Chapter 6

Immobilization of Candida antarctica lipase B on polystyrene nanoparticles

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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.

6.1. Introduction

Among lipases, Candida antarctica lipase B (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.\cite{1-3} By enzyme immobilization,
catalysts can be developed with significant advantages relative to free enzyme.\cite{4-6} Many literature reports describe the high utility of immobilized Cal-B for chemical transformations of low molar mass compounds,\cite{7,8} and polymerization reactions.\cite{9,10,11} Adsorption of an enzyme onto a surface can induce conformational changes which affect the rate and specificity of the catalyst.\cite{12} Therefore, immobilization research has largely focused on matrix selection and on optimizing immobilization conditions.\cite{13-16} For example, work has addressed support surface hydrophobicity\cite{17,18} and enzyme solution pH.\cite{15,19,20} These parameters have large influence on the total amount of enzyme loading and enzyme-catalyst activity.\cite{21,22} Hydrophobic binding of lipases by adsorption has proved successful due to its affinity for water/oil interfaces. Sugiura \textit{et al.} have demonstrated that lipases were adsorbed on any hydrophobic interface such as those originated by air-aqueous media,\cite{23} solvent-aqueous media\cite{24} and the surfaces of hydrophobically activated solids.\cite{25} Many catalysts based on lipase adsorption on hydrophobic supports have been developed and their effects on the activity reported. Sarada \textit{et al.} described that pancreatic lipase showed a 500-fold increase in its activity in the presence of hydrophobically coated glasses.\cite{26} A hyperactivation of lipase from \textit{Chromobacterium} after the immobilization of the lipase on the same materials was reported.\cite{27} It was also shown that lipases entrapped in hydrophobic sol-gels performed increased activity.\cite{28} Thus, the present work deals with the synthesis of polystyrene (PS) nanoparticles and its use as a support for the immobilization of \textit{Candida antarctica} lipase 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.

\textbf{6.2. Experimental section}

\textbf{6.2.1. Materials}

Cal-B in the form of a dried powder was purchased from Codexis® (Pasadena, CA, USA). Novozyme 435 was provided by Novozymes®. Polystyrene (M₉=100,000) was received from Avocado Research Chemicals, UK, and Pluronic® F-68 was received from Sigma-Aldrich, Germany. As solvent tetrahydrofuran p.a. (THF) from Merck, Germany, was used. All chemicals were used as supplied.
6.2.2. Synthesis of polystyrene nanoparticles

PS nanoparticles were prepared by nanoprecipitation process.\textsuperscript{[29]} PS (M\textsubscript{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 particle formation, the suspension was filtered 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 microscopy (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.

6.2.3. Enzyme immobilization by physical adsorption

Enzyme support, polystyrene nanoparticles (40 mg) were 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 shaken for 24 hours at 30 °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.

6.2.4. Hydrolytic activity

A 1,4-dioxane solution (5 ml) containing p-nitrophenyl acetate (pNPA) (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 (pNP) was determined by UV/VIS (PYE UNICAM SP8-200 UV/VIS spectrophotometer) at the \( \lambda_{\text{max}} \) (304 nm) of pNP. Hydrolytic activity for crude Cal-B powder, Cal-B physically adsorbed on polystyrene nanoparticles and Novozyme 435 are defined herein as the
nanomoles of pNPA hydrolyzed in 1,4-dioxane per time per unit of weight of enzyme (nmol of pNP/min/mg).

6.3. Results and discussion

6.3.1. SEM micrographs

Polystyrene was synthesized in the shape of nanoparticles. Figure 6.1. illustrates the scanning electron microscopy (SEM) micrographs and shows the spherical shape of nanoparticles.

Fig. 6.1. Scanning electron micrographs of polystyrene nanoparticles: (a) scale bar 1 μm; (b) scale bar 0.5 μm.

6.3.2. Effect of pH on lipase loading and hydrolytic activity of physically adsorbed lipase

The hydrophobic nature of the polystyrene nanoparticles used as support implies that the enzyme adsorption is governed by hydrophobic interactions.[30] 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.[31,32]

By analyzing the data shown in Table 6.1, no important differences in the amount of bound protein were observed for the different values of pH studied, supporting the hypothesis of hydrophobic interactions being the driving force of the immobilization process.
Table 6.1. Loading of Candida antarctica lipase B on polystyrene nanoparticles, obtained at different pH of the buffer of the immobilization solution.

<table>
<thead>
<tr>
<th>pH</th>
<th>enzyme loading$^A$ (μg/mg)</th>
</tr>
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<tbody>
<tr>
<td>5.0</td>
<td>231.2</td>
</tr>
<tr>
<td>6.8</td>
<td>248.0</td>
</tr>
<tr>
<td>8.0</td>
<td>241.4</td>
</tr>
<tr>
<td>9.5</td>
<td>240.7</td>
</tr>
<tr>
<td>11.5</td>
<td>243.1</td>
</tr>
</tbody>
</table>

$^A$Loadings were calculated from triple determinations within ±5% agreement.

Nevertheless, the pH of the buffer of the lipase solution affects the activity of the immobilized biocatalyst significantly, as can be observed in Figure 6.2.

![Figure 6.2](image)

**Fig. 6.2.** Hydrolytic enzyme activity of Cal-B physically adsorbed on polystyrene nanoparticles versus pH of the buffer solution used during the immobilization process.

The highest enzyme activity was obtained when adsorption was conducted near the enzyme isolectric point (pI 6.0)$^{[33]}$ suggesting that, at this pH, the enzyme is immobilized in its active configuration. Similar results were obtained by other authors$^{[34,35]}$ when immobilizing a *Candida rugosa* lipase on poly(acrylonitrile-co-maleic acid) hollow fibre and *Mucor javanicus* lipase on 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.\[^{21}\]

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 activity obtained for Cal-B immobilized on polystyrene nanoparticles. Figure 6.3 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).\[^{37}\]

![Fig. 6.3. Hydrolytic enzyme activity of Cal-B catalysts.](image)

### 6.4. Conclusions

*Candida antarctica* lipase B was successfully immobilized on polystyrene nanoparticles synthesized by nanoprecipitation. Although the amount of Cal-B adsorbed on polystyrene nanoparticles was independent of the pH of adsorption, indicating that hydrophobic interactions are 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 pNPA in organic media by the immobilized enzyme was assayed and compared with those of the free enzyme and Novozyme 435. The activity of the enzyme was improved tremendously with immobilization on polystyrene nanoparticles.

6.5. References