Final Draft
of the original manuscript:

Miletic, N.; Abetz, V.; Ebert, K.; Loos, K.:
Immobilization of Candida antarctica lipase B on Polystyrene Nanoparticles
In: Macromolecular Rapid Communications (2009) Wiley

DOI: 10.1002/marc.200900497

Immobilization of *Candida antarctica* lipase B on Polystyrene Nanoparticles

Nemanja Miletić, Volker Abetz, Katrin Ebert, Katja Loos*

K. Loos, N. Miletić

Polymer Chemistry Department, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Fax: +31-50-363-4400; E-mail: K.U.Loos@rug.nl

V. Abetz, K. Ebert

GKSS Research Centre Geesthacht GmbH, Institute of Polymer Research, Max-Planck-Str. 1, 21502 Geesthacht, Germany

Summary/Abstract

Polystyrene (PS) nanoparticles were prepared via a nanoprecipitation process. The influence of the pH of the buffer solution used during the immobilization process on the loading of *Candida antarctica* lipase B (Cal-B) and on the hydrolytic activity (hydrolysis of *p*-nitrophenyl acetate) of the immobilized Cal-B was studied. The pH of the buffer solution has no influence on enzyme loading, while immobilized enzyme activity is very dependent on the pH of adsorption. Cal-B immobilized on polystyrene nanoparticles in buffer solution pH 6.8 performed higher hydrolytic activity than crude enzyme powder and Novozyme 435.

Introduction

Among lipases, Cal-B is one of the most recognized biocatalysts because of its high degree of selectivity in a broad range of synthetic applications of industrial importance, including kinetic resolutions, aminolysis, esterification and transesterification.[1-3] By enzyme immobilization, catalysts can be developed with significant advantages relative to free enzyme.[4-8] Many literature reports describe the high utility of immobilized Cal-B for chemical transformations of low molar mass compounds,[9,10] and polymerization reactions.[11,12,13]

Adsorption of an enzyme onto a surface can induce conformational changes which affect the rate and specificity of the catalyst.[14] Therefore, immobilization research has largely focused on matrix selection and on optimizing immobilization conditions.[15-18] For example, work has addressed support surface hydrophobicity/hydrophobocity[19,20,21] and

enzyme solution pH.[17,22,23] These parameters have large influence on the total amount of enzyme loading and enzyme-catalyst activity.[24,25]

Hydrophobic binding of lipases by adsorption has proved successful due to its affinity for water/oil interfaces. Thus, the present work deals with the synthesis of PS nanoparticles and their use as a support for the immobilization of Cal-B. The effect of the pH of the immobilization solution on lipase loading as well as the hydrolytic activity of the corresponding preparation was studied, and the results were compared to the crude enzyme powder and commercially available preparation, Novozyme 435.

Experimental Part

Materials

Cal-B in the form of a dried powder was purchased from Codexis® (Pasadena, CA, USA). Novozyme 435 (consists of 20 % w/w of Cal-B)[26] was provided by Novozymes®. Polystyrene (M_w = 100,000) was received from Avocado Research Chemicals, UK, and Pluronic® F-68 was received from Sigma-Aldrich, Germany. As solvent tetrahydrofurane p.a. (THF) from Merck, Germany was used. All chemicals were used without further purification.

Synthesis of Polystyrene Nanoparticles

PS nanoparticles were prepared by a nanoprecipitation process.[27] PS (M_w = 100,000) was dissolved in THF to a final concentration of 0.45 wt %. Concentration of Pluronic® F-68 in the aqueous phase was 2.5 g/L. The aqueous phase was continuously stirred at a

stirrer rate of 600 rpm. The polymer phase was introduced into the aqueous phase by a syringe pump (Medipan Typ 610 BS, Poland) at a feed rate of 53 mL/h.

Immediately after the particle formation the suspension was filtrated through a metal filter of 32 µm mesh size and the solvent was removed from the suspension under vacuum in a rotating evaporator (Büchi Rotavapor EL 131) at 30 °C to a final volume of about 30 ml. Purification of the suspension was performed by filtration on Sepharose Cl-4B (Aldrich, 40-165 µm). Subsequently freeze drying was performed (Vaco I, Zirbus Technology GmbH).

The shape of the nanoparticles was observed using a scanning electron microscope (JEOL TSM 6320F) operating at 3 kV. The samples were made conductive by evaporating a layer of 2 nm Platinum/Palladium alloy onto the surface.

Enzyme Immobilization by Physical Adsorption

Enzyme support, polystyrene nanoparticles (40 mg), was placed in 4 mL capped vials. The vials were filled with 2.5 ml of an enzymatic solution 4.0 mg/ml in 100 mM PBS buffer (pH 5.0, 6.8, 8.0, 9.5 and 11.5). All the vials were rotated for 24 hours at 30 $^{\circ}$C. The suspension was centrifuged and the mother liquor was removed from each vial. The solid was washed with adequate PBS buffer and distilled water, centrifuged and the liquid removed. This procedure was repeated until no protein was detectable any more in the washing solution. The mother liquor and the resulted washing solutions were collected and using the bicinchoninic acid (BCA) protein assay, the amount of enzyme that is immobilized could be estimated. The resulting nanoparticles with immobilized Cal-B were freeze dried for 48 hours and then used for hydrolytic activity tests.

Hydrolytic Activity

A 1,4-dioxane solution (5 ml) containing *p*-nitrophenyl acetate (*p*NPA) (40 mM) and methanol (80 mM) was added to 20 ml vials containing 0.100 mg of enzyme. The assay reactions were carried out for 50 minutes at 35 °C (300 rpm) and were terminated by removal of the enzyme by centrifugation. The concentration of the reaction product *p*-nitrophenol (*p*NP) was determined by UV/VIS (PYE UNICAM SP8-200 UV/VIS spectrophotometer) at the λ_{max} (304 nm) of *p*NP. Hydrolytic activity for crude Cal-B powder, Cal-B physically adsorbed on polystyrene nanoparticles and Novozyme 435 are defined herein as the nanomoles of *p*NPA hydrolyzed in 1,4-dioxane per time per unit of weight of enzyme (nmol of *p*NP/min/mg Cal-B).

Results and Discussion

Polystyrene (PS) nanoparticles were prepared via a nanoprecipitation process. The scanning electron microscopy (SEM) micrographs (Figure 1) illustrate the spherical shape of nanoparticles.

The hydrophobic nature of the polystyrene nanoparticles used as support implies that the enzyme adsorption is governed by the hydrophobic interactions.[28] Therefore, those interactions should not be affected by changes in the pH adsorption. On the other hand, if electrostatic forces are important, changes over the isoelectric point of lipase will have a large impact on the binding constants.[29,30]

The data shown in Table 1 suggest no important differences in the amount of bound protein for the different values of pH studied. Hence, these results are clear evidence to

support the hypothesis that hydrophobic interactions are the driving force of the immobilization process.

Commercially available polystyrene resins were used for Cal-B adsorption by Gross *et al*.[31,32] Enzyme loadings varied between 5.1 and 8.7 μg/mg, that is around thirtyfold less than our obtained results for enzyme loading (comparison might be inappropriate, since the commercially polystyrene particle diameters range from 35 to 710 μm, that is much bigger than polystyrene nanoparticles we used for our studies).

Nevertheless, the pH of the buffer of the lipase solution affects the activity of the immobilized biocatalyst significantly, as can be observed in Figure 2. The highest enzyme activity was obtained when adsorption was conducted near the enzyme isoelectric point (pI 6.0).[33] It suggests that the enzyme is immobilized in its active configuration at this pH. Similar results were obtained by other authors[34,35] when a *Candida rugosa* lipase was immobilized on poly(acrylonitrile-*co*-maleic acid) hollow fibre and *Mucor javanicus* lipase on the SBA-15 mesoporous silica. According to them, lipase immobilization in pH range of 5.0 – 6.5 provided relatively high activity values because enzyme conformation, vital for enzymatic activity, changed with pH. The ionization state of the active site of the lipase molecule is affected by the pH of the buffer used in the immobilization process and activity is very sensitive to the pH of the solution during the binding step.[36] Inhibiting lipase activity by increasing the pH values is probably due to an unfavorable charge distribution on the amino acid residues, that produces a further activity decrease.[24]

Since the highest hydrolytic activity was observed when lipase adsorption was conducted at pH 6.8, all other steps of this work were carried out under these conditions.

Hydrolytic activity assay was also performed for crude enzyme powder and commercially available Novozyme 435 (0.772 mg of Cal-B was used) and results were compared with the activity obtained for Cal-B immobilized on polystyrene nanoparticles. Figure 3 shows that Cal-B immobilized on polystyrene nanoparticles performs much higher activity (4422.7 nmol *p*NP/min/mg Cal-B) than crude enzyme powder and Novozyme 435 (2396.0 and 3795.0 nmol *p*NP/min/mg Cal-B, subsequently).[37]

Conclusion

Cal-B was successfully immobilized on polystyrene nanoparticles synthesized by nanoprecipitation.

Although the amount of Cal-B adsorbed to polystyrene nanoparticles was independent of the pH of adsorption, indicating that hydrophobic interactions were the driving force of the immobilization process, immobilized enzyme activity was dependent on the pH of adsorption. The highest activity was obtained when lipase adsorption was conducted near the enzyme isoelectric point (pI 6.0), due to a favorable charge distribution on the amino acid residues, which prevented a possible change of enzyme conformation.

The hydrolysis reaction of *p*NPA in organic media by the immobilized enzyme were assayed and compared with those of the free enzyme and Novozyme 435. The activity of the enzyme was improved with immobilization on polystyrene nanoparticles.

Acknowledgement: The authors thank Regina Just for the preparation of the particles and Harry Nijland for recording the SEM micrographs.

Keywords: *Candida antarctica* lipase B; enzymes; nanoparticles; polystyrene

[1] A. Ghanem, *Tetrahedron* **2007,** *63*, 1721.

[2] E.M. Anderson, M. Karin, O. Kirk, *Biocatal. Biotransform.* **1998,** *16*, 181.

[3] R.D. Schmid, R. Verger, *Angew. Chem. Int. Ed.* **1998,** *37*, 1609.

[4] D. Goradia, J. Cooney, B.K. Hodnett, E. Magner, *J. Mol. Catal. B: Enzym.* **2005,** *37*, 231.

[5] E. Dumitriu, F. Secundo, J. Patarin, L. Fechete, *J. Mol. Catal. B: Enzym.* **2003,** *22*, 119.

[6] Y.X. Bai, Y.F. Li, Y. Yang, L.X. Yi, *Process Biochem.* **2006**, *41*, 770.

[7] N. Miletić, R. Rohandi, Z. Vuković, A. Nastasović, K. Loos, *React. Funct. Polym.* **2009**, *69*, 68.

[8] N. Miletić, K. Loos, *Aust. J. Chem.* **2009**, *62*, 799.

[9] A. Kumar, R.A. Gross, D. Jendrossek, *J. Org. Chem.* **2000,** *65*, 7800.

[10] A. Mahapatro, A. Kumar, B. Kalra, R.A. Gross, *Macromolecules* **2004,** *37*, 35.

[11] J. Hu, W. Gao, A. Kulshrestha, R.A. Gross, *Macromolecules* **2006,** *39*, 6789.

[12] J. Peeters, A.R.A. Palmans, M. Veld, F. Scheijen, A. Heise, E.W. Meijer, *Biomacromolecules* **2004,** *5*, 1862.

[13] L.W. Schwab, R. Kroon, A.J. Schouten, K. Loos, *Macromol. Rapid Commun.* **2008**, *29*, 794.

[14] P. Roach, D. Farrar, C.C. Perry, *J. Am. Chem. Soc.* **2005,** *127*, 8168.

[15] A.M. Dessouki, K.S. Atia, *Biomacromolecules* **2002,** *3*, 432.

[16] D. Duracher, A. Elaissari, F. Mallet, C. Pichot, *Langmuir* **2000,** *16*, 9002.

[17] G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Palomo, C. Mateo, A. Bastida, J. Coca, T. Haramboure, O. Hernandez-Justiz, M. Terreni, J.M. Guisan, *J. Mol. Catal. B: Enzym.* **2001,** *11*, 649.

[18] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernandez-Lafuente, J. Huguet, J.M. Guisan, *Biotechnol. Bioeng.* **1998,** *58*, 486.

[19] G.B. Sigal, M. Mrksich, G.M. Whitesides, *J. Am. Chem. Soc.* **1998,** *120*, 3464.

[20] S. Koutsopoulos, J. van der Oost, W. Norde, *Langmuir* **2004,** *20*, 6401.

[21] K. Loos, S.B. Kennedy, N. Eidelman, Y. Tai, M. Zharnikov, E.J. Amis, A. Ulman, R.A. Gross, *Langmuir* **2005**, *21*, 5237.

[22] S.M. Pancera, H. Gliemann, T. Schimmel, D.F.S. Petri, *J. Phys. Chem. B* **2006,** *110*, 2674.

[23] A.I.S. Brigida, A.D.T. Pinheiro, A.L.O. Ferreira, L.R.B. Goncalves, *Appl. Biochem. Biotechnol.* **2008,** *146*, 173.

[24] A. Salis, D. Meloni, S. Ligas, M.F. Casula, M. Monduzzi, V. Solinas, E. Dumitriu, *Langmuir* **2005,** *21*, 5511.

[25] A. Vaidya, E. Miller, J. Bohling, R.A. Gross, *Polym. Prep.* **2006,** *47*, 247.

[26] Y. Mei, A. Kumar, R.A. Gross, *Macromolecules* **2003**, *36*, 5530.

[27] O. Thioune, H. Fessi, J.P. Devissaguet, F. Puisieux, *Int. J. Pharm.* 1997, *146*, 233.

[28] D.S. Rodrigues, G.P. Cavalcante, A.L.O. Ferreira, L.R.B. Gonçalves, *Chem. Biochem. Eng. Q.* **2008,** *22*, 125.

[29] W, Norde, *Adv. Colloid Interface Sci.* **1986,** *25*, 267.

[30] T. Gitlesen, M. Bauer, P. Adlercreutz, *Biochim. Biophys. Acta* **1997,** *1345*, 188.

[31] B. Chen, M.E. Miller, R.A. Gross, *Langmuir* **2007**, *23*, 6467.

[32] B. Chen, E.M. Miller, L. Miller, J.J. Maikner, R.A. Gross, *Langmuir* **2007**, *23*, 1381.

[33] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, *Structure* **1994,** *4*, 293.

[34] P. Sabuquillo, J. Reina,G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, *Biochim. Biophys. Acta* **1998,** *1388*, 337.

[35] J.M.S. Rocha, M.H. Gil, F.A.P. Garcia, *J. Biotechnol.* **1998,** *66*, 61.

[36] P. Ye, Z.K. Xu, Z.G. Wang, J. Wu, H.T. Deng, P. Seta *J. Mol. Catal. B: Enzym.* **2005,** *32*, 115.

[37] N. Miletić, Z. Vuković, A. Nastasović, K. Loos, *J. Mol. Catal. B: Enzyme* **2008,** *69*, 68.

Table of Contents

Enzyme immobilization is a well known route for improving enzyme performance in non-natural environments. Here, we show that *Candida antarctica* lipase B immobilized on polystyrene nanoparticles, synthesized via a nanoprecipitation process, performed significantly higher activity than crude enzyme powder.

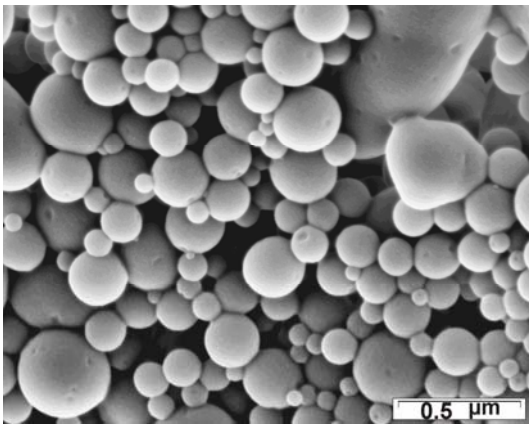

Table 1. Loading of Cal-B on polystyrene nanoparticles, obtained at different pH of the buffer of the immobilization solution.

pH	Enzyme loading[a)] [μg/mg]
5.0	231.2
6.8	248.0
8.0	241.4
9.5	240.7
11.5	243.1

[a)]Loadings were calculated from triple determinations within ±5% agreement

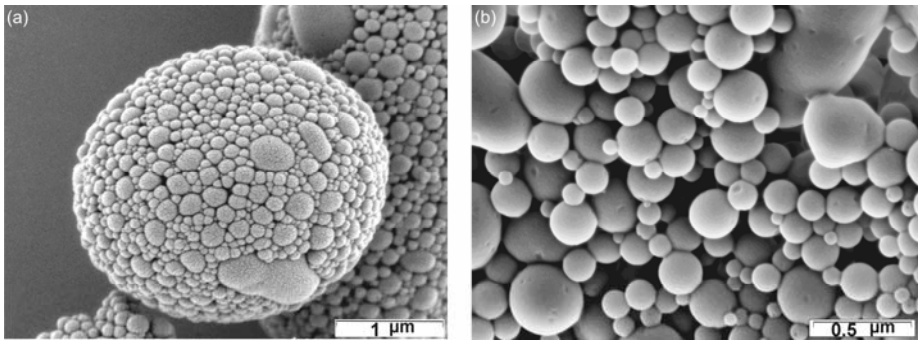

Figure 1. Scanning electron micrographs of polystyrene nanoparticles: (a) scale bar 1 µm; (b) scale bar 0.5 µm.

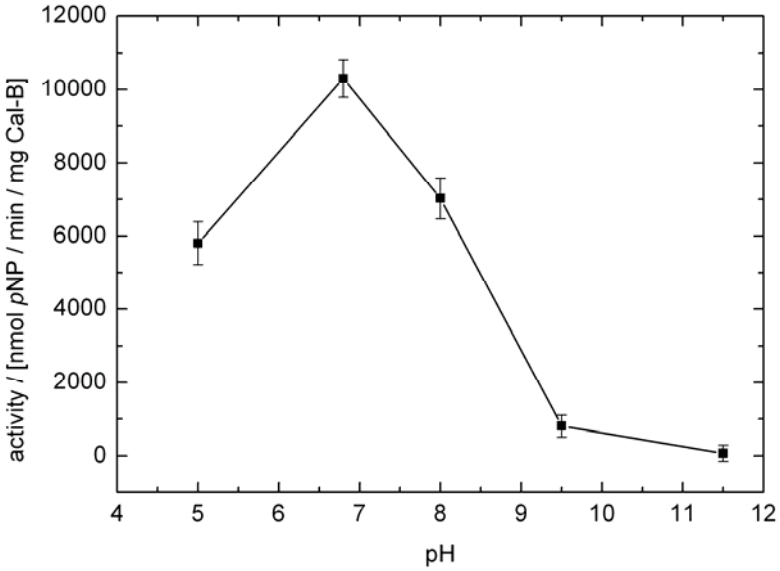

Figure 2. Hydrolytic enzyme activity of Cal-B physically adsorbed on polystyrene nanoparticles versus pH of the buffer solution used during the immobilization process.

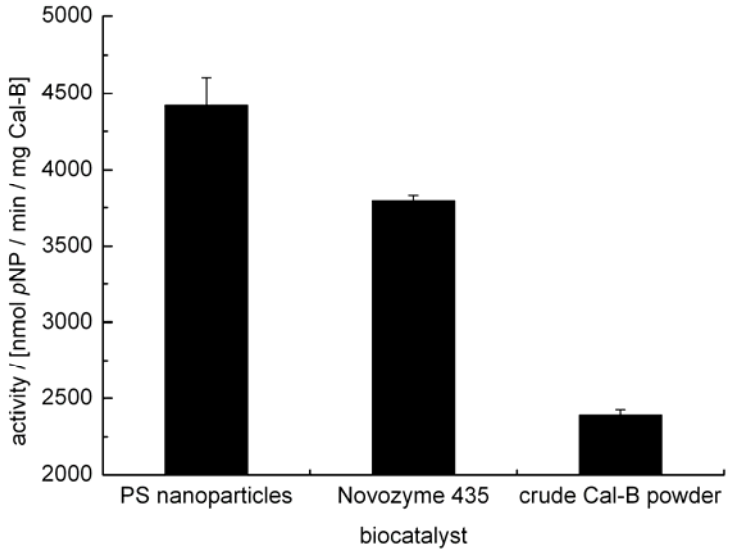

Figure 3. Hydrolytic enzyme activity of Cal-B catalysts.