Activity, conformation and dynamics of cutinase adsorbed on poly(methyl methacrylate) latex particles

R.P. Baptista a, A.M. Santos b, A. Fedorov b, J.M.G. Martinho b, C. Pichot c, A. Elaïssari c, J.M.S. Cabral a, M.A. Taipa a,*

a Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
b Centro de Química-Física Molecular, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
c Unité Mixte CNRS-BioMérieux, UMR-2142, ENS Lyon, 46 Allée d’Italie, 69364 Lyon Cedex 07, France

Received 5 August 2002; received in revised form 17 January 2003; accepted 27 January 2003

Abstract

The adsorption of a recombinant cutinase from Fusarium solani pisi onto the surface of 100 nm diameter poly(methyl methacrylate) (PMMA) latex particles was evaluated. Adsorption of cutinase is a fast process since more than 70% of protein molecules are adsorbed onto PMMA at time zero of experiment, irrespective of the tested conditions. A Langmuir-type model fitted both protein and enzyme activity isotherms at 25 °C. \( \Gamma_{\text{max}} \) increased from 1.1 to 1.7 mg m\(^{-2}\) and \( U_{\text{max}} \) increased from 365 to 982 U m\(^{-2}\) as the pH was raised from 4.5 to 9.2, respectively. A decrease (up to 50%) in specific activity retention was observed at acidic pH values (pH 4.5 and 5.2) while almost no inactivation (\( \eta_{\text{act}} \approx 87\%\)) was detected upon adsorption at pH 7.0 and 9.2. Concomitantly, far-UV circular dichroism (CD) spectra evidenced a reduction in the \( \alpha \)-helical content of adsorbed protein at acidic pH values while at neutral and alkaline pH the secondary structure of adsorbed cutinase was similar to that of native protein. Fluorescence anisotropy decays showed the release of some constraints to the local motion of the Trp69 upon protein adsorption at pH 8.0, probably due to the disruption of the tryptophan–alanine hydrogen bond when the tryptophan interacts with the PMMA surface. Structural data associated with activity measurements at pH 7.0 and 9.2 showed that cutinase adsorbs onto PMMA particles in an end-on orientation with active site exposed to solvent and full integrity of cutinase secondary structure. Hydrophobic interactions are likely the major contribution to the adsorption mechanism at neutral and alkaline pH values, and a higher amount of protein is adsorbed to PMMA particles with increasing temperature at pH 9.2. The maximum adsorption increased from 88 to 140 mg cutinase per g PMMA with temperature raising from 25 to 50 °C, at pH 9.2.

Keywords: PMMA; Cutinase; Adsorption; Lipolytic activity; Circular dichroism; Fluorescence

1. Introduction

Protein interactions with solid surfaces can affect the performance of many materials and...
processes, in areas ranging from medicine to biochemical engineering. Particularly, adsorption of proteins on microspheres (monodisperse latex particles) is of great interest for biomedical applications such as artificial tissues and organs, biosensors, drug delivery systems and solid-phase immunoassays (Whalgren and Arnebrant, 1991; Yoon et al., 1996; Sivakumar and Panduranga, 2002). The mechanism underlying protein adsorption is determined by many variables including pH, ionic strength, temperature, the properties of protein molecules and solid matrix, and the nature of the solvent and other components in the medium. A major factor influencing protein adsorption is the surface energy, and it has been frequently reported that hydrophobic surfaces adsorb more protein molecules than hydrophilic ones (Whalgren and Arnebrant, 1991; Kleijn and Norde, 1995; Gitlesen et al., 1997). Carboxylated microspheres can be used as model colloids for studying protein adsorption because they can involve a complex interplay of van der Waals forces, hydrogen bonding and electrostatic and hydrophobic interactions (Yoon et al., 1996). In practice, the interactions by themselves are reversible, however, several authors have shown that protein adsorption is irreversible (Yoon et al., 1996; Gitlesen et al., 1997; Norde and Zoungrana, 1998) and protein does not desorb from the carrier without the use of detergents (Gitlesen et al., 1997) and/or high salt concentrations. Adsorption isotherms model protein–sorbent interactions, quantifying properties such as the affinity of the protein to the adsorption matrix, and the maximum number of protein molecules in the adsorption layer. These features determine the adsorption yield, which depends on spatial distribution, lateral interactions, and conformation–orientation of adsorbed protein molecules.

Although adsorption on solid surfaces has been widely investigated for a diversity of systems, information on the conformational changes upon adsorption is not often addressed. Upon adsorption, proteins undergo conformational rearrangements dependent on the type and strength of interaction established with the support. Biophysical methods provide the means to unravel and understand at a molecular level the mechanisms underlying the interactions between biomolecules and adsorption surfaces. Correlation of structural data with biological activity measurements allows valuable structure–function relationships for a given system.

The focus of this work is the study of the effect of pH and temperature on the adsorption of recombinant cutinase from *Fusarium solani pisi* onto PMMA nanospheres bearing carboxylate groups at the surface. Cutinase is a protein with 197 residues with $M_W \sim 22$ KDa (Carvalho et al., 1999) and $pI$ 7.6 (Koops et al., 1999). It is an enzyme of industrial interest, owing to its potential use in synthesis of fats and oils and in the detergent industry (Carvalho et al., 1999). Cutinase structure is composed of a central $\beta$-sheet surrounded by five $\alpha$-helices and contains one single tryptophan residue (Trp$_{69}$) which is located in the vicinity of a disulphide bridge (Cys$_{31}$–Cys$_{109}$) (Longhi and Cambillau, 1999). Retention of biological activity of cutinase upon adsorption onto PMMA was assessed and correlated with structural data. Time-resolved tryptophan fluorescence was used to probe molecular dynamics of cutinase upon adsorption, and the overall secondary structure content of protein along the adsorption process was monitored by circular dichroism (CD) measurements.

2. Materials and methods

2.1. Protein

Recombinant cutinase was cloned in pMa5-L (Lauwereys et al., 1991) and over-expressed in *Escherichia coli* WK-6. Cutinase was purified through osmotic shock, acid precipitation, dialysis and two sequential anionic chromatographic steps, followed by a final dialysis (Sebastião et al., 1996). A lyophilized powder (purity > 95% w/w) was obtained. Stock solutions of cutinase (2 mg ml$^{-1}$) ($\varepsilon_{280} = 10.853$ M$^{-1}$ cm$^{-1}$) were prepared in 10 mM buffer pH 4.5–9.2.
2.2. Sorbent

PMMA microspheres of 100 nm diameter (determined by dynamic light scattering-DLS) and bearing carboxylic groups were produced by emulsion polymerization (Santos et al., 2003). A reactor of 50 ml capacity equipped with a glass anchor-type stirrer (300 rpm), condenser and nitrogen inlet was used. The initiator 4,4'-azo-bis(4-cyanopentanoic acid) (ACPA) (0.05 g) and sodium dodecyl sulfate (0.03 g) were added to methyl methacrylate (5 ml). The reaction was performed in an aqueous buffer solution (10 mM solution of NaHCO₃, pH values between 8.5 and 8.9) at 70°C. Polymerization was allowed for around 4 h. The particles were electrostatically stabilized by carboxylic groups at the surface coming from the initiator ACPA.

2.3. Protein assay

The amount of cutinase in solution was measured by the bicinchoninic acid (BCA) method patented by Pierce (Smith et al., 1985) in a microplate assay. Samples of 50 µl were added to 200 µl detection reagent, the plate was then incubated during 1 h at 37°C and absorbance read at 595 nm in a BIORAD 3550 microplate reader. A standard curve with bovine serum albumin in a concentration ranging from 0 to 1 mg ml⁻¹ was included in all microplate assays.

2.4. Enzyme activity assay

Lipase activity of cutinase was determined titrimetrically by the ‘pH-stat’ method (Brocklehurst, 1995) with a Metrohm Titirino 702-SM titrator, at pH 8.5 and room temperature. The reaction mixture consisted of 30 ml of 3 mM Tris–HCl with Arabic gum 3% (w/v) as emulsifying agent and 75 mM of tributyrin (obtained from Fluka) as substrate. The reaction was initiated with the addition of free or adsorbed cutinase solution to achieve an initial enzyme concentration of 75 nM. The amount of NaOH added was then recorded every 10 s. One enzyme activity unit (U) was defined as 1 µmol of fatty acid released per milliliter per minute on standard assay conditions.

2.5. Cutinase adsorption

The adsorption of cutinase onto PMMA latex particles was performed with PMMA dispersions of 0.5% (w/v). Latex particles were washed with Milli-Q water and centrifuged at 9844 × g, during 1 h, at 10°C. Adsorption was carried out in a 10 mM buffer solutions at pH 4.5, 5.2, 7.0 and 9.2 in a total volume of 10 ml, at room temperature (≈ 25°C). Two concentrations of cutinase were used: 1.2 mg m⁻² (0.33 mg ml⁻¹) and 1.5 mg m⁻² (0.40 mg ml⁻¹). The time course of cutinase adsorption was followed during ~ 60 h. The PMMA–protein dispersions were centrifuged during 30 min at 25°C and the amount of cutinase in the supernatant was quantified by the BCA method as described above. The amount of adsorbed cutinase was then calculated by the difference between the amount of cutinase in the solution before adsorption and the cutinase remaining in the supernatant after adsorption.

Adsorption isotherms were obtained by the following procedure: known amounts of cutinase were incubated in the presence of 0.5% (w/v) of PMMA during 4 h in an orbital shaker (80 rpm) under temperature control. Thereafter, the protein–latex solutions were centrifuged at 8944 × g during 30 min, at the temperature of the assay, and the protein content in the supernatant was determined. The lipolytic activity of adsorbed cutinase was measured after redispersion of latex particles in the same volume of fresh buffer solution. The amount of protein and lipolytic activity adsorbed per unit area of PMMA was then plotted versus the equilibrium protein concentration in solution. The experimental curves were fitted with Langmuir-type isotherms using Eqs. (1a) and (1b), for protein and activity, respectively:

\[
\Gamma_{ads} = \frac{\Gamma_{max} \times C_{eq}}{K + C_{eq}} \quad (1a)
\]

\[
U_{ads} = \frac{U_{max} \times C_{eq}}{K + C_{eq}} \quad (1b)
\]

where \(\Gamma_{ads}\) and \(U_{ads}\) are the amount and lipolytic activity of adsorbed cutinase per unit area of PMMA, \(\Gamma_{max}\) and \(U_{max}\) are the maximum amount...
of protein and lipolytic activity adsorbed per unit area of PMMA, \( C_{eq} \) is the equilibrium protein concentration in liquid phase and \( K \) is the equilibrium constant.

### 2.6. Circular dichroism

The effect of adsorption onto PMMA in the integrity of secondary structure of cutinase was studied by analysis of CD spectra, measured during 4 h of incubation at 25 °C, at different pH values. CD spectra were obtained with a JASCO spectropolarimeter, model J-720, at room temperature. A cylindrical quartz cell with 0.1-cm path length was used for far-UV CD spectra. The results were expressed in terms of mean residue ellipticity (\( [\Theta]_{\text{MRW}} \)) (in \( \circ \text{ cm}^2 \text{ dmol}^{-1} \)) and were determined according to Eq. (2):

\[
[\Theta]_{\text{MRW}} = \frac{MRW(\theta)}{10lC}
\]

where \( MRW \) is the mean residue weight (114.8), \( \theta \) is the observed ellipticity (in degrees), \( l \) is the path length (in cm), and \( C \) is the protein concentration (in g ml\(^{-1}\)). The spectrum of adsorbed cutinase was the average of three measurements after subtraction of buffer and PMMA baselines.

### 2.7. Fluorescence measurements

The fluorescence decay curves were obtained by the single photon timing technique using picosecond laser excitation. The 300 nm excitation pulse (5–6 ps) was achieved by doubling the laser output light of a Coherent 701-2 dye (Rhodamine 6G) laser synchronously pumped by a mode-locked Coherent Innova 400-1 argon ion laser. The light beam was expanded before entering the sample solution and neutral density filters were used in order to minimize the photoreactions of cutinase. The fluorescence was selected by a Jovin–Yvon HR320 monochromator with a grating of 100 lines mm\(^{-1}\) (20 nm bandwidth) and detected by a Hamamatsu 2809U-01 microchannel plate photo-multiplier. The fluorescence decays were made using polarized vertical light being the fluorescence selected by a vertical or horizontal polarizer for anisotropy measurements and at the magic angle (54.7°) for the fluorescence decays.

### 3. Results

#### 3.1. Cutinase adsorption onto PMMA

The time course of cutinase adsorption onto PMMA microspheres was evaluated at different pH values (4.5, 5.2, 7.0 and 9.2) (Fig. 1). More than 70% of cutinase molecules were adsorbed onto PMMA at time zero of experiment, at all pH values. The adsorption was faster at pH 9.2, being saturation achieved after 10 h of incubation time as compared with much higher saturation times at pH 4.5 and 5.2. No significant effects of the initial protein concentration in the liquid phase were observed at pH 7.0 and 9.2. On the contrary, at pH 4.5 and 5.2 a slight increase in protein bulk concentration (from 0.35 to 0.40 mg ml\(^{-1}\)) lead to a slight decrease of the adsorption yield. At acid pH values it is expected that adsorption of cutinase (\( pI = 7.6 \)) is driven by electrostatic attractions because the carboxyl groups at the surface of PMMA are ionized (\( pK_a = 4.5 \)). Indeed, the adsorption yield after 4 h of incubation decreased significantly (around 80%) as NaCl concentration was increased from 0 to 300 mM, at pH 4.5 and 5.2 (data not shown). The high adsorption yields observed at pH 7.0 (pH \( \approx pI \)) and pH 9.2 can only be explained by dominant hydrophobic interactions. This was confirmed by more than 90% decrease in the adsorption yield in the presence of 1% (v/v) of Triton X-100 at pH 7.0 and 9.2 (data not shown).

Adsorption isotherms at different pH values were obtained after 4 h of incubation at pH 4.5, 5.2, 7.0 and 9.2, at 25 °C (Fig. 2). During this period the PMMA latex particles are stable at all pH values (even at acidic pH, the latex dispersions are stable for about 6 h). Maximum amount of adsorbed cutinase (\( \Gamma_{\text{max}} \)) and lipolytic activity (\( U_{\text{max}} \)) increased with pH. \( \Gamma_{\text{max}} \) increased from 1.1 to 1.7 mg m\(^{-2}\) (220 to 340 mg g\(^{-1}\)) and \( U_{\text{max}} \)}
increased from 365 to 982 U m\(^{-2}\) as the pH was increased from 4.5 to 9.2 (Table 1). Protein adsorption mass and activity isotherms showed well-defined plateaus of adsorption for equilibrium cutinase concentrations in solution, \(C_{eq}\) lower than 0.3 mg ml\(^{-1}\), which are typical values for adsorption of globular proteins (Kleijn and Norde, 1995). A Langmuir type model fitted both the mass and activity of adsorbed cutinase onto PMMA particles. The values obtained for the

![Graph 1](image1)

**Fig. 1.** Time course of adsorption of 1.2 mg m\(^{-2}\) (open symbols) and 1.5 mg m\(^{-2}\) (closed symbols) of cutinase onto 0.5\% (w/v) of PMMA, at pH 4.5 (△, ■), 5.2 (○, ●), 7.0 (◇, ◆) and 9.2 (□, □).

![Graph 2](image2)

**Fig. 2.** Langmuir adsorption isotherms of cutinase onto PMMA at pH 4.5 (△, ■), 5.2 (○, ●), 7.0 (◇, ◆) and 9.2 (□, □), at 25 °C. Protein (A) and lipolytic activity (B) of cutinase adsorbed per PMMA area were plotted against the protein concentration in the bulk, at equilibrium.
Equilibrium constant $K$ (Table 1) were similar at all tested conditions.

The dimensions of cutinase are $45 \times 30 \times 30$ Å$^3$ (Carvalho et al., 1999), which leads to a monolayer density of close-packed protein molecules adsorbed in an end-on orientation, $\Gamma_{\text{mono}} \approx 1.2$ mg m$^{-2}$. This calculation was done on the assumption that the surface of cutinase adsorbed corresponds to the minor radius of cutinase molecule (15 Å) and that the PMMA surface is flat. Adsorption saturation at pH 7.0 and 9.2 was 1.7 mg m$^{-2}$, which is compatible with a monolayer of native-like molecules adsorbed in a molecular rearrangement close to an end-on orientation. At acidic pH values, saturation was slightly below the monolayer density ($\sim 1.2$ mg m$^{-2}$). Only 45 and 56% of the initial specific activity was achieved after adsorption at pH 4.5 and 5.2, respectively (Table 1). On the contrary, almost no enzyme inactivation ($\eta_{\text{act}} \geq 87-94\%$) was observed at pH 7.0 and 9.2.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\Gamma_{\text{max}}$ (mg m$^{-2}$)</th>
<th>$K \times 10^3$ (m$^{-2}$ mg$^{-1}$)</th>
<th>$U_{\text{max}}$ (U m$^{-2}$)</th>
<th>$n$</th>
<th>Activity retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>1.1±0.08</td>
<td>5.9±2.0</td>
<td>365±125</td>
<td>0.7±0.1</td>
<td>44.5±11.6</td>
</tr>
<tr>
<td>5.2</td>
<td>1.4±0.06</td>
<td>4.9±4.1</td>
<td>405±150</td>
<td>0.8±0.2</td>
<td>55.9±19.0</td>
</tr>
<tr>
<td>7.0</td>
<td>1.7±0.12</td>
<td>5.1±2.2</td>
<td>921±374</td>
<td>1.2±0.1</td>
<td>86.4±16.6</td>
</tr>
<tr>
<td>9.2</td>
<td>1.7±0.09</td>
<td>6.5±3.0</td>
<td>982±374</td>
<td>1.2±0.1</td>
<td>94.3±20.2</td>
</tr>
</tbody>
</table>

Table 1
Saturation of protein mass ($\Gamma_{\text{max}}$) and lipolytic activity ($U_{\text{max}}$) of cutinase adsorbed onto PMMA at pH 4.5, 5.2, 7.0 and 9.2

Equilibrium constant $K$ was calculated according to Eqs. (1a) and (1b). An end-on orientation was assumed for the calculation of the adsorbed protein layers ($n$) ($\Gamma_{\text{mono}} \approx 1.2$ mg m$^{-2}$). Structure–function correlation was accessed by retention of cutinase activity upon adsorption.

The effect of temperature on adsorption saturation ($\Gamma_{\text{max}}$), number of adsorbed layers ($n$) and specific activity retention of cutinase at pH 9.2 (Table 2).

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>$\Gamma_{\text{max}}$ (mg m$^{-2}$)</th>
<th>$n$</th>
<th>Activity retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>1.7±0.09</td>
<td>1.2±0.10</td>
<td>100</td>
</tr>
<tr>
<td>28</td>
<td>1.9±0.02</td>
<td>1.4±0.08</td>
<td>110</td>
</tr>
<tr>
<td>32</td>
<td>2.1±0.03</td>
<td>1.5±0.02</td>
<td>110</td>
</tr>
<tr>
<td>40</td>
<td>2.1±0.02</td>
<td>1.5±0.02</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>2.6±0.11</td>
<td>1.9±0.10</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2
Effect of temperature in adsorption saturation ($\Gamma_{\text{max}}$), number of adsorbed layers ($n$) and specific activity retention of cutinase at pH 9.2

in the absence of latex particles, after 4 h of incubation time at all tested temperatures.

3.3. Conformation and dynamics of adsorption

The adsorption of cutinase onto the surface of PMMA microspheres was also probed by far-UV CD and by the intrinsic fluorescence of its single tryptophan residue (Trp$_{\text{ex}}$). The integrity of secondary structure of cutinase was monitored by far-UV CD during incubation. At neutral and alkaline pH, the helical content in protein structure was followed along the incubation time by changes in the mean residual molar ellipticity value at 222 nm. Adsorption saturation was 1.7 to 2.6 mg m$^{-2}$ as the incubation temperature was raised from 25 to 50 °C (Table 2). A higher amount of protein was adsorbed onto PMMA surface at 50 °C and no inactivation of cutinase was observed in the absence of latex particles, after 4 h of incubation time at all tested temperatures. The adsorption of cutinase onto the surface of PMMA microspheres was also probed by far-UV CD and by the intrinsic fluorescence of its single tryptophan residue (Trp$_{\text{ex}}$). The integrity of secondary structure of cutinase was monitored by far-UV CD during incubation. At neutral and alkaline pH, the helical content in protein structure was followed along the incubation time by changes in the mean residual molar ellipticity value at 222 nm. Adsorption saturation was 1.7 to 2.6 mg m$^{-2}$ as the incubation temperature was raised from 25 to 50 °C (Table 2). A higher amount of protein was adsorbed onto PMMA surface at 50 °C and no inactivation of cutinase was observed in the absence of latex particles, after 4 h of incubation time at all tested temperatures. The adsorption of cutinase onto the surface of PMMA microspheres was also probed by far-UV CD and by the intrinsic fluorescence of its single tryptophan residue (Trp$_{\text{ex}}$). The integrity of secondary structure of cutinase was monitored by far-UV CD during incubation. At neutral and alkaline pH, the helical content in protein structure was followed along the incubation time by changes in the mean residual molar ellipticity value at 222 nm. Adsorption saturation was 1.7 to 2.6 mg m$^{-2}$ as the incubation temperature was raised from 25 to 50 °C (Table 2). A higher amount of protein was adsorbed onto PMMA surface at 50 °C and no inactivation of cutinase was observed in the absence of latex particles, after 4 h of incubation time at all tested temperatures.

3.3. Conformation and dynamics of adsorption

The effect of temperature on adsorption isothersms was studied at pH 9.2 (Table 2). At this pH value, more cutinase molecules are adsorbed to PMMA as compared with acidic pH values and the thermal stability of protein structure is high, $T_m = 53$ °C (Baptista et al., 2000). Adsorption saturation ($\Gamma_{\text{max}}$) increased from 1.7 to 2.6 mg m$^{-2}$ as the incubation temperature was raised from 25 to 50 °C (Table 2). A higher amount of protein was adsorbed onto PMMA surface at 50 °C and no inactivation of cutinase was observed in the absence of latex particles, after 4 h of incubation time at all tested temperatures. The adsorption of cutinase onto the surface of PMMA microspheres was also probed by far-UV CD and by the intrinsic fluorescence of its single tryptophan residue (Trp$_{\text{ex}}$). The integrity of secondary structure of cutinase was monitored by far-UV CD during incubation. At neutral and alkaline pH, the helical content in protein structure was followed along the incubation time by changes in the mean residual molar ellipticity value at 222 nm. Adsorption saturation was 1.7 to 2.6 mg m$^{-2}$ as the incubation temperature was raised from 25 to 50 °C (Table 2). A higher amount of protein was adsorbed onto PMMA surface at 50 °C and no inactivation of cutinase was observed in the absence of latex particles, after 4 h of incubation time at all tested temperatures.
one for native cutinase after 4 h of incubation. At pH 7.0 the decrease observed in the value of $[\Theta]_{222}$ points to a recovery of secondary structure after an initial loss upon adsorption. No significant changes were observed in CD spectra of adsorbed cutinase at pH 9.2.

The fluorescence spectra of cutinase free in solution and when adsorbed onto PMMA latex particles by excitation at 300 nm was typical of tryptophan. The fluorescence decay curves $I_F(t)$ were fitted with a sum of four exponentials (Eq. (3a)):

$$I_F(t) = \sum_{i=1}^{4} a_i \exp(-t/\tau_i)$$

where $a_i$ are the pre-exponential factors and $\tau_i$ the lifetimes. At room temperature, the lifetimes for cutinase are $\tau_1 = 0.05$ ns, $\tau_2 = 0.4$ ns, $\tau_3 = 2.5$ ns and $\tau_4 = 7.0$ ns, slightly dependent on pH value (Martinho et al., 2003). The short component is due to the hydrogen bond tryptophan (Trp69) with alanine (Ala32) that is strongly quenched by a disulphide bridge in proximity, while the others are due to tryptophan conformers. Upon cutinase adsorption the pre-exponential factors and lifetimes of the components vary, suggesting that the tryptophan interacts with the PMMA surface. The tryptophan fluorescence anisotropy $r(t)$ was fitted with a sum of two exponentials according to Eq. (3b):

$$r(t) = \beta_1 \exp(-t/\theta_1) + \beta_2 \exp(-t/\theta_2)$$

where $\beta_{1,2}$ are the pre-exponential factors and $\theta_{1,2}$ are the correlation times. The anisotropy at time zero for the free protein at pH 8.0 and 22°C is 0.25, a value typical of tryptophan in solution and in proteins (Fig. 4). This value decreases substantially for the adsorbed cutinase due to the dispersion of the fluorescence light by the latex particles. The long relaxation time ($\theta_2 = 14$ ns) was attributed to the rotation of the protein as a whole while the short lifetime ($\theta_1 = 0.5$ ns) is due to the local motion of single tryptophan residue of cutinase (Trp69). Upon cutinase adsorption the long lifetimes goes to infinity due to the immobilization of the protein, while short lifetime decreases suggesting that tryptophan residue becomes more mobile upon cutinase adsorption (Fig. 4).

4. Discussion

Protein adsorption onto solid surfaces is controlled by the properties of the support surface, the nature of protein molecule and the solution conditions. The surface of cutinase is complex in nature, with differences in characteristics such as hydrophobicity and charge within the pH range 4.5–9.2 (Petersen et al., 2001). The presence of titrable carboxyl groups in the surface of PMMA,
makes the adsorption mechanism of cutinase into a compromise between hydrophobic and electrostatic interactions, while the contribution of van der Waals and hydrogen bond interactions are likely to be relatively small.

Adsorption of cutinase onto PMMA particles is a fast process, owing to its high affinity to PMMA particles within the pH range 4.5–9.2, at 25°C (Fig. 1). Saturation was compatible with a close-packed monolayer of cutinase molecules adsorbed at all pH values. Nevertheless, more protein molecules were adsorbed near the isoelectric point of cutinase ($pI = 7.6$). This could be due to (i) minimum intramolecular and/or lateral repulsions of adsorbed protein molecules in the neighborhood of the protein isoelectric point (Whalgren and Arnebrant, 1991) allowing a more compact monolayer in sorbent surface, and also to (ii) different orientations of protein molecules upon adsorption to PMMA at different pH values.

A considerable extent of electrostatic interactions is likely the driving force for adsorption at acid pH values. At pH 4.5, cutinase surface displays a positive net charge due to the ionization of aspartic and glutamic acid residues (PDB entry 1AGY). The negatively charged carboxylate groups at PMMA surface can interact with positively charged residues on the surface of cutinase allowing electrostatic attractions. The addition of 300 mM NaCl also confirms that electrostatic forces play an important role in cutinase adsorption at acid pH values. The decrease in specific activity at acidic pH values could be due to structural rearrangements of cutinase upon adsorption or prevalent adsorption of enzyme molecules in a side-on orientation, with subsequent hindrance of substrate accessibility to the active site. A combination of both effects may explain the lower amount of adsorbed protein and the significant inactivation of adsorbed cutinase at acidic pH values. The decrease in $\alpha$-helical content monitored by far-UV CD upon adsorption at pH 4.5 and 5.2 may be related to a side-on orientation of cutinase molecules upon adsorption as its secondary structure is composed of a central $\beta$-sheet (five parallel strands) surrounded by four $\alpha$-helices (Longhi and Cambillau, 1999).

At neutral and more alkaline pH values all carboxyl groups present in PMMA surface are ionized and cutinase net charge becomes negative. A significant contribution of hydrophobic interactions in the adsorption mechanism seems to explain the higher adsorption saturation ($\Gamma_{max}$) observed at 7.0 and 9.2 (Table 1) as well as the increase of the amount of protein adsorbed onto PMMA at higher temperatures (Table 2). The fluorescence studies confirmed that tryptophan residue interacts with the PMMA surface with a high local mobility upon cutinase adsorption at pH 8.0. The release of some constraints to the local
motion of the tryptophan is probably due to the disruption of the tryptophan–alanine hydrogen bond (Prompers et al., 1999) when the tryptophan interacts with the PMMA surface. The results of anisotropy measurements at pH 8.0 correlate well with adsorption data showing a more compact monolayer of cutinase molecules on PMMA particles at neutral and alkaline pH values (Table 1). The Trp69 residue is located in the opposite side of the active catalytic triad Ser$_{120}$—Asp$_{175}$—His$_{188}$ (Longhi and Cambillau, 1999). Adsorption in an end-on orientation is compatible with the secondary structure integrity, and with a full exposure of the active site leading to a high biological activity retention of adsorbed cutinase at pH 7.0 and 9.2.

References


