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ORIGINAL ARTICLE

Enzymatic removal of protein fouling from self-assembled cellulosic nanofilms: experimental and modeling studies

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Abstract

Protein fouling is a serious problem in many food, pharmaceutical and household industries. In this work, the removal of rubisco protein fouling from cellulosic surfaces using a protease (subtilisin A) has been investigated experimentally and mathematically. The cellulosic surfaces were prepared using self-assembled monolayers (SAMs) on a surface plasmon resonance biosensor (chip) surface after conjugating cellulose to α-lipoic acid. Rubisco adsorption on the prepared cellulosic SAMs was found to be irreversible, leading to the creation of a tough protein fouling. The heterogeneous enzymatic cleansing of such tough fouling involves enzyme transfer to the surface and the subsequent removal of the rubisco via protease activity. In this work, these two processes were decoupled, allowing enzyme transfer and enzymatic surface reaction to be parameterized separately. Mathematical modeling of the enzymatic cleaning of protein fouling from cellulosic SAMs revealed that enzymatic mobility at the interface is an important factor. The approach presented in this work might be useful in designing better protein fouling-resistant surfaces. It could also be used to guide efforts to screen and gauge the cleaning performance of detergent–enzyme formulations.

Keywords Self-assembly of monolayers (SAMs) · Surface plasmon resonance (SPR) · Protein fouling · Enzymatic reactions · Mathematical modeling

Introduction

Contact between solid surfaces and proteins, such as in the case of food and pharmaceutical processing, surgical and dental tools, contact lenses, fabrics and dishes, leads to the deposition of undesirable protein films. For hygienic reasons, these protein films must be efficiently removed from the contaminated surfaces. Harsh thermal and/or chemical treatments (e.g., with acid or alkali) might remove protein fouling; however, harsh treatments are not always feasible, particularly for household applications (e.g., fabric and dish cleaning). Traditional detergent products, despite being safe for household cleaning processes, are not effective in removing tough protein fouling. Accordingly, modern detergent formulations incorporate enzymes (Fulton et al. 2015), especially proteases such as subtilisin A (also called subtilisin Carlsberg) (Blanch and Clark 1996; Donlon 2007; El Hadj-Ali et al. 2007; Haring and Schreier 1998; Maurer 2004; Naganthran et al. 2017; Vojcic et al. 2015; Watson 2006), to improve the removal of protein fouling from solid surfaces.

Enzymes are biocatalysts capable of degrading protein molecules into smaller fragments that are easily washed off the surface by a flow of cleaning solution. Since enzymes are usually expensive relative to other detergent ingredients, the amount of enzyme to be added to a detergent formulation is a key economical factor (Zhang et al. 2014). Optimizing the amount of enzyme in a detergent formulation requires understanding of its transport to the surface-bound protein fouling and the subsequent proteolysis of the fouling (Onaizi et al. 2009a, 2010). Such processes depend to a certain extent on the level of the enzyme in the cleaning solution. However, there is a threshold enzyme level, beyond which extra amounts of the enzyme in the cleaning solution will not bring any further significant benefit. The current practice in detergent formulations is mainly based on experience rather than scientific models (Schlijper 2003). The complexity of enzymatic cleaning of protein foulings from solid surfaces might have contributed to the lack of science-informed optimization of enzyme

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levels in detergent formulations. Thus, the key objective of the current work is to uncover some fundamental knowledge of the removal of tough protein fouling from cellulosic surfaces (representing cotton fabrics) using a powerful enzyme (subtilisin A) through a combined experimental and theoretical (i.e., modeling) approach.

Although, there have been a number of attempts (Esker et al. 2000; Fang et al. 2005; Foose et al. 2008; Lee et al. 2005; Nayak et al. 2007) to model the removal of protein fouling from solid surfaces using enzymes, these attempts were either over-simplified or inadequately formulated. Some of them ignored enzyme transfer to the surface-bound protein fouling (Foose et al. 2008; Nayak et al. 2007) while others did not differentiate between enzyme transfer and enzyme–protein complex formation (Fang et al. 2005; Lee et al. 2005); the latter is physically and chemically very different from enzyme adsorption. The enzyme transfer to the surface-bound protein fouling can be represented by the following ordinary differential equation (ODE), assuming that enzyme adsorption barrier rather than mass transfer is limiting, which is a reasonable assumption since adsorption parameters have been observed to vary with the chemistry of the surface underneath the protein films under the same hydrodynamic conditions (Onaizi et al. 2009a, 2010):

$$\frac{\mathrm{d}\Gamma_{\mathrm{E}}}{\mathrm{d}t} = k_a C_{\mathrm{E}}\left(\Gamma_{\mathrm{E},\infty} - \Gamma_{\mathrm{E}}\right) - k_{\mathrm{d}}\Gamma_{\mathrm{E}}. \tag{1}$$

The above Langmuir-based adsorption rate equation is transformed into the following Langmuir adsorption isotherm upon adsorption equilibrium (when $\frac{\mathrm{d}\Gamma_{\mathrm{E}}}{\mathrm{d}t} \to 0$):

$$\Gamma_{\mathrm{E}} = \frac{C_{\mathrm{E}}\Gamma_{\mathrm{E},\infty}}{C_{\mathrm{E}} + K_{\mathrm{diss}}}, \tag{2}$$

In the above equations, Γ_{E} is the surface concentration of the adsorbed enzyme, $\Gamma_{\mathrm{E},\infty}$ is the maximum (at surface saturation) surface concentration of the adsorbed enzyme, C_{E} is the bulk concentration of the enzyme, K_{diss} $(=k_{\mathrm{d}}/k_{\mathrm{a}})$ is the dissociation constant, k_{a} is the adsorption rate constant and k_{d} is the desorption rate constant. k_{d} can be estimated from the desorption data (washout of the enzyme using buffer), after setting C_{E} to zero, using the following equation:

$$\frac{\mathrm{d}\Gamma_{\mathrm{E}}}{\mathrm{d}t} = -k_{\mathrm{d}}\Gamma_{\mathrm{E}}. \tag{3}$$

With the known values of K_{diss} and k_{d}, k_{a} can be calculated.

The second process in enzymatic cleaning of protein fouling from solid surfaces is the surface proteolysis. The following lumped reaction equation is used to describe the proteolysis reaction (Onaizi et al. 2009a, 2010):

$$E + S \xrightarrow{k_c} E + P \tag{4}$$

where E, S, P and k_{c} refer to the enzyme, protein fouling, proteolysis product, and proteolysis reaction rate constant, respectively.

To predict the amount of protein fouling remaining on a surface (i.e., extent of cleaning achieved), the following ODE has to be solved simultaneously along with Eq. (1):

$$\frac{\mathrm{d}\Gamma_{\mathrm{S}}}{\mathrm{d}t} = -k_{\mathrm{c}}\Gamma_{\mathrm{S}}\Gamma_{\mathrm{E}}. \tag{5}$$

Since enzyme activity loss is inevitable in many enzymatic systems, mainly due to autolysis (Brode et al. 1994; Stoner et al. 2004), only active enzyme molecules can degrade and thus remove the surface-bound protein molecules. The fraction of active enzyme at the interface and also in bulk, assuming the same activity loss rate in bulk and at the interface, can be written as: $x = \Gamma_{\mathrm{EA}}/\Gamma_{\mathrm{E}} = C_{\mathrm{EA}}/C_{\mathrm{E}}$, where the subscript EA refers to active enzyme.

With the further assumption that the enzyme activity loss is a first-order ODE, which is likely to be the case (Baki et al. 1996; Banerjee and Ray 2017; Onaizi et al. 2009a, 2010; Pantoliano et al. 1987, 1989; Wells and Powers 1986), the following equation is obtained:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = -k_{\mathrm{de}}x, \tag{6}$$

where k_{de} is the enzyme activity loss (deactivation) constant.

The above model Eqs. (1, 5, 6) have to be solved simultaneously to regress the experimental surface proteolysis data. However, the concurrence of enzyme adsorption and surface reaction requires the decoupling of these two processes to provide reliable estimates of the five model parameters ($\Gamma_{\mathrm{E},\infty}$, k_{a}, k_{d}, k_{c} and k_{de}). Such decoupling can be achieved by masking the surface reaction (i.e., the rate of proteolysis approaches zero) while parameterizing enzyme adsorption, as described in Sect. 2. Under the condition of no surface reaction, Eq. (2) can be used to regress the equilibrium data of enzyme adsorption on the surface-bound protein fouling, while Eq. (3) can be used to fit the kinetic enzyme desorption data. These regressions will provide estimates for the three adsorption parameters ($\Gamma_{\mathrm{E},\infty}$, k_{a} and k_{d}). After obtaining the enzyme adsorption parameters, the surface proteolysis can be parameterized by regressing the experimental proteolysis data (using Eqs. 1, 5, 6) with the known values of the adsorption parameters. Such regression will provide estimates of the proteolysis reaction rate constant (k_{c}) and enzyme deactivation rate constant (k_{de}).

Materials and methods

Extraction and purification of rubisco

Rubisco, which is one of the most abundant proteins in nature (He et al. 2011), was extracted from spinach leaves according to the protocol published elsewhere (Onaizi et al. 2007). Briefly, the leaves were ground in a grinding buffer [50 mM *N*,*N*-bis(2-hydroxyethyl)glycine, 1 mM ethylenediaminetetraacetic acid, 10 mM 2-mercaptoethanol and 2 wt% polyvinylpolypyrrolidone] and then the spinach juice was filtered through Whatman filter papers. After that, the filtrate was centrifuged followed by the addition of PEG-3000 to the collected supernatant (final PEG-3000 concentration was 18 wt%). The solution was vigorously stirred during the addition of PEG-3000. Following the addition of PEG-3000, the solution was centrifuged and then 20 mM $MgCl_2$ was added to the obtained supernatant followed by centrifugation. Then, the collected supernatant was applied to a 5 mL ion-exchange HiTrap Q FF column for further purification. The collected rubisco fractions from this chromatographic purification were loaded onto an SDS–PAGE, run with MOPS buffer, stained with Coomassie Blue, and imaged on an electrophoresis imaging densitometer. The purity of every fraction was estimated from the intensities of the large and small subunits of rubisco relative to the summation of the intensities of all bands within every lane using Kodak 1D Image Analysis Software. Only highly pure (> 95%) samples obtained from the chromatographic purification were used in the subsequent experiments.

Preparation of cellulosic self-assembled monolayers (SAMs)

Cellulosic SAMs were prepared by chemical coupling (Onaizi and Leong 2011) to the gold surface of the SPR sensor chip. This was achieved through the modification of microcrystalline cellulose (MCC) with α-lipoic acid (ALA) as described elsewhere (Onaizi 2017). MCC is a powdery cellulosic material with a polymerization degree between 150 and 300. Briefly, 40 mL of *N*,*N*-dimethylacetamide (DMAc) was added to a 1.5 g MCC powder. To dissolve MCC in DMAc, the mixture was placed in an oven and heated at a slow rate (~ 2 °C min^{-1}) until the temperature reached 160 °C. Then, the mixture was left at room temperature to cool down for about 2 h. Following this cooling step, the MCC-DMAc solution was added to 4.5 g lithium chloride (LiCl) and the resultant mixture was stirred overnight at room temperature. Another solution was prepared by mixing 15 mL of DMAc, 0.95 g ALA and 0.75 g *N*,*N*′-carbonyldiimidazole (CDI) and then this solution was added to MCC-DMAc-LiCl solution with overnight stirring at room temperature and under nitrogen gas. The mixing of these two solutions promoted the covalent attachment of ALA to MCC. The resultant product (ALA–MCC) was recovered from the reaction medium through precipitation upon the addition of HPLC grade acetone. The precipitated ALA–MCC powder was removed from the liquid phase via filtration using metallic mesh (made from stainless steel) with a mesh size of roughly 2 µm. The obtained ALA–MCC powder was washed with HPLC grade acetone several times. After drying, the produced ALA–MCC powder was stored at –20 °C until use.

To prepare cellulosic SAMs on SPR chips, the chips were first cleaned according to the cleaning protocol published elsewhere (Onaizi et al. 2009a, b, 2010, 2014b, 2015, 2016b) and then they were immersed in 1 mg mL^{-1} solution of ALA–MCC in dimethyl sulfoxide (DMSO) for at least 1 day. The contact between ALA–MCC and the gold surface of the chips leads to the formation of covalent bonds between the sulfur atoms of the ALA in the conjugated ALA–MCC and the gold layer on the SPR biosensor chips (Onaizi and Leong 2011). These S–Au bonds anchor cellulose molecules (pointing outwards from the surface) permanently on the SPR chips. The cellulosic SAMs surfaces prepared using the above protocol were used as a model to immobilize rubisco protein molecules on these cellulosic films, followed by studying the removal of the immobilized protein molecules experimentally and theoretically (via a modeling approach) as will be described in subsequent sections.

Rubisco immobilization (fouling)

Rubisco has shown irreversible adsorption on a number of different surfaces (Onaizi et al. 2009a, b, 2010) and, thus, an adsorption approach was used to immobilize rubisco on cellulosic SAMs surfaces. The immobilization was monitored using SPR in real time. Before commencing rubisco immobilization, the SPR biosensor chip modified with cellulosic SAMs was mounted on the SPR flow cell and then the chip was flushed, at a flow rate of 5 mL min^{-1}, with 10 mL DMSO, 10 mL HPLC grade ethanol and then with 30 mL deionized water (DIW). After that, the chip was equilibrated in buffer (20 mM sodium phosphate buffer, pH 8) until a stable baseline was established (a stable baseline is established when the SPR reflectivity signal does not change with time). Typically, this equilibration step takes about 3–5 min at a buffer flow rate of 1 mL min^{-1}. After the creation of the baseline in buffer, reflectivity signal monitoring was paused and an angular scan in buffer (flowing at 1 mL min^{-1}) was performed and the obtained SPR angle was recorded. Following this step, the reflectivity signal monitoring was

resumed and the protein solution (0.5 mg mL^{-1} rubisco in the above buffer) was brought in contact with the cellulosic SAMs surface using a peristaltic pump. The flow rate of the protein solution was kept constant at 1 mL min^{-1}. To minimize the wastage of the protein sample, recycling was initiated after 5 min, which is enough to displace the protein-free buffer from the flow path (the volume of the flow path is 0.4 mL). Fresh rubisco sample was used in every experiment.

After 1 h of contact between the rubisco solution and the cellulosic SAMs surface, the reflectivity signal monitoring was paused again and another angular scan was performed. The two angular scans (before and after rubisco adsorption) were used to calculate the amount of rubisco immobilized on the surface while the changes in reflectivity signal with time were used to obtain rubisco adsorption kinetics on the cellulosic SAMs surface. Following the second angular scan, the reflectivity signal monitoring was resumed and the protein was removed from the flow path via washing (at flow rate of 1 mL min^{-1}) with protein-free buffer for 10 min. This wash step would also remove any weakly bound protein molecules at the interface.

Enzyme adsorption isotherm on immobilized rubisco

Once enzyme molecules transfer from bulk and bind on the immobilized rubisco film, they start cleaving the immobilized protein molecules. Under such a scenario, it is impossible to reliably estimate the enzyme adsorption parameters using SPR. To overcome this problem, the surface reaction must be negligible while parameterizing the enzyme adsorption. To mask the surface reaction, subtilisin A was treated with an enzyme inhibitor, phenylmethanesulfonyl fluoride (PMSF), to deactivate the enzyme. However, such treatment only resulted in a partial enzyme deactivation and the surface reaction was still significant. Treating the enzyme thermally or with other enzyme inhibitors was less effective. Thus, additional treatment was performed to eliminate proteolysis reaction during enzyme adsorption. This additional treatment was through cross-linking the immobilized rubisco molecules with glutaraldehyde (GA), reducing the enzyme accessibility to the surface-bound protein film. The combination of enzyme treatment with PMSF and protein cross-linking with GA resulted in a negligible proteolysis of the surface-bound rubisco film, allowing the enzyme adsorption to be parameterized in the absence of any interference from surface reaction.

The enzyme adsorption parameterization was achieved by first immobilizing the protein on cellulosic SAMs surface as described above, treating the adsorbed rubisco film with GA for 10 h and then pumping (at a flow rate of 1 mL min^{-1}) the PMSF-treated enzyme into the GA-treated rubisco film. Before pumping the enzyme solution, a baseline in phosphate buffer was determined and then an angular scan was conducted. Another angular scan was conducted after the injection of the highest enzyme concentration into the SPR flow cell. These angular scans along with the reflectivity signal recorded throughout the time course of the enzyme adsorption on the protein film were used to provide quantitative measurements of the amount of the enzyme bound on the protein film at any time using Winspall (v 2.20, MPI-für Polymerforschung, Mainz, Germany) simulations. The initial bulk enzyme concentration was 2 ppm. Once an adsorption equilibrium at this enzyme concentration is reached, the enzyme concentration in bulk was increased, providing another equilibrium data point. Several equilibrium data points were collected and used to establish the experimental adsorption isotherm. This experimental adsorption isotherm was regressed using Eq. (2) to provide an estimate for the maximum adsorbed amount ($\Gamma_{E,\infty}$) of the enzyme on the protein film at saturation conditions and also to provide an estimate for the dissociation constant (K_{diss}).

After collecting sufficient equilibrium data points for subtilisin A adsorption on immobilized rubisco film, the enzyme was washed off the flow path using enzyme-free buffer. The time-course data of the buffer wash step were used to estimate the enzyme desorption rate constant (k_d) from the immobilized rubisco film using Eq. (3) after setting the bulk enzyme concentration (C_E) to zero. With known values of K_{diss} and k_d, the adsorption rate constant (k_a) was calculated.

Rubisco proteolysis

After parametrizing the enzyme adsorption on the immobilized rubisco film, the degradation, and thus the removal, of the surface-bound protein molecules from the cellulosic SAMs surface was investigated. In these proteolysis experiments, fully active enzyme (i.e., not treated with PMSF) and not cross-linked rubisco were used. In each experiment, the protein was first immobilized on the cellulosic SAMs surface via adsorption as mentioned before and then the protein solution was removed from the flow path with protein-free buffer wash. Following that, an enzyme solution with a specific enzyme concentration was injected at flow rate of 1 mL min^{-1} and the proteolysis reaction (i.e., degradation and removal of protein fouling) was monitored for 1 h followed by 10 min wash (at 1 mL min^{-1}) with enzyme-free buffer to remove the enzyme from the flow path. Three enzyme concentrations (0.05, 0.5 and 5 ppm) were utilized. The results obtained from these experiments were regressed using the model equations (Eqs. 1, 5, 6) with the known enzyme adsorption parameters to estimate the proteolysis rate (k_c) and the enzyme deactivation rate (k_{de}) constants. The regression was performed using user-defined program

codes, based on the fourth-order Runge–Kutta method, in SigmaPlot.

Raw experimental data analysis

For each run, the raw data obtained from SPR measurements were first converted into an average film thickness using Winspall simulations. In these simulations, the refractive indices of rubisco and subtilisin A were assumed to be 1.45 (Davies 1996). The average film thickness is then converted into surface mass concentration (mg m^{-2}) by multiplying the average film thickness by rubisco/subtilisin A density. The densities of subtilisin A and rubisco were assumed to be 1.33 g cm^{-3}, which is the same as that reported in the literature for proteins (Hook et al. 2002; Voros 2004).

Results and discussion

After preparing the cellulosic SAMs films, the adsorption of rubisco protein on those films was studied. Figure 1 shows rubisco adsorption on the prepared cellulosic SAMs. The adsorption took place from 0.5 mg mL^{-1} protein solution in 20 mM sodium phosphate buffer, pH 8. Before injecting rubisco solution, the cellulosic SAMs surface was equilibrated in the buffer until a stable baseline was attained. The first couple of minutes in Fig. 1 represent the truncated baseline. Upon adding rubisco solution, the protein started

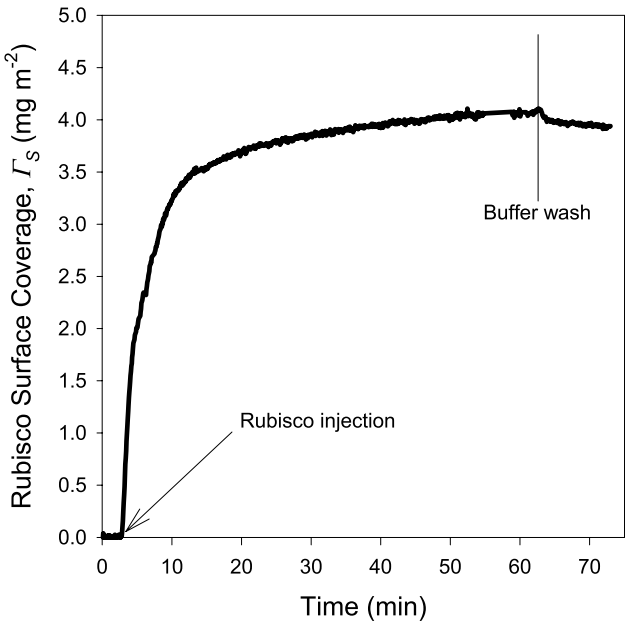

Fig. 1 Rubisco adsorption on a cellulosic SAMs surface. Adsorption took place from a rubisco solution of 0.5 mg mL^{-1} in 20 mM sodium phosphate, pH 8. The injection of the protein solution and the commencement of the buffer wash are indicated

accumulating on the cellulosic SAMs surface. Following the fast initial stage of adsorption, a second stage of slow adsorption took place. The amount of rubisco per unit area of the surface approached 4.1 mg after 1 h contact between the protein solution and the cellulosic SAMs surface.

The isoelectric point of rubisco is about 6 (Ru et al. 2000), whereas cellulose has an isoelectric point equivalent to 2.7 (Madan and Shrivastava 1975). Thus, both interactors (the protein and the surface) will carry a net negative charge at pH 8; all experiments in the current work were conducted at pH 8. Despite the expected net repulsive interaction between the protein and the cellulosic SAMs surface, the amount of rubisco bound to the surface is reasonably high. Rubisco is a bulky protein [molecular weight about 560 kDa (Onaizi et al. 2007; Ru et al. 2000)] carrying several positively charged amino acids on each molecule at pH 8. Thus, even though the net charge on the rubisco molecule is negative at pH 8, its positively charged amino acids at this pH are still appreciable; these positively charged amino acids are likely to be the key driving force for rubisco adsorption on cellulosic SAMs. Onaizi et al. (2009a) studied rubisco adsorption on different non-cellulosic surfaces and reported that rubisco adsorbed more on ALA, which is a negatively charged surface at pH 8, relative to its adsorption on the uncharged hydrophobic octadecanethiol (ODT) and the uncharged hydrophilic β-mercaptoethanol (BME) surfaces. The authors attributed such higher adsorption to the strong Coulombic (charge–charge) interaction between the positively charged amino acids on the protein molecules and the negatively charged ALA surface.

After 1 h contact between the protein solution and the cellulosic SAMs surface, the protein was removed from the flow path through the wash with protein-free buffer for 10 min. This desorption process resulted in a negligible reduction of the rubisco amount bound to the cellulosic SAMs surface (see Fig. 1), highlighting that rubisco adsorption on cellulosic SAMs is extremely irreversible (i.e., tough fouling). Such irreversibility was also observed for rubisco adsorption on other non-cellulosic surfaces with different characteristics (Onaizi et al. 2009a, b, 2010). Partial or completely irreversible adsorption of other proteins at solid–liquid (Adamczyk 2000; Koutsopoulos et al. 2004; Wertz and Santore 1999) and air–liquid (Graham and Phillips 1979; Malcolm et al. 2006; Onaizi et al. 2007; Svitova et al. 2003; Wang and Narsimhan 2005) interfaces has been also reported in the literature.

After creating a tough rubisco fouling on the cellulosic SAMs surface via irreversible adsorption, the biocatalytic degradation and, thus, the removal of adsorbed rubisco from the cellulosic SAMs surface was studied using subtilisin A, which is a protease enzyme with a wide use in detergent formulations (Blanch and Clark 1996; Donlon 2007; El Hadj-Ali et al. 2007; Haring and Schreier 1998;

Maurer 2004; Niyonzima and More 2015; Paul et al. 2016; Watson 2006). In such a heterogeneous catalytic process, enzyme molecules adsorb from the bulk liquid on the protein fouling and then start cleaving the protein molecules into smaller fragments that are easily washed away with the running enzyme solution. However, the concurrence of enzyme adsorption and protein removal makes it difficult to decouple these two processes, resulting in an over-parameterized system. To enable reliable estimates of the enzyme adsorption and surface reactions, the two processes (enzyme transfer to the protein fouling on the surface and the degradation reaction of the protein molecules) must be parameterized separately. In other words, the reaction must be masked during the enzyme adsorption process to reliably estimate the enzyme adsorption parameters. To achieve this objective, the enzyme was deactivated with PMSF and the adsorbed protein molecules on the cellulosic SAMs surface were cross-linked with GA as described in Sect. 2. The combination of the enzyme treatment with PMSF (i.e., partial deactivation of the enzyme) and the cross-linking of the protein film with GA resulted in a non-reactive SA-rubisco system, enabling the estimation of the enzyme adsorption parameters on the rubisco fouling.

In each enzyme adsorption experiment, sodium phosphate (20 mM, pH 8) buffer containing a specific concentration of the enzyme (initial concentration was 2 ppm) was injected using a peristaltic pump at flow rate of 1 mL min^{-1} and the adsorption of the enzyme on the protein fouling on the biosensor surface was monitored until an equilibrium in adsorption was reached (see Fig. 2). Once the enzyme adsorption on the protein film leveled off, the enzyme concentration in the buffer was increased to achieve another equilibrium data point. The collected equilibrium data points are plotted in Fig. 3. The regression of these data points using the Langmuir adsorption isotherm (Eq. 2) provided estimates for two adsorption parameters, the maximum adsorbed amount per unit area of the surface at the surface saturation condition ($\Gamma_{E,\infty}$) and the dissociation constant (K_{diss}). Although there are more advanced and more complex models for estimating protein adsorption parameters (Biesheuvel et al. 2005; Schaaf et al. 2000; van Tassel et al. 2000), for the sake of simplicity, the Langmuir adsorption isotherm was used in this work. Several published studies [see for example (Onaizi et al. 2009a, 2010, 2014a, b, 2016a, b; Roach et al. 2005; Scopelliti et al. 2010)] on the adsorption of biomolecules at different interfaces have shown the adequacy of the Langmuir adsorption isotherm model in estimating the adsorption parameters of biomolecules, despite some of the assumptions underlining the development of this model might not be fully met. The reasonably good fit of the enzyme adsorption using the Langmuir adsorption isotherm (see Fig. 3) and noticing the complete desorption of the enzyme from the rubisco protein fouling (see the inset of Fig. 2), suggest that the use of the Langmuir adsorption isotherm is acceptable.

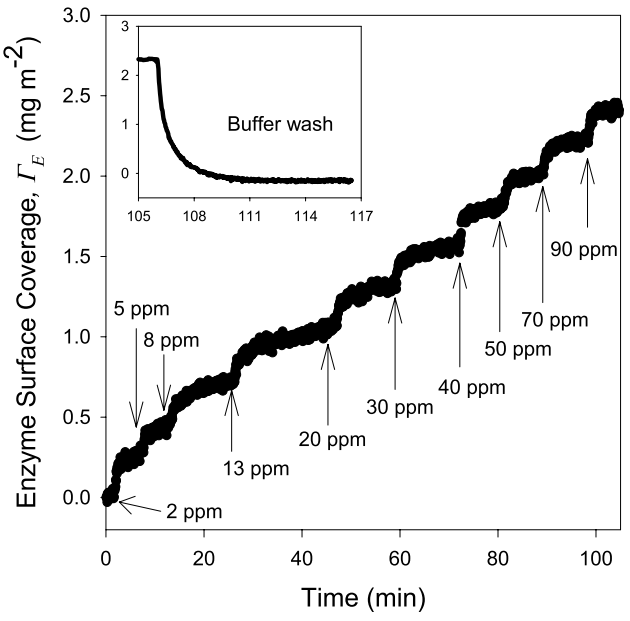

Fig. 2 Adsorption of subtilisin A as functions of time and the bulk enzyme concentration on rubisco film under the condition of negligible surface reaction. The enzyme-free buffer washout (i.e., enzyme desorption) is shown in the inset

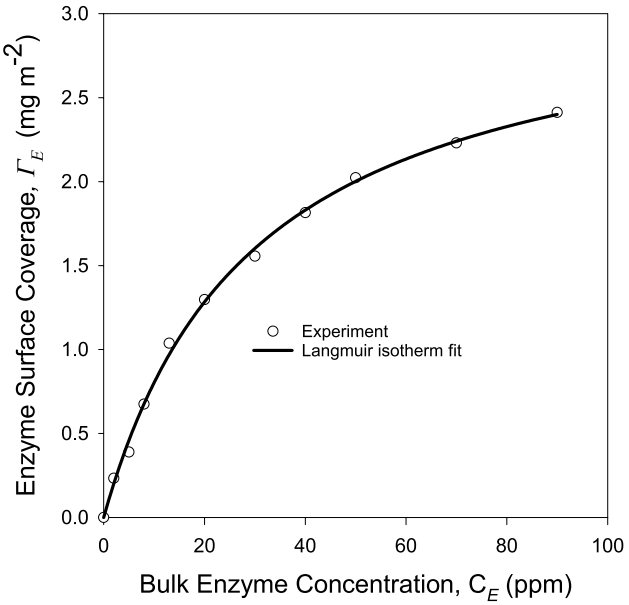

Fig. 3 Experimental and Langmuir adsorption isotherms of subtilisin A on rubisco fouling on cellulosic SAMs surface. The estimated values of the maximum adsorbed amount per unit area of the surface at the surface saturation condition ($\Gamma_{E,\infty}$) and the dissociation constant (K_{diss}) are 3.1 ± 0.2 mg m^{-2} and 29.0 ± 3.6 ppm, respectively

The estimated $\Gamma_{E,\infty}$ and K_{diss} from the Langmuir fit of the experimental data are 3.1 ± 0.2 mg m^{-2} and 29.0 ± 3.6 ppm, respectively. The estimated $\Gamma_{E,\infty}$ is very close to the expected close-packed full monolayer coverage of the protein film by the enzyme molecules, which is 3.3 mg m^{-2} (Foose et al. 2008). The coverage of rubisco film by subtilisin A has been reported to be a function of the chemistry of the surface underneath the protein film (Onaizi et al. 2009a, 2010). For example, $\Gamma_{E,\infty}$ for subtilisin A adsorption on rubisco films immobilized on ODT, ALA and cibacron blue F3GA (CB) has been reported to be 2.4 ± 0.4, 2.3 ± 0.3 and 3.0 ± 0.2 mg m^{-2}, respectively (Onaizi et al. 2009a, 2010). The $\Gamma_{E,\infty}$ value obtained in the current work for subtilisin A adsorption on rubisco fouling on a cellulosic SAMs surface is in line with the above values. The dissociation constant (K_{diss}) estimated herein is also within the range (14–102 ppm) of K_{diss} for subtilisin A adsorption on rubisco films immobilized on different surfaces (Onaizi et al. 2009a, 2010).

The dissociation constant represents the ratio of desorption rate to adsorption rate constant (k_d/k_a). The adsorption and desorption rate constants are required inputs in parameterizing the surface proteolysis reaction. With known K_{diss}, the estimation of either k_a or k_d will allow the estimation of the other. k_d can be estimated from the regression of the enzyme-free buffer wash data using Eq. (3) by setting the enzyme concentration (C_E) to zero. The regression of the buffer wash data is shown in Fig. 4. The estimated k_d value is 1.60 ± 0.30 min^{-1}, which is on the same order of magnitude as those reported for subtilisin A desorption from rubisco films immobilized on different non-cellulosic surfaces (Onaizi et al. 2009a, 2010). Another feature in Fig. 4 is the complete desorption of the enzyme from the protein film surface upon washing with enzyme-free buffer. Complete subtilisin A desorption from cross-linked BSA protein film when the film was washed with enzyme-free buffer has been reported in the literature (Foose et al. 2008). Furthermore, complete subtilisin A desorption from rubisco films immobilized on different non-cellulosic surfaces was also reported in the literature (Onaizi et al. 2009a, 2010).

The estimated k_d value along with the previously estimated K_{diss} were used to estimate the adsorption rate constant (k_a) as 0.057 ± 0.014 min^{-1} ppm^{-1}. Onaizi et al. reported smaller k_a values for subtilisin A adsorption on rubisco films immobilized on ALA (0.048 ± 0.005 min^{-1} ppm^{-1}) and ODT (0.016 ± 0.007 min^{-1} ppm^{-1}) surfaces (Onaizi et al. 2009a). However, the k_a value for subtilisin A adsorption on rubisco film immobilized on CB has been reported to be 0.152 ± 0.025 min^{-1} ppm^{-1} (Onaizi et al. 2010), which is almost threefold higher than the k_a value obtained in this work.

After parametrizing the enzyme adsorption on the rubisco fouling on the cellulosic SAMs surface in the absence of

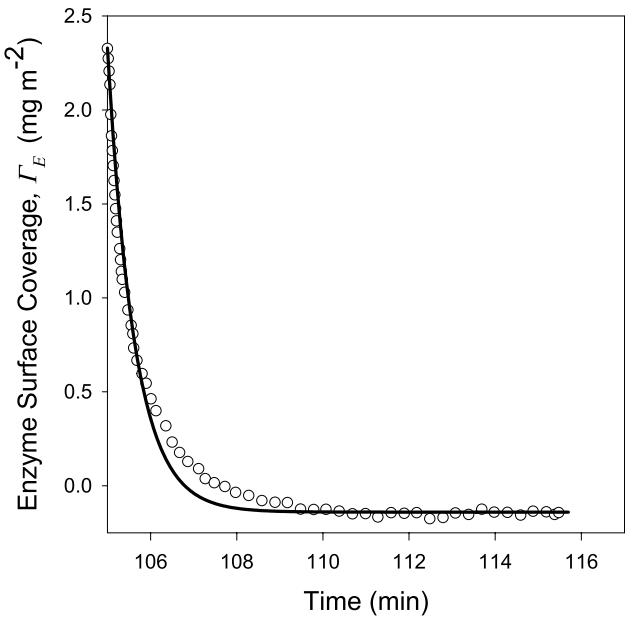

Fig. 4 Enzyme-free buffer washout (i.e., enzyme desorption) of subtilisin A from rubisco fouling on cellulosic SAMs surface. The estimated enzyme desorption rate constant (k_d) is 1.60 ± 0.30 min^{-1}

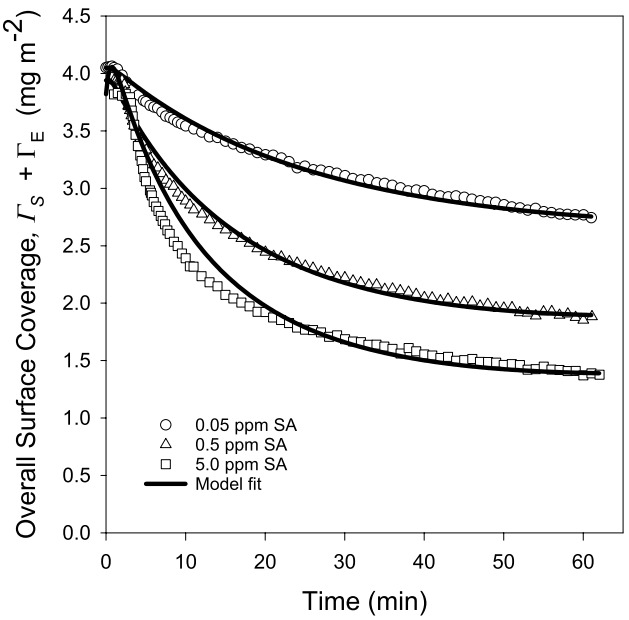

Fig. 5 Proteolysis removal of rubisco fouling from cellulosic SAMs surface as a function of subtilisin A concentration and enzyme-protein fouling contact time. The protein fouling was aged for 70 min (including 10 min wash with protein-free buffer) before introducing the enzyme solution. The solid curves show the calculated surface proteolysis time course using the mathematical model (Eqs. 1, 5, 6). The estimated proteolysis reaction (k_c) and the enzyme deactivation rate (k_{de}) constants are 1.70 ± 0.10 m^2 mg^{-1} min^{-1} and 0.03 ± 0.01 min^{-1}, respectively

surface proteolysis, rubisco fouling removal was studied using non-PMSF-treated subtilisin A and non-cross-linked rubisco films. The experimental proteolysis data of the surface-bound rubisco films and the model fit of the data (the solid curves) are shown in Fig. 5 for three bulk enzyme concentrations (0.05, 0.5 and 5 ppm). The regression of the experimental proteolysis data using the coupled model Eqs. (1, 5, 6) provided estimates for the surface proteolysis rate (k_c) and enzyme activity loss (deactivation) rate (k_{de}) constants as 1.70 ± 0.10 m^2 mg^{-1} min^{-1} and 0.03 ± 0.01 min^{-1}, respectively. The estimated values of k_c and k_{de} are the same as those reported for the proteolysis of rubisco films immobilized on different non-cellulosic surfaces (Onaizi et al. 2009a, 2010), suggesting that rubisco proteolysis is independent of the surface characteristics on which the protein is immobilized. This finding suggests that the surface reaction is not the limiting step in the overall process of removing rubisco fouling from cellulosic SAMs. The changes in the values of the adsorption and desorption rate constants with the variation in surface characteristics suggest that the mass transfer of the enzyme molecules to the interface is also not limiting since in all cases the hydrodynamics of the enzyme transfer to the protein fouling were the same. Accordingly, the enzyme adsorption barrier seems to be the limiting step in the overall rubisco fouling removal from the studied surfaces.

The extents of rubisco removal after 1 h treatment with 0.05, 0.5 and 5 ppm subtilisin A were 32, 54 and 76%, respectively. In previous studies (Onaizi et al. 2009a, 2010), the removal of rubisco from different surfaces was found to be related to the enzyme mobility at the interface. Enzyme mobility can be qualitatively predicted from the adsorption and desorption rate constants. Higher values of the adsorption and desorption rate constants imply that the enzyme is rapidly jumping on (for the case of adsorption) and off (for the case of desorption) the surface, resulting in an enhanced rate of protein degradation and, thus, its wash off the surface. This assertion is confirmed by comparing the extent of rubisco removal from different surfaces to the values of k_a and k_d (see Table 1). As shown in Table 1, the removal of rubisco from CB is the highest amongst all the studied surfaces. Subtilisin A adsorption and desorption rate constants in the case of rubisco film immobilized on the CB surface are also the highest. The removal of rubisco fouling from ALA and cellulosic SAMs surfaces is almost identical but lower than its removal from the CB surface. Comparing the ratio of k_a and k_d values for rubisco-ALA and rubisco–cellulosic SAMs reveals that (k_a)rubisco–cellulose/(k_a)rubisco-ALA is about 1.2 while (k_d)rubisco–ALA/(k_d)rubisco–cellulose is about 1.1. These ratios suggest that the enzyme mobility in both cases (rubisco–ALA and rubisco–cellulosic SAMs surface) is almost the same, resulting in almost identical values for rubisco removal from these two surfaces. Finally, despite the values of the enzyme desorption rate constant from the protein films immobilized on ODT and cellulosic SAMs being similar, the absolute adsorption rate (i.e., the adsorption rate constant) from the rubisco–cellulosic SAMs surface is almost fourfold higher than the absolute adsorption rate for rubisco-ODT. Such a higher rate of adsorption (and thus higher overall mobility) resulted in a significant increase in protein removal from the cellulosic SAMs surface relative to the ODT surface.

Conclusions

Rubisco contact with cellulosic SAMs surfaces resulted in a tough protein fouling that could not be altered by washing with enzyme-free buffer. Introducing subtilisin A to the protein surface led to the removal of adhered proteins; however, the extent of the removal was a function of the enzyme concentration and contact time. This heterogeneous

Table 1 Extents of rubisco fouling removal from cellulosic SAMs surface relative to its removal from different non-cellulosic surfaces using subtilisin A as a function of the enzyme mobility (i.e., adsorption and desorption rate constants) at the interface

Parameter	Cibacron blue F3GA	α-lipoic acid	Octadecanethiol	Cellulosic SAMs
k_c (m^2 mg^{-1} min^{-1})	1.70 ± 0.10	1.70 ± 0.10	1.70 ± 0.10	1.70 ± 0.10
k_{de} (min^{-1})	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
k_a (min^{-1} ppm^{-1})	0.152 ± 0.025	0.048 ± 0.005	0.016 ± 0.007	0.057 ± 0.014
k_d (min^{-1})	2.16 ± 0.05	1.75 ± 0.05	1.60 ± 0.15	1.60 ± 0.30
K_{diss} (ppm)	14.18 ± 1.98	36.2 ± 10.7	102.0 ± 22.8	29.0 ± 3.6
$\Gamma_{E,\infty}$ (mg m^2)	3.0 ± 0.2	2.3 ± 0.3	2.4 ± 0.4	3.1 ± 0.2
MQW contact angle (degree)	15	52	93	29
% Rubisco removal				
Using 0.5 ppm subtilisin A	82	53	32	54
Using 5 ppm subtilisin A	97	73	58	76
Reference	Onaizi et al. (2010)	Onaizi et al. (2009a)	Onaizi et al. (2009a)	Current work

cleaning process was successfully modeled and the utilized mathematical model was able to track the experimental data. Enzyme transfer to the protein fouling and surface reactions (proteolysis and enzyme activity loss) were taken into consideration while obtaining the values of the model parameters. Enzyme mobility (i.e., enzyme adsorption and desorption rates) at the interface played the major role in the enzymatic cleaning of rubisco fouling from cellulosic SAMs surface. Unlike surface reactions, which were insensitive to the surface chemistry underneath the rubisco protein fouling, enzyme mobility showed a strong dependence on the characteristics of the surface upon which the protein film was immobilized.

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Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

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