymerizations as well as the preparation of the buffer solutions, distilled water was used.

Hydrogel synthesis

Synthesis of hydrogels using water as a solvent, at 25 °C, in a nitrogen atmosphere was performed by the free radical copolymerization. The NiPAAm/IA ratio and the crosslinking agent concentration were varied in the samples. The concentrations of initiator and accelerator for all the samples were 1 wt%, with respect to the initial monomer content. The duration of hydrogel synthesis was 48 h for all the samples. After the polymerization, hydrogels were cut into discs and kept in distilled water which was replaced daily, during 7 days, to remove unreacted substances. The discs were left to dry to constant weight in vacuum, at room temperature. The determination of unreacted substances showed that the conversion was practically

complete. The samples were labeled as follows: the first number indicates the wt% of NiPAAm, the second corresponds to the wt% of IA, and the third is related to the wt% of the crosslinking agent, MBA.

Lipase immobilization and hydrogel-binding capacity

This preliminary study included the investigation of the effect of immobilization temperature on the immobilized lipase activity. The fresh lipase solution, with concentration of 1 mg of lipase per 1 mL of phosphate buffer of pH = 6.00 ± 0.01, was made before the immobilization process. This pH was higher than both pK_a values of the IA which ensured the maximum swelling of hydrogels [20] and was adequate to sustain lipase activity. The immobilization of lipase was performed by immersing xerogels into lipase solution and allowing gels to swell to the equilibrium to ensure the maximum amount of lipase adsorption. The immobilization was performed at different temperatures: 5, 25, and 37 °C. After reaching the equilibrium, the samples were withdrawn and left to dry in vacuum to constant weight at 25 °C, followed by washing out thoroughly with plenty of water until no free lipase was detected in the washing solution. The residual lipase solution was used to determine the content of the immobilized lipase, as the difference of lipase concentration in the initial and the residual solution, together with the lipase concentration of the sample washing steps. The amount of CRL immobilized into hydrogels was determined by measuring the enzyme concentration in the washing solution, using BCA protein assay [21], and the binding capacity was calculated using following equation:

$$C_B = (W_l - C \cdot V)/W_s \quad (1)$$

where C_B represented the hydrogel-binding capacity, W_l is the amount of lipase introduced into the system by hydrogel swelling to the equilibrium, C and V the lipase concentration and total volume of the washing solution, respectively, and W_s the weight of the hydrogel.

Swelling study

The swelling behavior of hydrogels immersed in water at 5, 25, and 37 °C was monitored gravimetrically during 3 days (2 days the measurements were performed for the first 8 h and third day for the first 5 h), and the degree of swelling was calculated using the following equation [22]:

$$q = \frac{W_t}{W_0} \quad (2)$$

where W_0 is the weight of the xerogel, and W_t the mass of the hydrogel swollen to equilibrium at time t. The hydrogel mesh size was calculated using the Eq. (3), which was derived by applying the swelling equilibrium theory [22]:

$$\xi = v_{2m}^{-1/3} \cdot \left(\frac{2C_n\bar{M}_c}{M_r}\right)^{1/2} \cdot l \quad (3)$$

where v_{2m} represents the volume fraction of polymer in the swollen state, M_r is the molar mass of the basic units of which the polymer chain is composed, $\bar{M}_c$ is the average molar mass of polymer chains between two crosslinking points, C_n is the Flory characteristic ratio [11], and l is the length of C–C bond (1.54×10^{-10} m for vinyl polymers) [13, 23].

Fourier transform infrared spectroscopy

Powdered xerogel/KBr pastilles were submitted to FT-IR analysis, and the spectra were obtained using a Bomem MB 100 FT-IR Spectrophotometer. The proper ratio (Sample:KBr = 1:50) was mixed and grounded and then compressed into a pellet under a pressure of 11 t, for about a minute, using Graseby Specac Model: 15.011. Spectra were obtained in the 4000–400 cm^{-1} wave number range, at 25 °C and at 4 cm^{-1} spectral resolution.

Scanning electron microscopy

Surface morphologies of the P(NiPAAm/IA) hydrogels were observed using JEOL JSM-5800 Scanning Electron Microscope. The swollen hydrogels, after being lyophylized, were frozen by plunging them into liquid nitrogen, and then broken. At the end, the samples were coated with platinum under vacuum using Polaron SC502 sputter coater.

Infinite focus microscopy (IFM)

For light microscopical evaluation, images of hydrogel surfaces were acquired using IFM in true color, reflected light mode (InifiniteFocus Alicona, Graz, Austria). For surface quantification, datasets within the metric sizes of 515 × 415 μm (×20) were analyzed. The samples were swollen to equilibrium in buffer solution of pH 7.0, at room temperature.

Enzyme leakage and immobilized lipase activity assays

Enzyme leakage measurement was carried out by placing hydrogel discs in a test tube filled with water at the temperature of 5, 25, and 37 °C for 96 h. The leakage percentage was calculated from the differences between the initial amount of lipase immobilized into hydrogels (amount at the beginning of time interval) and the amount of lipase released from the samples divided to the initial amount. For the measurement of released protein, 2 mL of sample were taken at specific time intervals, and returned back to the beaker after the spectrophotometric analysis, to maintain the same conditions throughout the experiment and by keeping both, sterile and sink conditions. The samples were analyzed at 225 nm, using Ultrospec

3300 pro UV/Visible Spectrophotometer, Biochrom Ltd. and bovine serum albumin as a standard. Each result, expressed as normalized average cumulative solution protein content values and the standard deviation, was a result of three independent measurements. The normalized average cumulative solution protein content values, calculated using Microsoft Excel (Redmond, WA, USA) software with ±5 % accuracy, were plotted as a function of time.

Immobilized enzyme activity

To determine the lipolytic activity of lipase immobilized into hydrogels, a standard Sigma lipase activity method, previously described [24, 25], was applied. Test tubes with 3.0 mL of Sigma substrate (Sigma Chemical Co., St. Louis, MO), 1.0 mL of 0.05 M tris–HCl buffer solution of pH 7.77 and 2.5 mL of distilled water were used. The reaction mixture was stirred and incubated for 20 min at 37 °C. Adequate masses of the samples crushed into powder were then added to the test tubes, stirred and again returned at 37 °C for the next 3 h. A blind probe was performed too. In order to terminate the enzymatic reaction, 3.0 mL of a methanol/phenolphthalein mixture were added to the reaction mixture and the fatty acids formation was quantified by titration with 0.1 M NaOH solution to the color change. The activities of the free or immobilized lipase were expressed in International Units (IU), where IU is defined as the amount of enzyme required to release 1 μmol of free fatty acid per minute under the specified conditions (pH = 7.77 and 37 °C). The efficiency of the immobilization was evaluated in terms of specific activity (SA) of the biocatalyst as follows:

$$\mathrm{SA}\left(\mathrm{IU}\times\mathrm{mg}^{-1}\right)=\left(\text{Activity of the immobilized lipase, IU}\right)/\left(\text{Amount of protein entrapped, mg}^{-1}\right). \quad (4)$$

Results and discussion

The effect of hydrogel composition on the swelling kinetics

Before immobilization, some properties of the synthetized hydrogels have been investigated and evaluated. The swelling studies of the synthetized hydrogels were performed at 5, 25, and 37 °C until the attainment of the equilibrium using distilled water as a medium. Figure 1 shows the effect of IA content, crosslinking agent content, and temperature on swelling kinetics of the investigated hydrogels. The *y*-axes are scaled differently according to the obtained hydrogels degrees of swelling to present results more transparently. The experiments were conducted during the period of 3 consecutive days (first 2 days measurements of the degree of swelling were performed during first 8 h in a queue, while the same measurements on the third day were performed during 5 h in a queue). This explains the gap between the groups of the data on Fig. 1 obtained for the samples degrees of swelling.

It is evident that the degree of swelling of P(NiPAAm/IA) hydrogels depends very much on temperature, decreasing drastically with increasing temperature, especially at

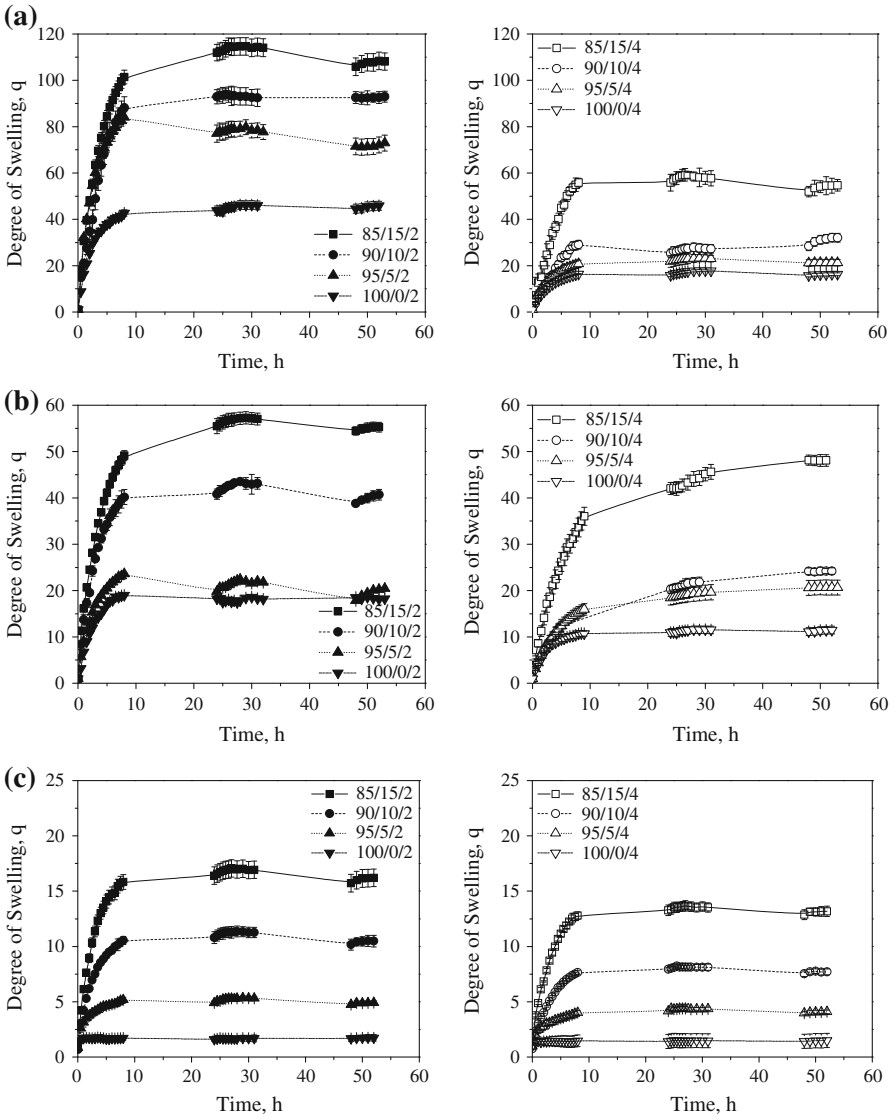

Fig. 1 The degree of swelling versus time dependences for the hydrogels with different IAs and crosslinking agent contents (*left*, 2.0 wt% of MBA; *right*, 4.0 wt% of MBA) at temperatures of **a** 5 °C, **b** 25 °C, and **c** 37 °C (The *y*-axes were given with a different scale to present results more transparently; first two numbers in the legend labels indicate the wt% of NiPAAm and IA, respectively, and the third is related to the wt% of MBA.)

37 °C, which is above the LCST value for PNiPAAm. The PNiPAAm LCST value is around 32 °C, but P(NiPAAm/IA) copolymers have higher LCST values, depending on IA content [12]. The degree of swelling of hydrogels increased with IA content and decreased with MBA content, which was expected. The dependence of the degree of

swelling on MBA content was less expressed in the case of samples with the same IA content at 37 °C (Fig. 1c). Comparing the results for the degrees of swelling, it can be seen that the lowest degree of swelling has PNiPAAm homopolymer, labeled as 100/0/4, at all temperatures. The addition of small amounts of IA (5, 10, and 15 wt%) during the synthesis of P(NiPAAm/IA) hydrogels increased the network hydrophilicity, which along with the electrostatic repulsion of COO^- groups, increased the degrees of swelling. The increase of the crosslinking agent concentration, in general, produced the increase in crosslinking network density and thereby reduced the mobility of polymer chains and the elasticity of the network.

FT-IR analysis

The FT-IR spectra of homo- and copolymer hydrogels of different compositions and degree of crosslinking were recorded, and were shown in Fig. 2.

FT-IR spectra of hydrogels are similar. Each spectrum shows a broad band in the wavenumber range between 3700 and 3100 cm^{-1} corresponding to O–H stretching vibrations of carboxylic groups of IA and N–H stretching vibration NiPAAm. Peak at 1720 cm^{-1} denotes to the typical vibrations of carbonyl groups of IA [26]. The characteristic amide I and amide II bands of NiPAAm occur at 1650 and 1540 cm^{-1}, respectively. Two typical C–H vibration bands, of almost the same intensity, at 1386 and 1379 cm^{-1} belong to the divided bands of symmetric $CH(CH_3)_2$ group. A band at 1174 cm^{-1} represents a C–C stretching of $CH(CH_3)_2$ group [27].

SEM and IFM analyses

Figure 3 shows the morphology of P(NiPAAm/IA) copolymeric hydrogels before CRL immobilization. The effect of the crosslinking agent is evident, appearing as the difference in pore size with the change of the crosslinking agent concentration, in samples of the same NiPAAm/IA ratio.

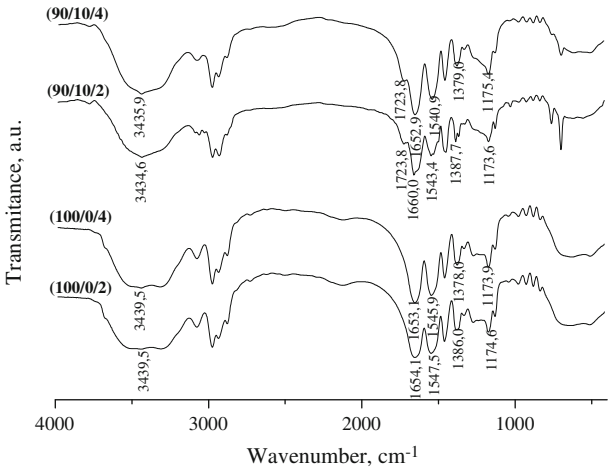

Fig. 2 FT-IR spectra of the hydrogels before CRL immobilization

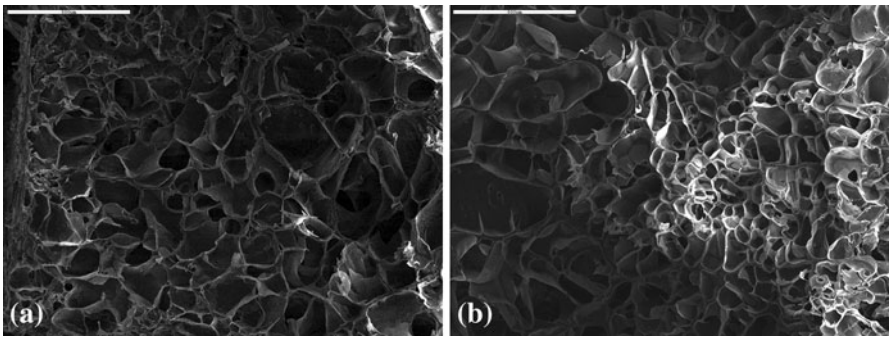

Fig. 3 SEM micrographs of hydrogels: **a** 90/10/2/0 (*bar* 500 μm, ×70) and **b** 90/10/4/0 (*bar* 500 μm, ×70) swollen to equilibrium at 25 °C in distilled water

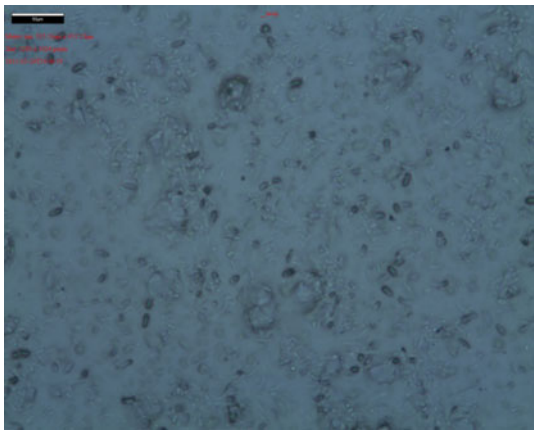

Fig. 4 The light microscopical evaluation image of the hydrogel 90/10/2

Larger pores were observed in samples with lower degree of crosslinking (2.0 wt%) which is in correlation with the results of swelling and with data from literature [28]. Using Infinite Focus Microscope, light microscopical evaluation showed rough surface structure for the hydrogel 90/10/2 (image taken before lipase immobilization), allowing generation of 3D topographical image for analysis and accurate measurement. The light microscopical image of the hydrogel was shown in Fig. 4.

The mesh size of the hydrogels at different temperatures is presented in Table 1. According to the calculated values for the pore size, the investigated hydrogels were classified as microporous, having pore size range from 0.01 to 0.1 μm [29] (Table 2).

The hydrogel-binding capacity and the immobilized lipase activity

Table 1 shows the data of the binding capacity of the hydrogels at different temperatures. As it can be seen from the presented results, the mesh sizes of the

Table 1 The mesh size (ξ) and the binding capacity (C_B) of the investigated hydrogels at different temperatures (The standard deviation values were <±3 % of the mean values in all cases)

Sample	$t = 5$ °C		$t = 25$ °C		$t = 37$ °C	
	ξ, nm	C_B, $mg_{enz}/g_{hydrogel}$	ξ, nm	C_B, $mg_{enz}/g_{hydrogel}$	ξ, nm	C_B, $mg_{enz}/g_{hydrogel}$
85/15/4	50.2	408.8	36.9	334.5	1.56	115.6
90/10/4	20.6	250.0	14.3	223.1	1.22	73.12
95/5/4	10.2	234.7	9.94	212.0	0.46	18.94
100/0/4	9.10	217.1	4.62	178.0	0.18	8.708
85/15/2	118.0	469.3	46.3	418.6	1.44	126.8
90/10/2	92.7	418.8	40.8	352.5	0.80	87.10
95/5/2	42.6	312.5	10.0	249.2	0.54	29.82
100/0/2	33.0	209.7	7.84	201.6	0.22	16.14

Table 2 The values of release rate constants (k), release exponents (n), and $t_{1/2}$ values for the P(NiPAAm/IA) hydrogels immersed in distilled water at 25 °C, with lipase immobilized at 5 °C

Parameter	Sample							
	85/15/2	90/10/2	95/5/2	100/0/2	85/15/4	90/10/4	95/5/4	100/0/4
k, h^{-1}	0.15	0.16	0.16	0.11	0.21	0.08	0.2	0.08
n	0.58	0.66	0.44	0.53	0.44	0.80	0.48	0.84
$t_{1/2}$, h	7.65	5.26	14.2	11.4	7.15	7.73	7.21	6.45

investigated hydrogels were adequate to enable the immobilization of CRL within the gel. The binding capacity of the immobilized enzyme was determined by using BCA protein assay, as previously described. The binding capacity was estimated to be in the range between 8.7 mg_{enz}/g hydrogel (sample 100/0/4, at 37 °C) and 469.3 mg_{enz}/g hydrogel (sample 85/15/2, at 5 °C), highly depending on hydrogel structure and temperature.

The results of immobilized lipase mass and SA of the immobilized lipase are also presented in Fig. 5a, b. The SA of free lipase is given as well, for the reason of comparison. It is evident that the mass of immobilized lipase (Fig. 5a) depends on the mesh size of the hydrogels and of the temperature at which lipase was loaded. The amount of immobilized lipase varied in the same manner as the mesh size values. It was higher for the samples with lower MBA and higher IA content and decreased with increasing temperature, as expected. Hydrogels 100/0/2 and 100/0/4, with the lowest mesh sizes, showed the lowest capacity for lipase immobilization. In these hydrogels, lipase was probably attached mainly to the surface of the hydrogel disc, due to steric hindrances for lipase diffusion inside the gel.

It can be seen that higher specific activities of immobilized lipase were achieved for the immobilization at lower temperatures. It is obvious that the immobilization at 5 °C allows lipase to preserve the highest percent of native lipase activity, while by raising the immobilization temperature the lipase activity percentage decreases. When the immobilization was performed at the temperature above the PNiPAAm

LCST value (37 °C), lipase retains very little of its initial activity (Fig. 5b). Hydrogels with higher IA content have greater mesh size which facilitates diffusion, providing higher immobilized enzyme activity. The same interpretation can be applied to the MBA content in hydrogels: if the hydrogels of the same acid content and different MBA contents are compared (i.e., 90/10/2 and 90/10/4) it can be concluded that an increase in crosslinking agent content leads to the decrease of the lipase SA.

The immobilized lipase activity yield was in the range between 31.0 and 86.5 %, which was comparable or much higher than the results achieved by other authors and by applying some different immobilization methods [30–32]. The highest amount of immobilized lipase and SA were achieved for hydrogels 85/15/2, 90/10/2, and 90/10/4. This led to conclusion that these were optimal compositions of P(NiPAAm/IA) hydrogels for lipase immobilization at 5 °C. The further examination was performed using these samples.

The lipase leakage study

A major shortcoming of all enzymes entrapped into hydrogels systems, such as lipase immobilized into P(NiPAAm/IA) hydrogels, is their low operational stability, resulting from facile enzymes leakage from the hydrogels. In contrast to the chemical methods, the physical forces involved in enzyme entrapment are too weak to keep enzyme stably retained within the hydrogel network. Subsequently, some lipase leakage is unavoidable. The possibility to investigate the potential use of immobilized lipase for biotechnological applications, in which room temperature is commonly used, was the reason of performing the release study at 25 °C [33]. The hydrogels with lipase loaded at 5, 25, and 37 °C were stored in dry state before the release experiments. These experiments were conducted to confirm that a relatively small quantity of immobilized lipase was released from the immobilized biocatalyst. After immersion of xerogels in water at 25 °C, the release of protein occurred (Fig. 6), but very little of immobilized lipase was released. It was also evident that

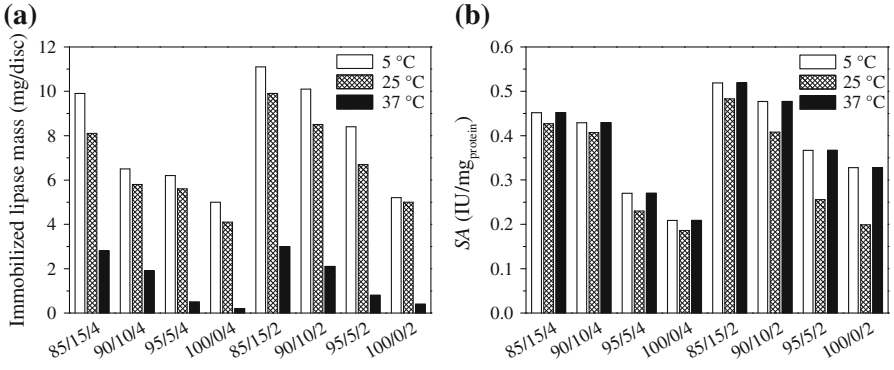

Fig. 5 The immobilized lipase mass (**a**) and the SA (**b**) at different temperatures. The SA of free lipase is 0.600 IU/mg_{enz} determined by standard Sigma lipase activity method

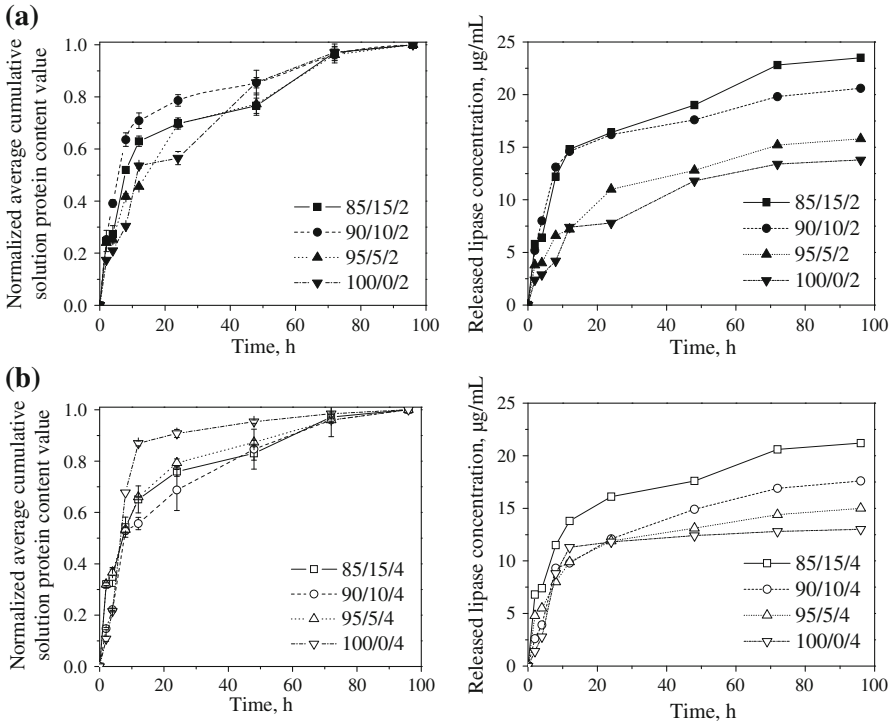

Fig. 6 Normalized average cumulative solution lipase content values versus time (*left* **a** and **b**) and concentration of released lipase (*right* **a** and **b**) at 25 °C (The samples with lipase were obtained by entrapment at 5 °C)

there was initial burst release of lipase from P(NiPAAm/IA) hydrogels. Korsmeyer et al. derived a simple relationship which described bioactive compounds release from a polymeric system. To find out the mechanism of enzyme release, first 60 % enzyme release data was fitted in Korsmeyer–Peppas model using following equation [34, 35]:

$$M_t/M_\infty = k \cdot t^n \tag{5}$$

where M_t/M_∞ is fraction of bioactive compound released at time t, k is the rate constant, and n is the release exponent indicative of mechanism of the compound release.

The Korsmeyer–Peppas release profile exponent (n) ranged between 0.435 and 0.840 showed a combination of diffusional and dissolutional mechanisms (anomalous transport), which indicated that the bioactive compound release from the hydrogel was controlled by more than one process. In this case of swelling polymers, diffusivities of loaded molecules were strongly affected by the degree of swelling and crosslinking agent content within the hydrogel. The rate constant values did not vary much for different samples, and the half time of the lipase release values ($t_{1/2}$) at 25 °C were in the range between 5.3 and 11.4 h (Fig. 6right a, b). It is also important to note

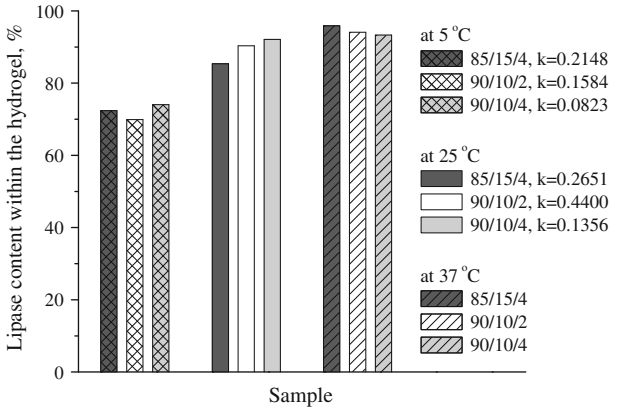

Fig. 7 The remained immobilized lipase content at 25 °C in hydrogels, in which lipase was immobilized at different temperatures (5 °C, 25 °C, and 37 °C) after 96 h (this values remain constant after 24 h)

that all samples exhibited very rapid release kinetics, but the quantity of released protein was small. This offered the possibility for lipase immobilized on these P(NiPAAm/IA) hydrogels to be employed as a biocatalyst.

For the practical application of biocatalysts, the lipase content that remains within hydrogel after the release experiments is of a great importance. Samples 90/10/2, 90/10/4, and 85/15/4, with lipase being immobilized at 5, 25, and 37 °C, were chosen for the release experiments to show the effect of hydrogel composition and temperature at which lipase was immobilized on the release kinetics. Figure 7a–c represents the amount of lipase (immobilized at three different temperatures) which remains immobilized in samples of P(NiPAAm/IA) versus time for the release study in water at 25 °C.

The highest amount of immobilized lipase remains when the enzyme is immobilized at 37 °C (Fig. 7c). But bearing in mind the results presented in Fig. 5, where much higher hydrogel binding capacity and SA for chosen samples were obtained for lipase immobilized at 5 and 25 °C (Fig. 7), it can be deduced that these samples show satisfactory results regarding the quantity of lipase which remains immobilized after the treatment in water at 25 °C for a very long period of time. Overall, these results suggest that CRL can be successfully immobilized by simple post-loading entrapment method.

Conclusion

Temperature and pH-sensitive poly(NiPAAm-*co*-IA) hydrogels, of different composition and degree of crosslinking, were synthesized to examine the immobilization of *C. rugosa* lipase by swelling the dry samples in lipase solution at 5, 25, and 37 °C. It was found that the content of both anionic component and crosslinking agent in the sample, as well as the entrapment temperature at which the immobilization process was performed, affected the hydrogel-binding capacity and activity of immobilized enzyme. The results suggested that CRL was

successfully immobilized by simple post-loading entrapment method. The release study was performed at 25 °C with an idea to investigate the potential use of immobilized lipase for biotechnological applications, in which room temperature is commonly used. The network parameters of the hydrogels 90/10/2, 90/10/4, and 85/15/4 were adequate to enable efficient immobilization of CRL within the gel and favorable release profiles. These samples exhibited very rapid release kinetics, with the initial burst release, but the quantity of released protein was small, offering the possibility for lipase being immobilized at 5 and 25 °C on P(NiPAAm/IA) hydrogels, with appropriate network parameters, to be employed as a biocatalyst.

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