Journal of Molecular Catalysis B: Enzymatic 70 (2011) 55–60

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Electrochemically synthesized polyaniline as support for lipase immobilization

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ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 30 January 2011
Accepted 1 February 2011
Available online 25 February 2011

Keywords:
Lipase
Amination
Polyaniline
Electrochemical synthesis
Galvanostatic technique

ABSTRACT

Electrochemical synthesis of polyaniline support for enzyme immobilization provides easier control over the properties of obtained polymer and reduced risk of biocatalyst inactivation with residues of toxic compounds. In the present study, immobilization of lipase from *Candida rugosa* on electrochemically synthesized PANI (activated with glutaraldehyde) resulted with high lipase loadings up to 93.7 mg of proteins per gram of dry support. The activation of support and immobilization were optimized, with respect to activity yield. The optimum concentration of glutaraldehyde was 2% (w/v) and optimum concentration of enzyme was 4 mg ml^{-1}. Modification of enzyme surface with carbodiimide and ethylenediamine was performed in order to increase concentration of amino groups. Aminated lipase exhibited higher specific activity (52%) and thermal stability (3 times) after immobilization, compared with non-modified lipase. Also, reusability of immobilized enzyme was significantly increased with amination, especially if immobilization was performed at pH 10, so in such a way obtained derivative retained 91% of activity after 15 reaction cycles.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) gain huge interest of researchers because they catalyze reactions at hydrophobic/hydrophilic interfaces (such as hydrolysis and transesterification of fats and oils, ester synthesis) usually with high enantioselectivity [1–4]. A number of immobilization supports and methods have been applied in lipase immobilization in order to extend reusability and increase their stability towards high temperatures and organic solvents [5,6].

Polyaniline (PANI) is a semi-flexible rod organic polymer, which recently has drawn attention of researchers in area of biotechnology. Due to its good electrical conductivity, low-cost monomers, ease of synthesis, good mechanical strength, and simple doping chemistry, PANI is very attractive polymer for constructing electrochemical biosensors [7–9]. Numerous investigations of PANI application in electrochemical biosensors has been reported, with vast majority focused on glucose sensors with imobilized glucose oxidase [9–12], but examples of PANI-based biosensors with immobilized lactate dehydrogenase [13], tyrosinase [14], cholesterol oxidase [15], lipase [7], and horseradish peroxidase [16] can also be found.

In last decade, PANI was recognized as excellent support for enzyme immobilization due to its high physical and chemical stability, large retention capacity, and good morphological characteristics [17,18]. Prior to immobilization PANI is usually activated using glutaraldehyde, in order to introduce carbonyl group and facilitate covalent immobilization *via* amino groups of enzymes. In such a way immobilized trypsin and horseradish peroxidase exhibited good activity and increased stability at extreme pH values [17,19]. In a study with glucoamylase, the increase of specific enzyme activity after immobilization was observed and immobilized enzyme showed high reusability, thermal and storage stability [20]. Important advantage of PANI-based supports is possibility of preparation of nanofibre structures, which ensure reduced mass transfer limitation, and simultaneously enables easier recovery than nanoparticles or carbon nanotubes [21]. In a study with lipase immobilized on polyaniline nanofibres by adsorption and subsequent glutaraldehyde cross-linking high stereo-selectivity and stability of immobilized enzyme was achieved. Also, it was demonstrated that lipase immobilized on polyaniline nanofibres can be easily separated by low-speed centrifugation [21] or even using magnets if iron-oxide was applied in preparation of nanofibres [22].

The aim of this study was to investigate prospects of using polyaniline obtained by electropolymerization as support for immobilization of lipase from *Candida rugosa*. Electrochemical syn-

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doi:10.1016/j.molcatb.2011.02.004

thesis using galvanostatic technique was reported to provide better control over reaction rate and morphological properties of obtained polymer and has proven to be acceptable for enzyme immobilization [9,23,24]. Also, it is performed with significantly lower amount of toxic compounds than ordinary organic synthesis, leading to reduced risk of enzyme inactivation during immobilization by residues of these compounds. In this study, the immobilization process was optimized with regard to glutaraldehyde concentration during activation, pH and enzyme concentration during immobilization, in order to promote covalent immobilization. In a final stage of study, chemical modification of lipase with 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) and ethylene diamine (EDA) was applied in order to introduce additional amino groups, promote multipoint covalent attachment of enzyme, and improve stability of immobilized enzyme.

2. Experimental

2.1. Materials

Lipase from *C. rugosa*, glutaraldehyde (25% solution), EDAC, hydroxylamine, and EDA were purchased from Sigma (St. Louis, MO, USA). Aniline and Coomassie protein assay reagent were purchased from Fluka (Buchs, Switzerland). For polishing electrode, polishing alumina (1 μm, Banner Scientific Ltd., Coventry, UK) and polishing cloths (Buehler Ltd., Lake Bluff, IL, USA) were used. *p*-Nitrophenyl palmitate was purchased from Alfa Aesar Gmbh (Karsluhe, Germany). Other reagents and solvents used are analytical grade.

2.2. Methods

2.2.1. Electrochemical synthesis of polyaniline

Electrochemical polymerization of PANI on graphite electrode was performed galvanostatically at constant current density of $j = 2.0\ mA\ cm^{-2}$ from aqueous solution of 1.0 mol dm^{-3} HCl containing 0.25 mol dm^{-3} aniline on both sides of the graphite electrode ($S = 100\ cm^2$) inserted between two stainless steel plates, serving as counter electrodes ($S = 50\ cm^2$). Prior to electrochemical synthesis, graphite electrode was first mechanically polished with fine emery papers (2/0, 3/0 and 4/0 respectively) and then with polishing alumina on polishing cloths, the traces of the polishing alumina were ultrasonically removed from the electrode surface during 5 min. Prior to use, aniline was distilled in argon atmosphere. Polymerization was performed in the prismatic polyethylene electrochemical reactor with a volume of 0.8 dm^3. After synthesis, polyaniline powder was mechanically detached from elctrode. PANI powder was rinsed repeatedly with distilled water to the negative reaction on chloride ions, and dried at 90 °C under vacuum. When the water insoluble PANI mass was determined, acetone soluble olygomers were removed in Soxhlet extractor during 3 h, than washed with distilled water and dried in the vacuum at 90 °C. Micrograph of the PANI deposit on graphite electrode was taken by optical microscope (Olympus CX41, Camera Olympus UC30), interfaced to PC.

2.2.2. Activation of PANI powder

The activation of support was performed by incubation of 0.5 g of polyaniline powder in 20 ml of glutaraldehyde (GA) solution in 20 mM sodium phosphate buffer (pH 7,0) for 1 h at 25 °C in orbital shaker at 150 min^{-1}. The concentration of GA was varied within range 1–5% (v/v). After incubation, activated polyaniline support was filtered and thoroughly rinsed in order to elute unbounded GA and avoid unwanted cross-linking.

2.2.3. Lipase immobilization on PANI powder

Then, activated support was transferred into flask with 10 ml of *C. rugosa* lipase solution in 10 ml of sodium phosphate buffer for 16 h at 25 °C in orbital shaker at 150 min^{-1}. The concentration of lipase was varied between 1 and 6 mg ml^{-1}.

2.2.4. Chemical modification of lipase

Amination of suspended lipase was performed using 1 M solution of EDA with 10^{-2} or 10^{-3} M of EDAC at pH 4.75. After 90 min of gently stirring at 25 °C, the modified preparations were filtered and incubated for 4 h with a 0.1 M hydroxylamine solution at pH 7 and 4 °C to recover EDAC-modified tyrosines [25].

2.2.5. The protein assay

The protein concentration was determined spectrophotometrically at $\lambda = 595$ nm (Ultrospec 330 pro, Amersham Biosciences) using standard Bradford assay [26].

2.2.6. Activity assay

Lipase activity was determined by method based on measuring the effects of the hydrolysis of *p*-nitrophenyl palmitate (pNPP). The absorbance was measured at 410 nm against substrate free blank. The standard graph was prepared by using *p*-nitrophenol. One international unit (IU) is defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per min under the assay conditions.

3. 3.Results and discussion

3.1. PANI powder morphology

After electrochemical synthesis, polyaniline was mechanically detached from graphite electrode and microscopic analysis of morphology and size of obtained PANI powder was performed. Obtained microphotographs (Fig. 1) show that electrochemical synthesis of PANI leads to formation of particles of non-uniform shape and size. It can be noticed that smallest particles are prevalently rod-shaped particles with size of several μm. Agglomeration leads to formation of irregular, drastically larger, conglomerate particles, with largest diameter of around 200 μm. On large conglomerates, pores with dimensions around 1 μm are noticeable (Fig. 1b), and within such pores lipase could also be readily immobilized, since *C. rugosa* lipase dimensions are approx. 7 nm × 5 nm × 7 nm [27].

3.2. The immobilization of lipase from C. rugosa

The initial part of our study was focused on optimization of key factors of support activation and lipase immobilization. PANI particles were modified previous to immobilization using GA, in order to introduce reactive carbonyl groups. Low concentration of introduced GA residues can lead to insufficient immobilization of enzyme, while conversely high concentration can lead to unwanted cross-linking of enzyme and distortion of catalytic conformation [28,29]. Therefore, the concentration of GA during activation of support was optimized with respect to obtained enzyme load and hydrolytic activity of obtained immobilized lipase. Immobilization was performed with 6 mg ml^{-1} in immobilization mixture. All experiments have been carried out in triplicate, so average values and standard errors are illustrated inFig. 2.

Obtained results show that variation of GA concentration between 1 and 5% insignificantly influenced concentration of lipase immobilized on support, resulting with concentrations in very narrow range between 88.3 and 93.7 mg g^{-1} of dry support. On the other hand, the effect on activity of immobilized lipase was very significant. The highest activity of immobilized lipase was achieved when support was activated with 2% solution of GA (7.5 IU g^{-1} of

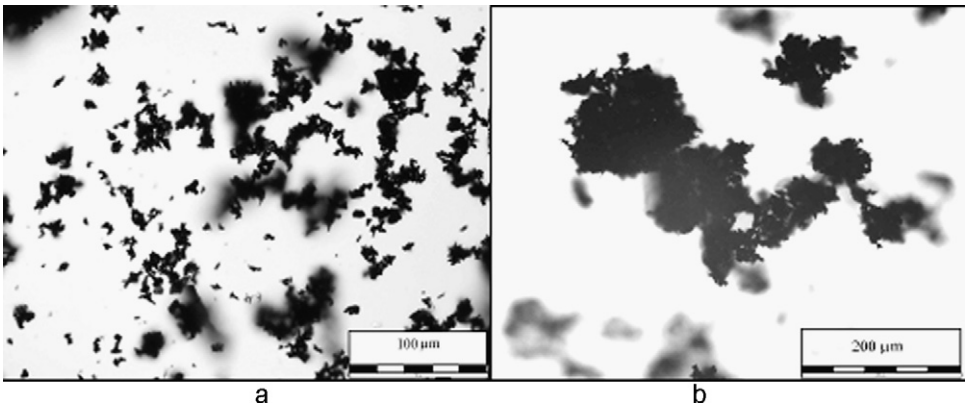

Fig. 1. Micrographs of the PANI powder obtained by electrochemical synthesis.

support), and further increase of GA concentration led to steep decrease of activity.

The chemistry of glutaraldehyde activation is not completely elucidated, since a variety of glutataldehyde structures can be formed at usual conditions of support activation, such as its mono- and dihydrates, as well as cyclic hemiacetal and oligomers [30]. Despite ambiguities in elucidation of the bond formation mechanism, GA activation has been successfully applied for long time and the effects of the most important process parameters on activation of support have been previously analyzed [29]. Thorough investigation of GA concentration effect on activation process was undertaken by Betancor et al. and conditions allowing formation of monolayer or bilayer of glutaraldehyde on support surface were established [28]. The activation time applied in our study was adequate for mild activation leading to formation of monolayer of GA on support. Reduced activity of immobilized lipase, which occurs at GA concentration above 2%, can be ascribed to resulting dense distribution of GA residues on surface of support and increased probability of cross-linking leading to disturbance of lipase catalytic active conformation. Almost insignificant effect of GA concentration on concentration of immobilized enzyme indicates that applied concentration of lipase was high enough to reach saturation at observed GA concentration range.

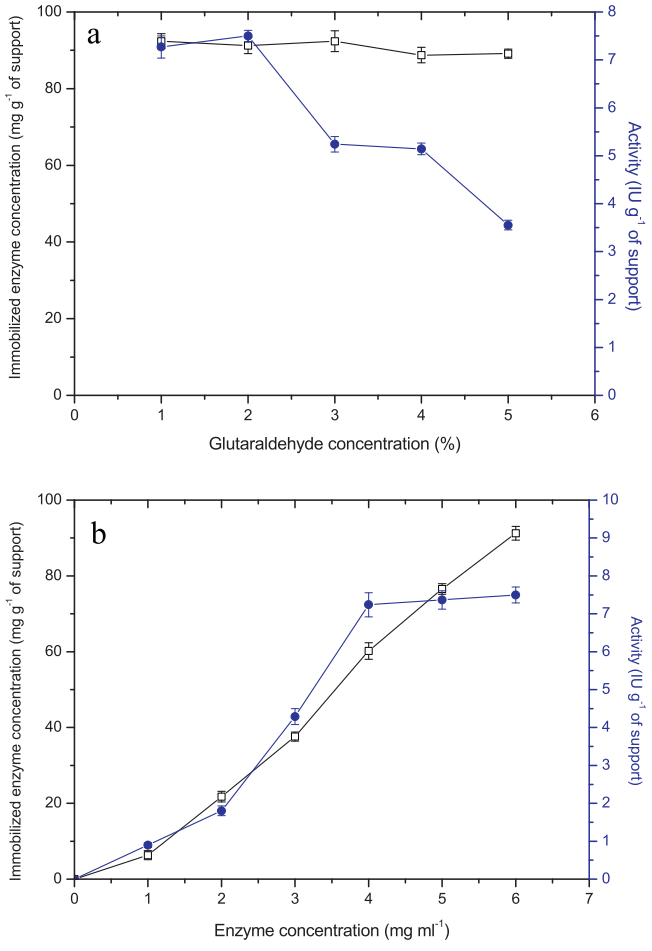

Fig. 2. The effect of (a) glutaraldehyde concentration during support activation and (b) initial lipase concentration in immobilization mixture on amount of immobilized enzyme (□) and activity of immobilized enzyme (●).

The effect of offered lipase amount was investigated by varying lipase concentration in solution in range 1–6 mg ml^{-1} in immobilization on PANI activated with 2% of GA. Experiments have been carried out in triplicate, and results are illustrated in Fig. 2b. It is obvious that the increase of offered enzyme led to more or less linear increase of enzyme load on support, reaching 91.2 mg g^{-1} of support. On the other hand, the activity of obtained derivatives was increasing rapidly until around 60 mg g^{-1} was immobilized and further increase of enzyme load cause only slight change of the immobilized activity. Therefore, the specific activity (calculated per mg of immobilized protein) reached highest values (98 IU mg^{-1}) when immobilization was performed with 4 mg ml^{-1} of enzyme preparation in immobilization solution.

Obtained results of enzyme load (almost 100 mg g^{-1}) indicate that electrochemically synthesized PANI support has high binding capacity. Nevertheless, it seems binding allows retention of catalytic conformation of lipase only in limited range of enzyme concentration, since binding of additional amount of enzyme left overall activity unchanged and specific activity reduced. It is plausible that, when immobilization is performed at low concentration of offered lipase, binding can occur with retaining catalytic conformation of enzyme and at positions on surface of support which allows easy access of support to active site of enzyme. In contrast, with higher concentration of offered enzyme in late stage of immobilization process lipase binding probably occurs in less favorable regions of support, maybe even in larger pores of PANI conglomerates (Fig. 1b), which can hinder diffusion of substrate during reactions.

3.3. Lipase amination and immobilization of modified lipase

In order to promote covalent immobilization and increase stability of immobilized lipase, enrichment of enzyme surface with amino groups through chemical amination of carboxylic groups of aspartic and glutamic side chains was performed. In Fig. 3 dis-

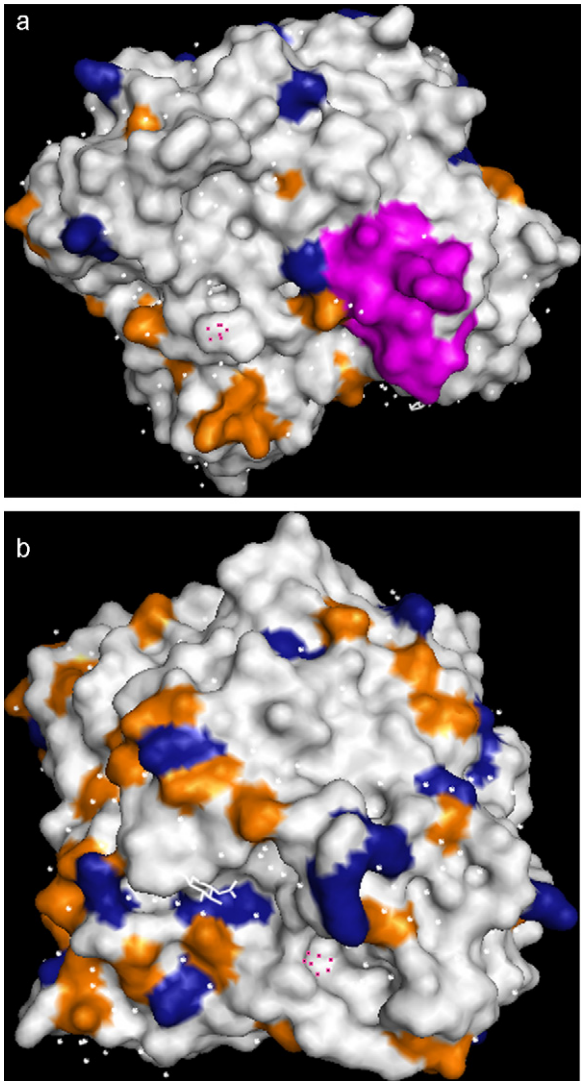

Fig. 3. Distribution of Lys, Asp and Glu residues on the surface of lipase from *C. rugosa*. Lys are shown in blue, Glu and Asp are shown in orange, and lid covering active site is shown in pink. (a) Front view and (b) 180° rotation of the front view, in *x*–*y* plane. The3D structure was obtained using Pymol vs. 0.99 and data obtained from Protein Data Bank (PDB). PDB code of *C. rugosa* lipase: 1TRH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

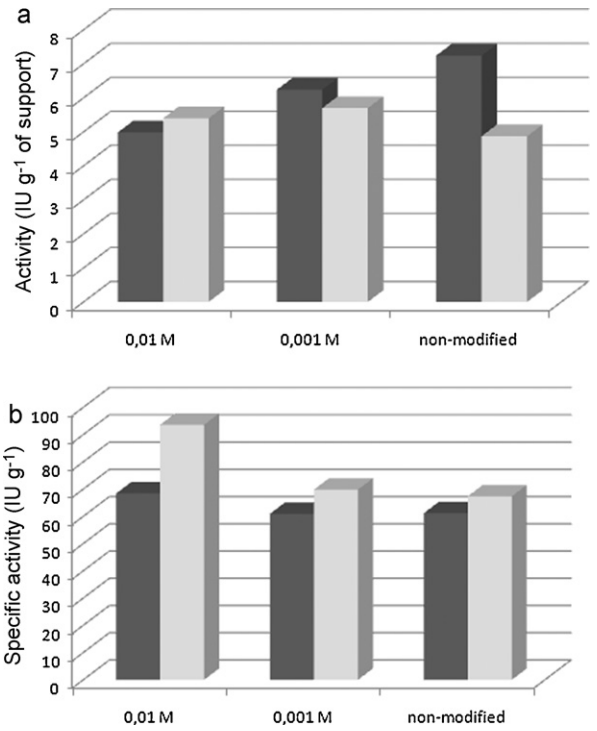

Fig. 4. The effect of amination, applied EDAC concentration, and immobilization pH on: (a) overall activity of immobilized lipase, and (b) specific activity of immobilized lipase. Immobilization pH was 7 (dark gray bars) or 10 (light gray bars).

tribution of lysines, aspartic and glutamic acids on surface of *C. rugosa* is illustrated. It can be observed that density of carboxylic groups is significantly higher than amino groups, which implies that amination of carboxylic groups can lead to drastic increase of amino groups density enabling multipoint covalents attachement. It was previously reported that chemical amination with EDA, after activation with EDAC, has been successfully applied with several industrially important lipases (lipases from *T. lanuginosa*, *B. thermocatenulatus* and *P. fluorescens*), after adsorbing it on hydrophobic support in order to protect undesired chemical changes in vicinity of lipase active site and activity loss [31–33].

In our study, prior to immobilization the effects of chemical amination of free suspended enzyme on retained activity were analyzed. The amination was performed with different concentrations of EDAC (0.01 M or 0.001 M), which has been previously reported to ensure complete or partial amination, respectively. It was found out that lipase activity was not drastically reduced (26% and 14%, respectively), so in following experiments amination was performed without previous adsorption of lipase in order to simplify immobilization procedure. It is plausible that only slight impact of chemical modification on activity of enzyme can be ascribed to lower density of side chains with carboxylic groups in vicinity of lid that covers catalytic triad (Fig. 3), compared with structures of previously reported aminated lipases [31,32].

Both, modified and non-modified lipases, were immobilized at pH 7 or pH 10. Immobilization at pH 10 was tested because p*K* of ε-amino groups of lysine is around 10, hence in basic conditions portion of non-dissociated groups is larger and this form is more prone to react with carbonyl groups [34]. Immobilization was performed with PANI support, activated with 2% GA, and in all experiments immobilization mixtures of equal lipase activities were applied. All experiments were carried out in triplicate and obtained average activities of immobilized derivatives are illustrated in Fig. 4a. The highest activity (7.1 IU g^{-1}) was achieved using non-modified lipase in immobilization performed at pH 7. Generally, it can be observed that, in experiments at pH 7, activities of immobilized lipases continuously decrease with increasing extent of modification, resulting with the lowest activity with lipase modified with 0.01 M of EDAC. At pH 10, immobilized activity of non-modified lipase is significantly reduced, to 4.8 IU g^{-1}. Conversely, alkaline conditions were adequate for immobilization of aminated lipases since only slight change of activities were achieved. Besides activities, concentration of immobilized proteins was also determined and used for calculation of specific activities, which are presented in Fig. 4b. The highest specific activity (93.7 IU g^{-1}) was measured for derivative obtained with lipase modified with 0.01 M EDAC immobilized at pH 10. Generally, enzyme loading was significantly lower when immobilization had been performed at pH 10, resulting with higher specific activities of these derivatives and different trend than in Fig. 4a. Also, lipase amination exhibited favorable effects on specific activity, since the increase of specific activity was observed with increase of applied EDAC concentrations.

Table 1
Half-lives ($t_{1/2}$) of different *C. rugosa* lipase preparations determined in stability assay at 60 °C.

Lipase	[EDAC] in amination, M	$t_{1/2}$, h
Immobilized at pH 7	–	5.3 ± 0.8
	0.001	5.7 ± 0.4
	0.01	5.9 ± 0.5
Immobilized at pH 10	–	6.9 ± 0.7
	0.001	13.1 ± 1.1
	0.01	15.9 ± 0.9
	–	1.2 ± 0.4
Free lipase	0.001	0.9 ± 0.4
	0.01	0.8 ± 0.3

The trend observed with derivatives obtained at pH 7 can be explained by prevalence of adsorption mechanism due to high concentration of charged lysine amino groups. The decrease of immobilized activity and enzyme loading with enzyme modification (Fig. 4a) can be ascribed to lower content of negatively charged residues on enzyme surface, which reduces adsorption of enzyme by electrostatic interactions with secondary amino groups on surface of polyaniline. On the other hand, at pH 10 amino groups are uncharged and covalent immobilization *via* carbonyl groups can readily occur. Therefore, immobilized activity increased after modification due to increased number of reactive amino groups on enzyme surface. Specific activities were significantly higher for derivatives obtained at pH 10. Since there is higher probability for bond formation *via* area containing highest density of reactive amino groups it is plausible immobilization occurs through area at rear part of molecule (Fig. 4b), with respect to active site of lipase. Therefore, observed increase of activity in immobilization at pH 10 could be due to more favorable orientation of enzyme during immobilization process resulting with immobilized enzyme with well-exposed active site, and consequently higher activity.

3.4. Thermal stability study

For better evaluation of immobilization support and methods it is necessary to compare stability of different enzyme derivatives. In our study, thermal stability assay was performed at 60 °C and pH 7. For easier comparison of stabilities half-lives of different derivatives were determined, and obtained results are presented in Table 1. The most illustrative results are depicted in Fig. 5.

It seems that both, pH of immobilization and chemical modification of lipase exhibited strong influence on stability of immobilized lipase. The immobilization at pH 10 resulted with derivatives of significantly higher stability (Fig. 5a). For non-modified lipase, immobilization at pH 10 led to increase of derivative half-life to $t_{1/2}$ = 6.9 h from $t_{1/2}$ = 5.3 h obtained at pH 7, while for lipase modified with 0.01 M EDAC the increase was even larger-from $t_{1/2}$ = 5.9 h to $t_{1/2}$ = 15.9 h. Lipase amination also has shawn clearly positive effect on stability of immobilized derivatives, particularly in immobilizations performed at pH 10 (Fig. 5b). Derivatives obtained using aminated lipase exhibited around two-times higher half-lives than derivatives obtained with non-modified lipases. Additionally, it is noticeable that amination at higher concentration of EDAC (0.01 M) resulted with significantly more stable derivative. These results indicate that approach that combines introduction of additional amino groups on lipase surface and immobilization at pH conditions, enabled making of lipase derivative with stability three-times higher in comparison with derivative obtained using conventional approach. Also, aminated lipase immobilized at pH 10 exhibited 16 times higher half-life than free lipase. Such a trend could also be explained with previously stated assumption that immobilization occurs *via* region containing higher density of amino groups. As a consequence, it seems that higher number of covalent bonds

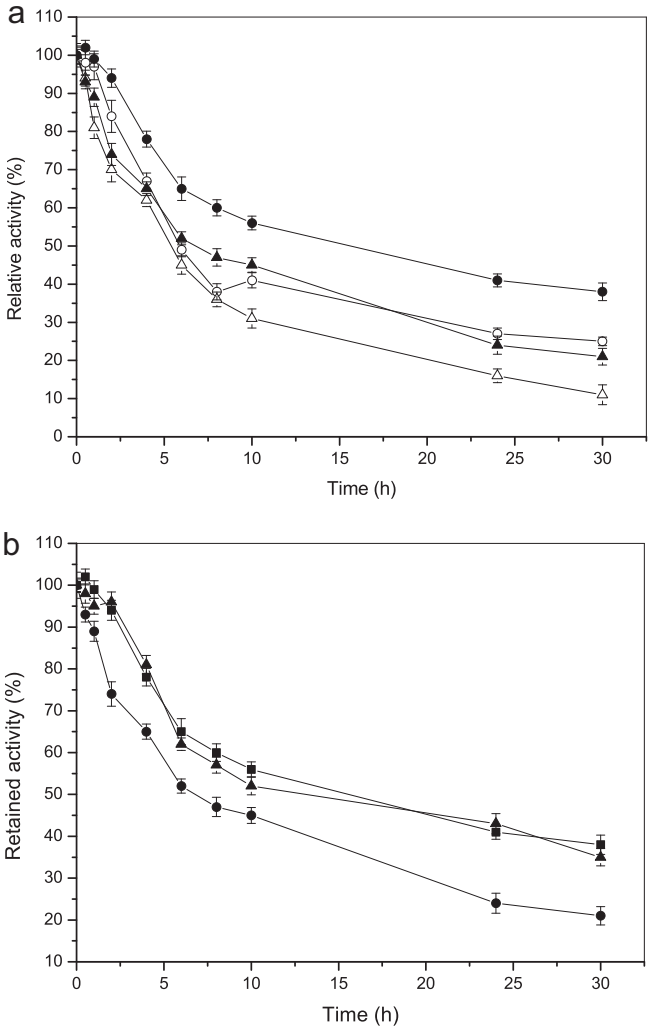

Fig. 5. Thermal stability assay: (a) the effect of immobilization pH. (△) lipase immobilized at pH 7; (▲) lipase immobilized at pH 10; (○) immobilization at pH 7 using lipase aminated with 0.01 M EDAC; (●) immobilization at pH 10 using lipase aminated with 0.01 M EDAC and (b) the effect of amination. The immobilization was performed at pH 10 using: (●) non-aminated lipase; (▲) lipase aminated with 0.001 M EDAC; (■) lipase aminated with 0.01 M EDAC.

with carbonyl groups of activated PANI support have been formed leading to significant increase of stability of immobilized lipase.

3.5. Reusability study

One of the most important advantages of immobilized enzymes is the possibility of repeated use through a number of reaction cycles, hence the prospect of recovering lipase immobilized on PANI supports from reaction mixture and repeated use of derivatives were analyzed. Immobilized derivative was separated using low-speed centrifugation (4000 × *g*) after each reaction cycle and re-suspended in fresh substrate. The effects of different strategies for lipase immobilization on reusability were tested by comparison of two derivatives: one prepared with non-modified lipase at pH 7 and other prepared with aminated lipase at pH 10 (Fig. 6).

Obtained results indicate that approach based on the increase of number of reactive amino groups on lipase surface and immobilization at basic conditions causes significant extension of immobilized enzyme reuse. Namely, in such a way obtained derivative retained 91% of initial activity after 15 reaction cycles, while derivative obtained using conventional approach (non-modified enzyme and immobilization at pH 7) retained 73% of initial activity. These

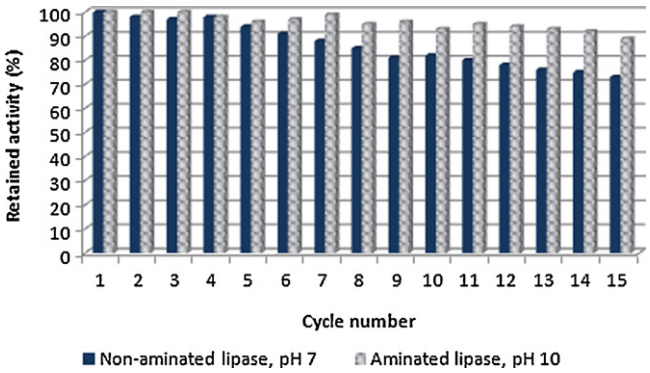

Fig. 6. The effect of lipase modification on reusability of PANI-immobilized lipases in hydrolysis of *p*-NPP.

results imply that promoting of covalent immobilization by amination and immobilization at pH 10 leads to reduced elution of enzyme from support and/or reduced flexibility leading to reduced chance of activity loss.

Previously presented results unambiguously indicate good prospect for application of electrochemically synthesized PANI as immobilization support, so in final stage preliminary evaluation of its potential for application in biosensor construction was performed. With this purpose, PANI was synthesized on surface of graphite electrode using same current density as in previous experiments and lipase was immobilized directly on PANI-coated graphite electrode. In this experiment lipase aminated using 0.01 M EDAC was applied and immobilization was performed at pH 10, due to highest stability of this derivative exhibited in previous experimental series. The activity of lipase immobilized on graphite electrode was 4.7 IU g^{-1} of PANI, 87% of activity achieved after immobilization of aminated lipase on PANI powder. Afterward, graphite electrode was applied in repeated reaction cycles of *p*-NPP hydrolysis in order to evaluate stability of immobilized enzyme and it was determined that it retained 95% of initial activity after 15 cycles. Results of this part of our study indicate that electrochemically synthesized PANI offers prospect of immobilization of enzymes directly on electrode with similar activity retention as in case of immobilization on PANI powder. Additionally, enzyme immobilized on PANI-coated electrode exhibited even higher usage stability than enzyme immobilized on PANI powder, which indicates that this material should be considered in development of biosensors.

4. Conclusions

The results clearly demonstrate that electrochemically synthesized PANI can be applied as immobilization support after activation with GA, since almost 100 mg of lipase from *C. rugosa* was immobilized per one gram of PANI powder with specific activities reaching 98 IU mg^{-1}. Dimensions and morphology of obtained PANI powder allows simple separation of immobilized derivative from reaction mixture by low-speed centrifugation. The stability of obtained immobilized lipases was significantly improved using approach which combines chemical modification of enzyme prior to immobilization and optimization of immobilization pH. Enrichment of lipase surface with amino group and performing immobilization at pH 10 has proven to be good strategy to increase thermal stability of immobilized enzyme for several times. Additionally, this strategy enabled important increase of operation stability resulting with immobilized lipase that retains 91% of initial activity after 15 reaction cycles. Even higher operational stability was observed with enzyme immobilized on PANI-coated electrode implying that applied combination of support preparation, enzyme pre-modification, and immobilization conditions leads to highly stable immobilized enzyme with good prospect in biosensor construction.

Acknowledgment

The authors are grateful for financial support from Serbian Ministry of Science (project III 46010).

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