

COVALENT IMMOBILIZATION OF LIPASE FROM *Candida rugosa* ON EUPERGIT®

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An approach is presented for the stable covalent immobilization of lipase from Candida rugosa on Eupergit® with a high retention of hydrolytic activity. It comprises covalent bonding via lipase carbohydrate moiety previously modified by periodate oxidation, allowing a reduction in the involvement of the enzyme functional groups that are probably important in the catalytic mechanism. The hydrolytic activities of the lipase immobilized on Eupergit® by two conventional methods (via oxirane group and via glutaraldehyde) and with periodate method were compared. Results of lipase assays suggest that periodate method is superior for lipase immobilization on Eupergit® among methods applied in this study with respect to both, yield of immobilization and hydrolytic activity of the immobilized enzyme.

KEY WORDS: Eupergit®; lipase; *Candida rugosa*; immobilization

INTRODUCTION

Lipase (E.C. 3.1.1.3.) gained enormous attention in scientific community during the last two decades due to a variety of commercial products that can be obtained in lipase-catalyzed reactions (1-3). The most important products are diglycerides, monoglycerides, fatty acids and glycerol (products of fat and oil hydrolysis), flavor esters and sugar esters (products of esterification) and purified enantiomers with improved physiological action. A significant advance in the application of lipases was the discovery of enhanced lipase activity and thermal stability in reactions carried out in organic solvents, especially those with low polarity (1). Another improvement occurred due to the great progress in the field of development new techniques and novel carrier materials for enzyme immobilization

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(2). Lipase stability and prospect for multiple utilization was enhanced by the application of various immobilization strategies, i.e. covalent attachment, adsorption, entrapment in polymer matrices, encapsulation, and entrapment in pores of membrane reactor (4).

The main advantage of covalent immobilization over other strategies is the absence of a barrier between the enzyme and reaction medium, and consequently lack of diffusional limitations. On the other hand, it is necessary to activate the enzyme or carrier to achieve covalent immobilization, because of low affinity towards forming covalent bonds of functional groups in both molecules. In order to retain high specific activity of the immobilized enzyme, the method of immobilization must be mild and should not affect enzyme active site. Therefore, activation of the carrier is usually method of choice because enzymes are prone to denaturation in the presence of aggressive chemical agents which are usually used for activation. Numerous chemical activators have been used for carrier activation in lipase immobilization, for instance cyanogen bromide for activation of polysaccharides, cyanuric chloride for modification of polysaccharides and their amino derivatives, tresyl chloride for activation of hydroxyl groups within inorganic carriers, glutaraldehyde for introducing carbonyl group in a carrier that contains amino group, and sodium periodate for oxidation of hydroxyl groups into carbonyl groups (4-9). Various inorganic materials (glass, metal oxides, ceramics), natural polymers (cellulose, chitin, starch and other polysaccharides), and synthetic polymers and copolymers (polymers of acrylic acid derivatives, polyamides, derivatives of polyvinyl alcohol) have been used as carriers for covalent immobilization. A carrier suitable for enzyme immobilization must have a large surface area with a high content of reactive groups, good mechanical and chemical stability and good flow properties. Macroporous polymers, such as polystyrene and Eupergit[®], meet these requirements.

Eupergit[®] (made by copolymerization of *N,N*-methylene-bis-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide) is particularly attractive for application because it can be subjugated to high shear forces and extreme pH values due to its excellent mechanical properties and chemical stability over a pH range from 0 to 14 (10). Enzymes bind Eupergit[®] via its oxirane groups, which react with amino groups of protein to form covalent bonds. Due to high density of oxirane groups on the Eupergit[®] surface, enzymes are bound at various sites of the particle, which ensures high operational stability of the enzyme (11). This high stability was confirmed in a study by Ivanov and Schneider (11) focused on ester synthesis catalyzed by lipase from *Pseudomonas cepacia* immobilized on five different carriers. Although celite-immobilized lipase showed higher activity, Eupergit[®] proved to be superior when stabilities were compared. High stability of lipase immobilized on Eupergit[®] was also reported in a study of enantioselective hydrolysis (12).

The main goal of this study was to investigate the possibility of immobilizing lipase on Eupergit[®] via lipase carbohydrate moiety previously modified by periodate oxidation. This method has shown very promising results in studies with other glycoproteins (invertase and glucoamylase), especially in a study with invertase, where the obtained specific activity was the highest for invertases immobilized on macroporous carriers, reported so far (13). The advantage of this method is the fact that it avoids formation of covalent bond in the protein part of the enzyme molecule, which could lead to changes in the enzyme conformation and, therefore decrease of the enzyme activity. In order to investigate the prospects of using this method for lipase immobilization, the activities of lipase from *Candida rugosa* immobilized on Eupergit[®] by two conventional methods (via oxirane group and via glutaraldehyde) and with periodate method were compared.

EXPERIMENTAL

Materials

Eupergit®C 250 L and Eupergit®C were kindly donated by Degussa (Rohm Pharma Polymers). Lipase substrate, which stands for stabilized olive oil emulsion (50% v/v), with 0.1% sodium azide as preservative, and Folin&Ciocalteu's Phenol Reagent was purchased from Sigma, St. Louis, USA. All other chemicals were purchased from Merck, Darmstadt, Germany.

Methods

Eupergit® modification: One portion of Eupergit® was modified prior to use with 1 M 1,2-diaminoethane at 60°C for 4 h at pH 10. After that, the polymer was washed three times with distilled water and ethanol, then dried.

Immobilization via epoxide: Unmodified Eupergit® was incubated with 1 mg/ml of native lipase in 0.1 and 1.0 M sodium phosphate buffer pH 7.0 for 48 h at 4°C. After that, the polymer was washed three times with 1 M NaCl and stored in sodium phosphate buffer at pH 7 at 4°C until use.

Immobilization by glutaraldehyde: Modified Eupergit® was incubated in 2.5 % (w/v) glutaraldehyde in sodium phosphate buffer at pH 8 for 2 h, and washed several times with the same buffer. Polymer was then incubated with native lipase in sodium phosphate buffer at pH 7.0 and 4°C for 48 h. After that, the polymer was washed three times with 1 M NaCl and stored in sodium phosphate buffer at pH 7 at 4°C until use.

Immobilization by periodate: Lipase was oxidized by periodate as described previously for invertase (14) by incubating 1 mg/ml of enzyme with 5 mM sodium metaperiodate in acetate buffer pH 5.0 for 6 hours in dark at 4 °C. The unreacted sodium periodate was then removed with 10 mmol/l ethylene glycol for 30 minutes. The oxidized lipase was then dialyzed against 50 mmol/l acetate buffer pH 5.0 for 18 h. Modified polymer was incubated with oxidized lipase in sodium acetate buffer at pH 5.0 and 4°C for 48 h. After that, the polymer was washed three times with 1 M NaCl and stored in sodium phosphate buffer at pH 7 at 4°C until use.

Lipase activity assays

Specific activities of enzyme preparations were determined in the hydrolysis of olive oil by previously described method (15). Hydrolysis of lipase substrate was carried out at 37°C for 3 h. The amount of produced fatty acid was determined by titration with 0.01 M NaOH. The activity was expressed as international units (U). One unit of activity is defined as the amount of enzyme needed to liberate 1 µmol of fatty acid per minute.

Determination of yield of immobilization

The amount of enzyme immobilized on carrier was determined by measuring the concentration of enzyme in filtrate, after separation of the immobilized enzyme. Enzyme concentration was determined by colorimetric measurement (at 500 nm) of concentration of protein dyed with Folin&Ciocalteu's phenol reagent. Yield of immobilization (%) was calculated according to Eq. [1].

$$\text{Yield (\%)} = 100 * \frac{(\text{enzyme input}) - (\text{enzyme in filtrate})}{(\text{enzyme input})} \quad [1]$$

RESULTS AND DISCUSSION

Lipase from *Candida rugosa* was immobilized by three methods on two types of Eupergit; Eupergit®C and Eupergit®C 250 L. The polymers have identical chemical structures, but Eupergit®C 250 L has larger pores (100 nm) in relation to Eupergit®C (10 nm) (16). Yields of lipase immobilization in each of 6 experiments are illustrated in Fig. 1. It is noticeable that the highest degree of immobilization (46.7 %) was achieved when lipase was immobilized on Eupergit®C 250 L by periodate method. Lipase immobilization on Eupergit®C by the same method showed significantly lower efficiency, since only 27.8 % of the enzyme was bound to the polymer. On the other hand, when the immobilization method included pretreatment of polymer with glutaraldehyde, Eupergit®C exhibited considerably higher yield (37 %) than the polymer with larger pores (16.5 %). Conventional method of immobilization on Eupergit®, without pretreatment, gave very low immobilization yields with both polymers.

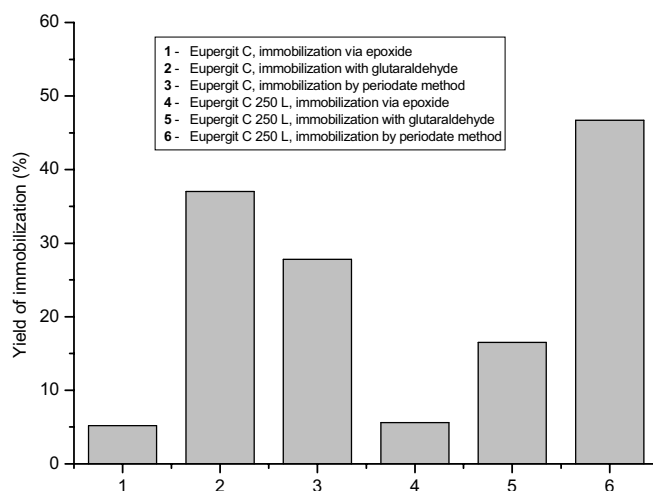


Fig. 1. Yields of lipase immobilization on two types of Eupergit® by various methods

The efficiency of immobilization was additionally checked by the determination of the lipolytic activity of the filtrates obtained after separation of the immobilized enzymes. Results are presented in Fig. 2. It is evident that the highest enzyme activity was measured in filtrates 1 and 4, which were obtained by immobilization procedure without pretreatment. After comparison of these results with the results of previous part of the study, it can be concluded that they are in good correlation, since the most active filtrates are obtained with the methods that gave the lowest immobilization yields. Therefore, the filtrates ob-

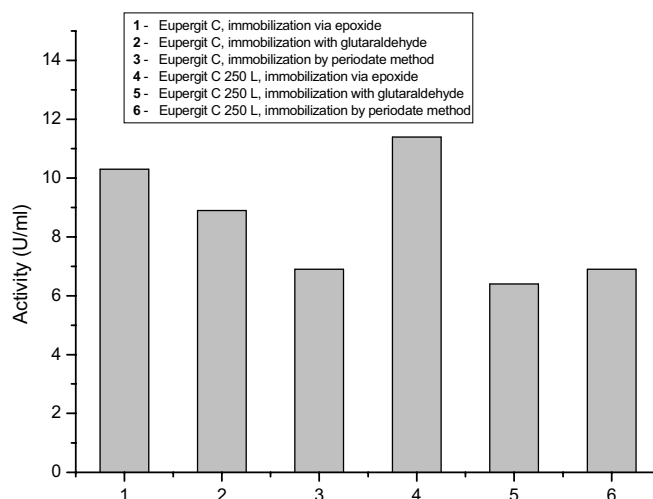


Fig. 2. Lipolytic activities of filtrates obtained after separation of immobilized lipase

tained in periodate immobilization showed lower activity, due to the fact that they contained less enzyme since larger portion was attached to the carrier. Additionally, it is of great importance that these filtrates showed measurable lipase activity, because this result implies that treatment of lipase with periodate did not destroy lipase activity. Similar results were obtained in previous studies focused on this method of lipase pretreatment with different enzymes (9, 15).

Finally, lipolytic activities of the obtained immobilized enzymes were determined. Results are illustrated in Fig. 3. The activity of lipase from *C. rugosa* immobilized by periodate method was higher than in the experiments where the other methods were applied. The maximum activity (16 U/g) was observed when Eupergit[®]C was employed as a carrier for immobilization, while Eupergit[®]C 250 L exhibited slightly lower lipolytic activity (14.7 U/g). By comparing Figs. 1 and 3 it is evident that the activity of lipase immobilized on Eupergit[®]C by periodate method is surprisingly high compared to the yield of immobilization. Therefore, the differences between activities of the two forms of Eupergit[®], are significantly lower than it could be anticipated after first part of the study. It is plausible to think that substantially larger portion of enzyme immobilized on Eupergit[®]C 250 L was entrapped in the intrinsic section of carrier due to its larger pores, which can hinder the approach of the substrate to the active site due to diffusional limitations and steric hindrance. In the case of Eupergit[®]C, mesoporous supports, the molecules of enzymes are immobilized on the surface and the substrate can easily reach the active site of lipase. Similar results were obtained by Moreno and Sinisterra in the case of different inorganic supports (6). Therefore, lipase immobilized on Eupergit[®]C probably showed higher activity even with lower lipase concentration. Nevertheless, the results proved that the attachment of lipase pretreated with sodium metaperiodate to both forms of Eupergit[®] by periodate method can be successfully performed. In both cases sufficient immobilization yields were achieved and obtained immobilized enzymes showed considerable activity in the hydrolysis of olive oil.

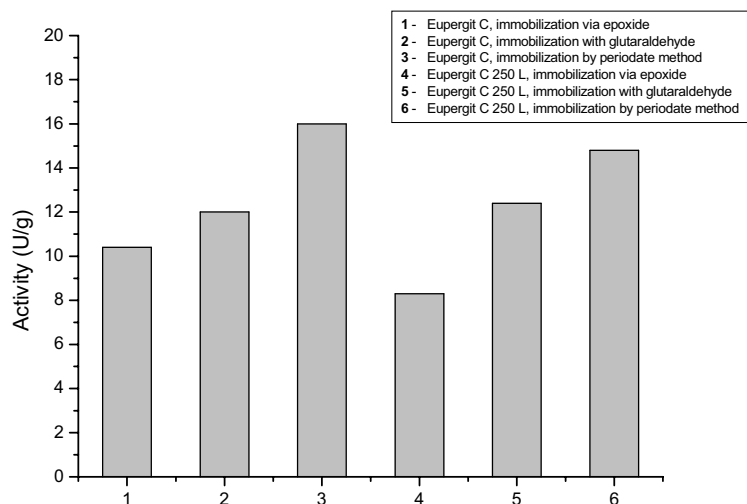


Fig. 3. Lipolytic activities of lipase immobilized on various supports

Lipase immobilized on a carrier pretreated with glutaraldehyde exhibited considerably lower lipolytic activity. Discrepancies between the activities of lipase in experiments with Eupergit[®]C and Eupergit[®]C 250 L were almost inconsiderable when this method was applied, 12 and 12.4 U/g, respectively. Even lower activities were observed with lipase immobilized without any pretreatment of enzyme or carrier. In this case, Eupergit[®]C showed somewhat better properties than Eupergit[®]C 250 L, in respect to activities. Hence, the results of lipase assays imply that periodate method is superior for lipase immobilization on Eupergit[®] among the methods applied in this study in respect of both yield of immobilization and lipolytic activity of the immobilized enzyme.

CONCLUSIONS

A novel periodate method for immobilization of enzymes on the one of the most popular commercial carriers, Eupergit[®], showed a remarkable effect in comparison with conventional methods for covalent attachment. The yield of immobilization was higher than in experiments when other methods were applied. More importantly, the obtained results imply that the method is mild since lipase retained its lipolytic activity. Activities of lipase attached to Eupergit[®] by periodate method are significantly higher than in the case of attaching lipase on Eupergit[®] pretreated with glutaraldehyde. It can be concluded that attachment of lipase via its carbohydrate moiety to Eupergit[®] proved to be a suitable method which resulted in the immobilized enzyme with high retained activity.

REFERENCES

1. Klibanov, A.M.: Enzymes that work in organic solvents. *Chem. Tech.* **16** (1986) 354-359.

2. Paiva, A.L., V.M. Balcao, F.X. Malcata: Kinetics and mechanisms of reactions catalyzed by immobilized lipases, Review. *Enzyme Microb. Technol.* **27** (2000) 187-204.
3. Balcao, V.M., A.L. Paiva, F.X. Malcata: Bioreactors with immobilized lipases: State of the art, Review. *Enzyme Microb. Technol.* **18** (1996) 392-416.
4. Knežević, Z. D., S. S. Šiler-Marinković, Lj. V. Mojović: Immobilized lipases as practical catalysts. *APTEFF* **35** (2004) 151-164.
5. Axen, R., J. Porath, S. Ernback: Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature* **214** (1967) 1302.
6. Moreno, J.M., J.V. Sinisterra: Immobilization of lipase from *Candida cylindracea* on inorganic supports. *J. Mol. Catal.* **93** (1994) 357-369.
7. Arroyo, M., J.M. Moreno, J.V. Sinisterra: Immobilization/stabilization, on different hydroxylic supports, of lipase from *Candida rugosa*. *J. Mol. Catal.* **83** (1993) 261-271
8. Stark, M.B., K. Holmberg: Covalent immobilization of lipase in organic solvents. *Biotechnol. Bioeng* **34** (1989) 942-950.
9. Prodanović, R., S. Jovanović, Z. Vujčić: Immobilization of invertase on a new type of macroporous glycidyl methacrylate. *Biotechnol. Lett.* **23** (2001) 1171-1174.
10. Katchalski-Katzir E., D. M. Kraemer: Eupergit® C a carrier for immobilization of enzymes of industrial potential. *J. Mol. Catal. B:Enzym.* **10** (2000) 157-176.
11. Mateo, C., O. Abia, R. Fernandez-Lafuente, M. J. Guisan: Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment. *Enzyme Microb. Technol.* **26** (2000) 509-515.
12. Ivanov, A. E., M. P. Schneider: Methods for the immobilization of lipases and their use for ester synthesis. *J. Mol. Catal. B:Enzym.* **3** (1997) 303-309.
13. Wirz, B., R. Barner, J. Huebscher: Facile chemoenzymatic preparation of enantiomerically pure 2-methylglycerol derivatives as versatile trifunctional C₄-synthons. *J. Am. Chem. Soc.* **58** (1993) 3980-3984.
14. Milosavić, N., R. Prodanović, S. Jovanović, I. Novaković, Z. Vujčić: Preparation and characterization of two types of covalently immobilized amyloglucosidase. *J. Serb. Chem. Soc.* **70** (2005) in press.
15. Knežević Z., Lj. Mojović, B. Adnađević: Palm oil hydrolysis by lipase from *Candida cylindracea* immobilized on zeolite type Y. *Enzyme Microb. Technol.* **22** (1998) 275-280.
16. Boller T., C. Meier, S. Manzler: Eupergit Oxirane Acrylic Beads: How to Make Enzymes Fit for Biocatalysis. *Org. Process Res. Dev.* **6** (2002) 509-519.

КОВАЛЕНТНА ИМОБИЛИЗАЦИЈА ЛИПАЗЕ ИЗ *Candida rugosa* НА EUPERGIT®

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У овом раду је испитана могућност примене методе за ковалентно везивање липазе из *Candida rugosa* за комерцијални полимерни носач Eupergit® којом се добијају стабилни и активни имобилисани ензими. Везивање се одвија преко угљенохидратне компоненте ензима, која је претходно модификована оксидацијом помоћу перјодата, а не преко протеинске компоненте, која је важна за каталитичку активност ензима. Хидролитичка активност на овај начин имобилисане липазе упоређена је са активностима липаза које су имобилисане помоћу две конвенционалне методе (везивање преко епоксидних група носача и везивање за носач модификован глутаралдехидом). Резултати овог истраживања показују да је перјодатна метода погоднија за имобилизацију липазе на Eupergit® са оба испитивана аспекта: приноса имобилизације и хидролитичке активности имобилисаног ензима.

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