Impact of external electric field on protein adsorption onto metal surface and the application to enzymatic cleaning

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ABSTRACT

The adsorption of protein to a solid surface often plays a vital role in various industrial fields and has been extensively and continuously investigated with regard to various factors, including protein nature (size, net charge, fragility, etc.), surface characteristics (materials, surface functional groups, etc.) and medium conditions (pH, ion strength, electrolyte type, etc.). As a result, the electrostatic interaction between protein and solid surface has been suggested to play an important role in the adsorption of protein onto a solid surface. Accordingly, it is naturally expected that the surface electric potential affects the process and characteristics of the protein adsorption. Since the surface potential of an electro-conductive materials in an aqueous system can be electrically controlled, it may be feasible to reduce or enhance the adsorption of protein onto metal (oxide) surfaces by controlling the surface potential. On the other hand, the removal of a solid surface fouled with proteinaceous soilings (cleaning) is extremely important in food and pharmaceutical industries, and one of the removal techniques for proteinaceous soilings is enzymatic cleaning. Since the binding of hydrolytic enzyme with surface-adhering protein initiates the hydrolysis of proteinaceous soilings into soluble fragments, the control of adsorption of hydrolytic enzyme by an external electric potential may possibly increase the effectiveness of the enzymatic cleaning. Hence, in this study, the impact of an external electric potential on the protein adsorption onto metal (oxide) surface was first explored. Next, the
influence of an external electric potential on the cleaning characteristics of protease was investigated.

At the outset, the adsorption behavior of a protein to a base metal surface in the presence of external electric field was investigated, using hen egg white lysozyme (LSZ) and six types of base metal plates (stainless steel SUS316L (St), Ti, Ta, Zr, Cr, or Ni) as the protein and adsorption surface, respectively. LSZ was allowed to adsorb on the surface under different conditions (surface potential, pH, electrolyte type and concentration, surface material), which was monitored using an ellipsometer. LSZ adsorption was minimized in the potential range above a certain threshold and, in the surface potential range below the threshold, decreasing the surface potential increased the amount of protein adsorbed. The threshold potential for LSZ adsorption was shifted toward a positive value with increasing pH and was lower for Ta and Zr than for the others. A divalent anion salt (K$_2$SO$_4$) as an electrolyte exhibited the adsorption of LSZ in the positive potential range while a monovalent salt (KCl) did not. A comprehensive consideration of the obtained results suggests that two modes of interactions, namely the electric force by an external electric field and electrostatic interactions with ionized surface hydroxyl groups, act on the LSZ molecules and determine the extent of suppression of LSZ adsorption.

Next, the author examined the influence of protein characteristics and structure on the protein adsorption onto base metal surfaces in the presence of external electric potential. Fifteen types of protein and six types of base metal surfaces were used as adsorbate protein and metal surfaces. The attained amounts adsorbed and the initial adsorption rates in the protein adsorption at different applied surface potentials were measured by using an in-situ ellipsometry. Results indicated that the relationship
Among the protein adsorption, the surface electric potential, and pH strongly depends on the balance of acidic and basic amino acid residues, namely, the pI value of a protein: In the adsorption onto a stainless steel surface at pH 5.6, the proteins with the pI value ≤ 9.3 exhibited the minimum adsorptive affinity at the negative surface potentials below a certain value and more highly adsorb at more positive surface potential; Basic proteins having the pI value >~10 significantly adsorbed at the negative potentials; α-Chymotrypsinogen and RNaseA, with the intermediate pI values, show roughly constant amount adsorbed in the tested surface potential range. On the other hand, as the pH increases, the threshold surface potential for the adsorption of acidic and basic proteins shifted positively and negatively, respectively. These results coincide with the protein adsorption mechanism based on the electrostatic interactions among protein ionized groups, the surface ionized hydroxyl groups, and the applied surface potential, as described above. Furthermore, from the differences among the surface potential dependences of the amount adsorbed onto different base metal surfaces, it was deduced that the ionization states of hydroxyl groups varied by the type of base metal. All these findings appear to support the view that a base metal surface can be controlled for the affinity to a protein by manipulating the surface electric potential as has been reported on some electrode materials.

Based on the above findings, the influence of an external electric field on the enzymatic cleaning of a metal surface fouled with a protein was investigated. The model fouling protein (bovine serum albumin (BSA) or lysozyme (LSZ)) was prepared on a stainless steel (St) surface, and the resulting surface subjected to enzymatic cleaning with an electric potential being applied to the St sample plate. Trypsin, α-chymotrypsin, and thermolysin were used as model proteases. The amounts of
protein fouling that remained on the plate before and during the enzymatic cleaning process were measured by means of a reflection absorption technique using Fourier transform infrared spectroscopy. In the case for BSA fouling, the cleaning efficacy of the protease tended to increase at more negative applied potentials. On the other hand, there was an optimum applied potential for removing of the LSZ fouling. Atomic force microscopy analyses indicated that applying an adequate range of electric potential enhanced the enzymatic removal of protein fouling inside scratches on the sample plate surface. These findings suggest the existence of two modes of electrostatic interactions for the external electric field, one with protease molecules and the other with digested fragments of the fouling protein.
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CHAPTER 1

Introduction

1. Outline of Adsorption of Protein onto a Solid Surface

ADSORPTION is defined as the adhesion of atoms, ions, or molecules from a gas, liquid, or dissolved solid to a surface [1]. One of the adsorption systems that have been continuously and extensively interested is the adsorption of protein molecules onto a liquid-solid interface. The protein adsorption often play crucial roles in wide variety of industrial fields: Preparation of immobilized enzyme for constructing a bioreactor is based on the adsorption of catalytic protein molecules onto the carrier particles [2,3]; In an Enzyme-linked immunosorbent assay (ELISA), antibody molecules are fixed on a plastic well surface, and certain types of protein such as BSA are also adsorbed to the well surface in order to avoid nonspecific binding of analyte molecule [4-6]; “Biosensor” generally is composed of electrode and the immobilized proteins having specific bioactivity [7]; Biocompatibility of artificial implant has been well known to closely relate to the adsorption of certain types of protein [8-10]; Also in the cultivation of cells, the adsorption of certain types of proteins are known to determine the cell viability [11];
Chromatographic purification of protein utilizes difference among the interaction affinities of different types of proteins with sorbent surface [12].

On the other hand, the protein adsorption is often related to industrially unwanted phenomena. In food and bio-industries, protein is a major component of fouling deposits in the product-manufacturing processes, and the protein adsorption layer formed on the manufacturing facility walls is known to form the foundation of the proteinaceous foulings [13-15]. The clogging of the separation membrane is also ascribed to the protein adsorption and subsequent formation of protein-fouling layer on the membrane [16].

1.1. Factors Affecting Protein Adsorption Characteristics

As described above, the protein adsorption onto a solid surface is a ubiquitous phenomenon and can affect various artificial and natural processes. Hence, to date, numerous researches on the protein adsorption onto solid surfaces have been conducted. As a result, the influences of several conditional factors on the protein adsorption characteristics have been elucidated.

(i) Solid surface

The adsorption substrates that had been investigated for the impact on the protein adsorption characteristics may be classified into three, including organic, inorganic, and metal materials. As for the organic sorbent, polystyrene (PS) surface has been extensively investigated as an adsorbent surface for protein adsorption in anticipation of the utilization for ELISA [17,18]. It has been suggested that protein adsorption onto a PS surface occurs through electrostatic, hydrophobic, and aromatic interaction [19].
The inhibition of protein adsorption onto a PS surface by arginine [19] was indicated, which suggests that the negative charges on a PS surface may be determinant for binding the protein molecules. The representative as one of the inorganic materials, well-investigated for the protein adsorption, is calcium phosphate. Calcium phosphate can have different crystal forms and elemental defects, which drastically varies the adsorptive affinity and adsorption selectivity [20].

The metal and metal oxides frequently encounter the opportunity to contact with protein molecules. Noble metals are generally used as a biosensor electrode, on which proteins/enzymes are immobilized [21-23]. Basic metals and stainless steel are building block of food- and drug-manufacturing systems and thus often subjected to the high protein concentration liquid and consequent formation of proteinaceous foulings. The protein adsorption isotherms onto metal and base metal surfaces have been determined for various proteins [24], from which many types of proteins exhibit irreversible mode of adsorption [24] (although the adsorption isotherm shows a Langmuir type [25]).

(ii) Temperature

A high temperature is thermodynamically supposed to be unfavorable for the adsorption both in gas and liquid phases by the increased entropy due to increasing temperature. On the other hand, it has been reported that the adsorption of protein onto a plastic surface tends to be increased with increasing temperature. This is explained by thermal denaturation of protein molecule and consequent surface aggregation of protein [26]. Also in the case for the adsorption onto stainless steel surface, the increasing temperature above a certain value was found to result in the
drastic increase in the adsorbed amount of protein, which was due to surface aggregation [27].

(iii) pH and ionic strength

Since a protein molecule contains many ionizable side-chain groups, the protein net charge is strongly varied depending on the solution pH. On the other hand, a solid surface also is charged, more or less, the net value of which depends on the pH of its environment. In especial, the oxide surface layer of metal is generally covered with hydroxyl groups in an aqueous system, and the hydroxyl groups are known to turn into cationized $-\text{OH}_2^+$ or anionized $-\text{O}^-$ at pHs below or above the pK value of the surface -OH group [28,29]. These charges of protein molecule and adsorbent surface largely contribute to the attraction or repulsion between the protein and the surface [30]. The electrostatic repulsion among the adsorbed protein molecules is naturally expected to determine the mean distance between the adsorbed protein molecules [31].

On the other hand, an ionic component serve to weaken both the electrostatic attraction and repulsion between protein molecule and adsorbent surface, which is attributed to the screening the charges of the protein and the surface [32]. The screening effect of the ionic component acts also on the adsorbed protein molecules [32]; The increase in the ionic strength works to decrease the occupied surface area of the adsorbed protein molecules.

(iv) Protein type

Till now, a huge number of proteins have been discovered. However, only limited types of proteins had been well investigated for the adsorption characteristics, and the
findings on the relationship between protein structure and adsorption characteristics are limited. One of the findings is that maximal protein adsorption occurs near its pI due to minimization of repulsive forces among adsorbed protein molecules [33-36]. On the other hand, Imamura et al. [24] measured and compared the adsorption isotherms of various proteins onto a metal (titanium) oxide surface at different pHs. It was indicated that the pH values associated with maximal amount adsorbed roughly tended to be higher with increasing pI of protein up to a certain value (~6) but became almost constant at around 6 for proteins with a pI of greater than 6 [24].

In the protein adsorption onto titanium oxide surfaces [24], furthermore, the impact on the reversibility/irreversibility of protein adsorption by the composition of amino acid residues was investigated. Results suggested that irreversible adsorption is significant in the adsorption of most acidic and neutral proteins in the pH range where protonated hydroxyl groups are present on the surface together with neutral and deprotonated groups. The adsorption of basic proteins, the pI of which were above 9, was largely reversible in the pH range of 3-10.

Another aspect of protein that can strongly affect the protein adsorption characteristics is the softness/hardness of the protein molecule [37,38]. The disulfide bond inside a protein molecule serves to lower the structural flexibility of the protein. The protein molecule with more intra-disulfide bonds is thus harder to change its conformation upon the adsorption onto a solid surface, resulting in less adsorption force [37,38].

1.2. Protein Adsorption Mechanisms

Many researchers have been engaged in elucidating the mechanism of the protein
adsorption onto a solid surface. However, most of their trials have resulted in demonstrating the diversity of the adsorption characteristics according to the adsorption conditions, including types of protein and adsorbent substrate. On the other hand, the protein adsorption onto a stainless steel surface was extensively investigated in the field of food engineering with intent to avoid the formation of foulings in the manufacturing processes and improve the effectiveness of cleaning the fouled surface. As a result, the following interpretation into the protein adsorption onto a stainless steel surface has been suggested [39,40]: As described above, the surface hydroxyl groups covering a stainless steel surface in an aqueous system is converted to cationized $-\text{OH}_2^+$ or anionized $-\text{O}^-$ at pHs below and above the pI ($\sim$6, [41,42]). On the other hand, a protein molecule contains ionizable side chains, namely, carboxylic groups of acidic amino residues and amino/guanisyl/imidazole groups of basic amino acid residues, the compositions of which are markedly varied by the protein type. The electrostatic attraction between ionized surface hydroxyl groups and protein side chains has been suggested to play an essential role in the protein adsorption onto metal oxide surface including stainless steel [39,40]. Namely, in the pH range below the surface pI, dissociated (anionic) carboxylic groups of protein and surface $-\text{OH}_2^+$ electrostatically interact, which triggers the protein adsorption; When the pH is above the surface pI, the cationized groups ($-\text{NH}_3^+$, $-\text{C(NH}_2)_2^+$, and $-\text{NH}_2^+\text{NH}_2^-$) of a protein molecules bind to the surface $-\text{O}^-$. It should be emphasized that the electrostatic interaction between protein $-\text{COO}^-$ and surface $-\text{OH}_2^+$ was found to be formed even at the pH below the pK of protein carboxylic groups, indicating that the surface $-\text{OH}_2^+$ groups may serve to induce the dissociation of protein $-\text{COOH}$ upon the protein adsorption [43]. Since the former $-\text{COO}^-/-\text{OH}_2^+$ interaction is stronger than the later interactions [43], the
irreversible adsorption is more likely to occur in the cases for acidic protein (having larger number of carboxylic groups) and low solution pH.

The protein adsorption onto a polystyrene (PS) also has been well studied for understanding the mechanism, especially, individual interactions directly leading to the adsorption [44,45]. According to the results, basic amino acid residues, arginine and lysine, has been indicated to be the interaction sites for the PS surface. However, the contribution of hydrophobic amino acid residues to the protein affinity to the PS surface has been known. Consequently, the peptide sequence, found as a PS-binding peptide tag for the protein immobilization, has been known to be comprised of both basic and hydrophobic amino acid residues [45].

1.3. Removal techniques for Protein Adsorbed on a Solid Surface

As describe above, an adsorbed protein on a solid surface often becomes the main component of “proteinaceous stain” or “protein fouling” in food manufacturing processes. Most of the food manufacturing facilities are made of metals including stainless steel, and proteins often have extremely strong adsorptive affinity to the metal oxide surface. Consequently, the cleaning of the metal (oxide) surface fouled with proteinaceous ingredients generally requires large amount of water, detergent, energy, and time. Hence, to date, there are many researches on the technique to remove the adsorbed protein from metal (oxide) surfaces.

Chemical cleaning is one of the commonly used techniques, using chemical agents such as acids, alkalis, detergents, and oxidants. Sodium hydroxide, potassium hydroxide and phosphate are totally effective for protein desorption by breaking down the peptide bonds and amino acids of protein through hydrolysis (acid or alkali) reaction
It has been generally known that the increasing temperature increases the effectiveness of the cleaning agents. Sodium dodecyl sulphate (SDS) is also often used for the removal of a biofilm [47]. The chemical cleaning technique is not only cheap in price but also save in time irrespective of some hazardous problems.

Enzymatic cleaning has become a common technique for removing stains and dirt from dishes and clothing and is available for cleaning the metal (oxide) surfaces fouled with proteinaceous soilings [48,49]: It was noticed that the effective cleaning efficiencies (> 90%) for the removal of whey fouling protein on membranes with alcalase was observed within only 20 min in a pH range of 6.6-9.7 under various operating conditions of pH of solution, added amount of alkali to adjust pH, enzyme concentration and cleaning period [50]. In the enzymatic cleaning, the enzyme molecule having hydrolytic activity is allowed to contact with fouling and/or stains on a solid surface and breaks down the surface-adhering substances into soluble fragments. The hydrolytic enzyme molecules quickly and repeatedly attack the substances to be removed, and the enzymatic hydrolysis reactions occurs under mild conditions (around room temperature and normal pressure). Therefore, the enzymatic cleaning might appear quite promising as a technique to remove proteinaceous soiling also in food and bio-industries. However, in the food manufacturing process, a large amount of foulings is often formed, and large amount of hydrolytic enzyme and considerably long reaction time are thus required for obtaining sufficient cleanness. The technique to improve the rate of the enzyme-catalyzed hydrolysis is necessary.

On the other hand, some new cleaning methodologies have been developed. One of the alternative cleaning technique is a H$_2$O$_2$-electrolysis cleaning [51]: A fouled metal surface is made to contact with an aqueous solution containing hydrogen peroxide and
supporting electrolyte. A slight negative potential (-0.2 ~ -0.8 vs. Ag/AgCl) is then applied into the metal. The •OHs are generated by the electrolysis of hydrogen peroxide on the metal surface and effectively attach to decompose the adsorbed organic soils. In addition to the H₂O₂-electrolysis cleaning, the blast cleaning, UV/ozone, UV/H₂O₂, megaHz sonification and so on have been developed and examined for the effectiveness and feasibility as an alternative cleaning technique [52].

1.4 Purpose of This Study

As described above, the electrostatic interaction between protein and solid surface has been suggested to play an important role in the adsorption of protein onto a solid surface. Especially, the irreversible adsorption of protein onto a metal (oxide) surface was demonstrated to be mainly ascribed to the protein-surface electrostatic interactions. On the other hand, a solid surface has a certain electric potential, depending on the chemical composition and the circumstances. It is naturally expected that the surface electric potential, more or less, affects the process and characteristics of the protein adsorption. The surface potential of an electro-conductive materials in an aqueous system can be electrically controlled. Hence, it may be feasible to reduce or enhance the adsorption of protein onto metal (oxide) surfaces by controlling the surface potential.

If possible to control the protein adsorption onto a metal oxide surface, the improvement of the cleaning effect of a hydrolytic enzyme may be possible. Namely, when the electric potential of protein-fouled metal (oxide) surface is controlled so as to attract the hydrolytic enzyme molecules, the enzyme concentration in the vicinity of the
metal (oxide) surface is increased and, as a result, the reaction frequency between the hydrolysis enzyme and fouling protein may possibly be increased.

Based on the above background, the impact of an external electric potential on the protein adsorption onto metal (oxide) surface was first explored in this study. Next, the influence of an external electric potential on the cleaning characteristics of protease was investigated.

1.5 References


CHAPTER 2

Adsorption of Lysozyme on Base Metal Surfaces in the Presence of an External Electric Potential

2.1. Introduction

The adsorption of protein to a solid surface often plays a vital role in various industrial fields, such as the construction of biosensors and biochips [1,2], immobilized enzyme preparations [3-5] manifestation of the biocompatibility of artificial implants [6,7], and proteinaceous fouling on manufacturing equipment surfaces in industrial settings [8-11]. Hence protein adsorption is a topic that has been extensively and continuously investigated for more than 50 years [8,9,11].

The factors that determine the protein adsorption behavior are generally classified into three, namely, the nature of the protein, surface characteristics, and the type of medium. Structural flexibility (or fragility) [12,13], isoelectric point (or net charge) [13], and molecular size [14] of proteins directly affect the extent and strength of the adsorptive interactions between a protein molecule and a solid surface as well as the surface area occupied by the adsorbed protein molecule; Metal [15], semiconductor [16,17], glass [18,19], and plastic materials [20-22] all have different adsorption characteristics and presumably different adsorption mechanisms are operative for these
materials; The pH of the adsorption medium as well as the electrolyte type and concentration naturally have effects on the ionization state of a protein and the nature of the solid surface [17,23-25], which can have a significant impact on protein adsorption behavior [17,25].

On the other hand, a solid surface always has some electric potential that varies depending on the material type and the contacting conditions. The surface electric potential is naturally considered to be one of the factors that affect protein adsorption behavior. In actual fact, protein adsorption can be altered by applying an external potential to the adsorptive surface, in which several electrode surfaces as adsorption surface have been used [2,26-32]. The adsorption of albumin, cytochrome c, and soybean peroxidase to a Au surface was reported to increase as the result of imposing both negative and positive external potentials [28-30], and a similar tendency was observed for the adsorption of fibrinogen to a platinum surface [26]. In the case for a carbon-based electrode as an adsorptive surface, increasing the surface potential from negative to positive resulted in a monotoneous decrease in the surface coverage by the adsorbed protein (bovine serum albumin and fibrinogen) while the adsorption rate showed a complicated dependence on the surface potential [26,31]. It was also reported that the rate and amount of protein adsorption on an optically transparent carbon electrode (OTCE) were increased when a positive external potential was imposed [26,31], which was more significant for a hard protein rather than for a soft one [32]. All these reports demonstrate that it is possible to control protein adsorption by adjusting the external electric potential [2,30].

Base metals, such as stainless steel and titanium, are important materials that are used in various industrial processes. The surface of a base metal is usually covered
with a thin oxide film, which also has a strong affinity for protein molecules [33-36], resulting in the formation of proteinaceous soiling [8,9,11]. For this reason, the necessity of controlling protein adsorption on base metal (oxide) surfaces has been a subject of intense interest for a long time [3,37]. However, only a few studies have been conducted on how and why the external electric potential affects the adsorption of a protein on the base metal (oxide) surface [27].

The aim of this study was to understand the impact of an external electric potential on the adsorption of a protein to base metal (oxide) surfaces. Six types of base metal substrates and hen egg-white lysozyme were used as an adsorptive surface and a protein, respectively. Protein adsorption in the presence of different external potentials were measured in situ under different conditions, including pH, electrolyte type and concentration, and substrate material, by means of ellipsometry. The attained thickness (amount) of the adsorbed protein and the initial adsorption rate were investigated for the surface potential dependencies. Based on the experimental results, a proposed mechanism for the impact of an external electric potential on the adsorption of a protein on a base metal (oxide) surface is discussed.

2.2. Materials and Methods

2.2.1 Materials

Six types of base metal plates (Table 1) (30x50x1 or 2 mm) that had been mechanically polished to a mirror sheen were purchased from Fruuchi Chemical Co. (Tokyo, Japan). Lysozyme from hen egg white (LSZ) (L-6876) was obtained from Sigma-Aldrich Co. (St. Louis, MO). Potassium chloride, magnesium chloride, and
potassium sulfate as an electrolyte and ca. 12 M hydrogen peroxide solution, for the regeneration of the protein-adsorbed base metal surfaces, were the products of Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

2.2.2. Protein adsorption onto base metal surfaces with applied electric potential

The adsorption of protein (LSZ) on the base metal surface in the presence of an external electric potential was conducted using the same experimental setup as was used in our previous studies [27,28]. The base metal plate was immersed in 20 mM electrolyte solution (pH 5.8, 550 mL) in a glass cell, and a prescribed electric potential was applied to the plate, using a potentiostat (HSV-100, Hokuto Denko Co., Tokyo, Japan). An Ag/AgCl electrode (immersed in saturated KCl solution) and a platinum sheet (5x5x0.1 mm) were used as reference and counter electrodes, respectively. Nitrogen gas was purged into the glass cell solution at a flow rate of 35 mL/min (throughout the adsorption experiment) to minimize gas dissolution and provide constant stirring. Several milliliters of the electrolyte solution in the glass cell was then replaced with the same volume of the LSZ stock solution (~10 mg/mL, in 20 mM KCl solution) so as to give a final protein concentration of 10 µg/mL. Immediately thereafter, the solution in the glass cell was vigorously stirred using a pencil-type mixer (GL. Sciences Co., Tokyo, Japan) with a hand-made rotating tip (8 mm width, 50 mm length) for 10 s, which initiated the adsorption of the LSZ on the sample plate surface. On the other hand, as described later, this study revealed that the application of certain range of potential to base metal surfaces except for Ta and Ti mostly avoided the adsorption of LSZ. Hence, alternatively, the LSZ adsorption was initiated by switching from the non-adsorptive surface potential to a prescribed adsorptive one after
Table 1. Refractive indexes, extinction coefficients, and water contact angles of base metal surfaces used in this study as well as those of the adsorbed lysozyme layer. Standard deviations (N≥10) for each value are also shown.

<table>
<thead>
<tr>
<th>Material</th>
<th>Refractive index (-)</th>
<th>Extinction coefficient (-)</th>
<th>Water contact angle (˚)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUS316L (St)</td>
<td>2.6±0.2</td>
<td>4.3±0.2</td>
<td>44±3</td>
</tr>
<tr>
<td>Titanium (Ti)</td>
<td>2.5±0.1</td>
<td>3.1±0.2</td>
<td>9±1</td>
</tr>
<tr>
<td>Zirconium (Zr)</td>
<td>2.2±0.1</td>
<td>2.7±0.05</td>
<td>66±3</td>
</tr>
<tr>
<td>Tantalum (Ta)</td>
<td>2.16±0.05</td>
<td>2.36±0.05</td>
<td>40±7</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>4.43±0.02</td>
<td>4.51±0.02</td>
<td>28±4</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>2.08±0.05</td>
<td>4.03±0.05</td>
<td>46±6</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.38 [27]</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
the sample plate was sufficiently (~1 h) immersed in the LSZ adsorption with applying the non-adsorptive potential. Both the procedures for the LSZ adsorption exhibited the same adsorption processes, which demonstrated that the 10-s agitation sufficiently homogenized the LSZ solution in the glass cell. The amount of the LSZ adsorbed on the sample plate surface during the LSZ adsorption was measured as the thickness of the layer of LSZ that was adsorbed using an ellipsometer (Mizojiri Optical Co. Ltd., Tokyo, Japan). In the ellipsometric measurement, the sample plate surface was irradiated with a He-Ne laser (633 nm, wavelength) through an optical glass window of the glass adsorption cell. The reflected ray was collected at 1.68-s intervals with a rotating light detector and then converted into the LSZ adsorption layer thickness. In the calculation of the adsorption thickness, the refractive index (RI) of the adsorbed LSZ layer was fixed to be 1.38 on the basis of our previous analyses [38]: Although this constant RI assumption overrode the information on the density of the adsorbed LSZ layer and converted the calculated thicknesses into the apparent ones, it provided a sufficient correlation between the adsorption layer thickness and the amount adsorbed [38]. The RI values and extinction coefficients for the bare base metal surfaces were measured in the LSZ-free aqueous solution using the ellipsometer before the initiation of the LSZ adsorption (Table 1) and used for the calculation of the adsorption layer thickness.

Alternatively, the adsorption experiments were conducted at pHs of 4 and 7 where the solution pH was adjusted by adding small amounts of HCl or KOH, respectively. The pH changes during the adsorption experiments were at most ±0.3.
2.2.3. Fourier transform Infrared spectroscopy of adsorbed LSZ on a base metal surface

The relationship between the measured thickness of LSZ adsorption layer and the amount of adsorbed protein were determined following procedures that were used previously [38,39]: (i) The calibration line between the amount of LSZ on the sample plate and infrared (IR) absorption intensity was determined for each sample plate material. Namely, several µL of a LSZ solution (~0.1 mg/mL, in electrolyte free water) were placed on the center of the sample plate and the sample was then dried to fix LSZ on the sample plate. The sample plate with a known amount of LSZ was then set on the sample stage of a reflection-absorption (RA) accessory (FT-80; SpectraTech, Shelton, CT) inserted in a Fourier transform spectrometer (Magna 560; Nicolet, Madison, WI), so that entire fixed LSZ stain was included inside the sample stage window (13 mm in diameter) to be irradiated by IR light. The IR spectra of different amounts of LSZ on the sample plates were obtained at a resolution of 8 cm⁻¹ with 64 scans. The area of the IR band at around 1650 cm⁻¹ due to carbonyl stretching vibration (amide I band) of the fixed LSZ was determined from the obtained IR spectrum and plotted against the surface LSZ density (mg-LSZ/m²-surface), derived from the amount of fixed LSZ divided by the area of the sample stage window; (ii) IR spectra of the sample plates, that were subjected to the LSZ adsorption (and for which the thickness of the LSZ adsorption layer was measured), were obtained from time to time using the same procedure as described above (i). The area of the IR absorption due to amide I band of the adsorbed LSZ was determined from the IR spectrum and converted into the amount of adsorbed LSZ (mg/m²) by using a calibration line obtained by procedure (i). Based on the data sets for the LSZ adsorption layer thickness and the amount of adsorbed LSZ for each sample plate material, and by assuming that the thickness of the adsorbed layer is proportional to the amount adsorbed [38,39], the
thickness of the LSZ adsorption layer was converted into the amount of adsorbed protein.

2.2.4. Regeneration of sample plates

All the sample plates were used repeatedly in the LSZ adsorption experiment. For the regeneration of a sample plate that had been used for LSZ adsorption as well as the pretreatment for the LSZ adsorption, the sample plate was subjected to a H$_2$O$_2$-electrolysis cleaning procedure [38,40] to completely remove the adsorbed LSZ. Namely, after the LSZ adsorption experiment, the LSZ solution in the glass adsorption cell was replaced with a protein-free 20 mM KCl solution containing 10 mM H$_2$O$_2$. A negative electric potential (-0.4~1.6 V vs Ag/AgCl) was then applied to the sample plate with the adsorbed LSZ using the same potentiostat system as was used in the LSZ adsorption experiment. The adsorbed LSZ on the sample plate was effectively removed from the sample plate by hydroxyl radicals generated due to the electrolysis of H$_2$O$_2$ (H$_2$O$_2$ + e$^-$ → •OH + OH$^-$) [40], which was monitored by an ellipsometer. The H$_2$O$_2$-electrolysis treatment was continued for more than 30 min until a further decrease in the adsorption layer thickness was negligible.

All of the above experiments were done at 25 ± 2 °C and repeated at least in duplicate for each condition.

2.2.5 Contact angle measurement

The contact angles of a sessile water on the base metal substrates used in this study were measured by means of a goniometer at 25±2°C to evaluate the hydrophobicity/hydrophilicity of the substrates. More than ten independent measurements were conducted at different locations of the surface for each base metal
substrate.

2.3. Results and Discussion

2.3.1 Adsorption characteristics of LSZ on electric potential applied St surface

Figure 1 shows the representative processes for the adsorption of LSZ on a base metal surface (St) at different applied electric potentials. Typically, the increase in the thickness of the LSZ layer proceeded via three steps, as shown in Fig. 1. Namely, (1) the LSZ adsorption layer thickness rapidly increased immediately after the start of the adsorption; (2) The increase in the adsorption layer thickness then slowed down, (3) followed by reaching a maximum value within ca. 3,000 s, although the LSZ adsorption layer thickness continued to increase throughout the experimental period (~7000 s) under certain conditions (curve for -0.3 V vs Ag/AgCl, in Fig. 1). Hence, the LSZ adsorption processes under different conditions were analyzed from the maximum amount of adsorbed LSZ and the initial adsorption rate at a given LSZ concentration.

2.3.2 Effect of electrolyte type and pH on LSZ adsorption

In Fig. 2(a), the amount of LSZ adsorbed on a St surface at pHs 4.0, 5.8, and 7.0 are shown as a function of the applied surface potential. In the cases of the consecutive adsorption, the amounts of LSZ adsorbed at a time point of 7,000 s were retrieved and are also plotted in Fig. 2(a) as closed keys. As shown in Fig. 2(a), increasing the surface potential decreases the amount of LSZ that is adsorbed, and the relationship between the amount of LSZ adsorption and the surface potential appears to be shifted toward a more positive potential with increasing pH. It should be noted that the adsorption of LSZ is minimized in potential ranges above certain thresholds.
In our previous study [27], the adsorption of β-lactoglobulin, which is acidic (pI 5.1 [41]), on a St surface was found to be largely prevented when a negative potential was applied to the St surface. On the other hand, as shown in Fig. 2(a), the adsorption of the basic LSZ was minimal in the positive potential ranges. These suggest that the protein adsorption onto a base metal surface also can be controlled by controlling the surface electric potential, as reported for those onto the electrode materials [2,26-32], and that the surface potential range for the protein adsorption control is varied depending on the net charge of the protein.

In an aqueous solution, the oxide surface on a base metal is covered with hydroxyl groups, and the surface –OH groups can acquire various charges, i.e., cationized (-OH₂⁺) or anionized (-O⁻) at pHs below or above the isoelectric point of the surface, respectively [23,24]. Our previous studies [13,35,42] revealed that these ionized surface hydroxyl groups serve as sites for electrostatic interactions with acidic and basic amino acid residues of a protein, which are largely responsible for protein adsorption on a base metal surface (without applying any electric potential). Acidic and basic proteins thus tend to adsorb on a base metal surface under acidic and basic conditions, respectively, where surface hydroxyl groups largely exist as –OH₂⁺ and –O⁻, respectively [23,24]. On the other hand, an electrically polarized surface would naturally attract or repel protein molecules. Such an electric field-based interaction was actually proposed as one of the main mechanisms for the adsorption of a protein on electrode surfaces in previous studies [2,26-32].
Fig. 1. Courses for the thickness of the adsorbed LSZ layer on externally a polarized stainless steel plate surface in a 20 mM KCl solution containing 10 μg/mL LSZ at 25±2°C.
Fig. 2. Amounts of LSZ adsorbed on St as a function of applied surface potential under different conditions, including (a) pH and (b) type of electrolyte (pH 5.8). The electrolyte concentration was 20 mM (a) or varied from 2 mM to 200 mM (b). LSZ concentration and temperature were 0.01 mg/mL and 25±2°C, respectively. Closed keys denote the amount adsorbed at 7000 s for the cases of consecutive adsorption. Error bars represent the highest and lowest values obtained for each condition.
2.3.3. Mechanisms of LSZ adsorption on base metal surface

Based on these considerations and according to the pI value of St surface (5.8 [43]), the dependences of the amount of LSZ that is adsorbed on pH and applied surface potential (Fig. 2(a)) can be explained as follows: In the case of a pH below the surface pI value, electrostatic repulsions likely occur between LSZ molecules and surface –OH$_2^+$ groups (upper cases in Fig. 3), and the range of negative applied potential used may not induce a sufficiently strong an electric field-based attraction to overcome the repulsion against surface –OH$_2^+$ groups at pH 4 (upper left in Fig. 3). As the pH becomes higher, the electrostatic repulsion between LSZ and surface hydroxyl groups is weakened by the conversion of surface –OH$_2^+$ to –OH or –O$^-$ (middle cases in Fig. 3), and it would follow that the adsorption of LSZ would occur due to an electric field-based attraction in the negative potential range (middle left in Fig. 3), as observed at pH 5.8 (Fig. 2(a)). When the pH increases above the surface pI value (bottom cases in Fig. 3), the electrostatic repulsion between LSZ and surface hydroxyl groups then turns into attractive forces. Therefore, a more positive surface potential (and thus a greater electric repulsion for LSZ) may be required to minimize LSZ adsorption by the increased electrostatic interactions between LSZ and surface –O$^-$ groups (bottom right in Fig. 3), as observed in Fig. 2(a).

As shown in Figs. 1 and 2(a), when the applied surface potential is lowered below a certain value, the adsorbed layer of LSZ continues to increase in thickness throughout the experiment (~7,000 s). A similar phenomenon has been observed in previous studies [31,32]. Benavidez and Garcia [31] proposed that an external electric potential may induce polarization of the protein; One end of the polarized protein binds to the surface (or adsorption layer) and the other end serves as an adsorption site(s) for
consecutive protein lamination (adsorption). However, from the fact that the LSZ adsorption does not occur on the positively polarized surface, it is considered that a LSZ molecule may not be polarized so as to provide the binding (negative) sites for the positively polarized surface. This conflicts with the above mechanism based on the intramolecular polarization of the adsorbed protein [31]. The other possible explanation is as follows: When the sample plate surface is extensively covered with the adsorbed LSZ, the screening of the surface potential by the adsorbed LSZ molecules would become significant. By the potential drop due to the electric resistance of the adsorbed LSZ layer, an additional potential may be applied to the sample plate, which would induce further LSZ adsorption via electric field-based attraction. Consequently, the adsorption of LSZ and amplification of the applied surface potential may continue to occur, resulting in the continuous adsorption of LSZ, as shown in Fig. 1.

The amounts of LSZ that were ultimately adsorbed (on a St surface at pH 5.8) were measured in the presence of different types of electrolytes (KCl, MgCl₂, K₂SO₄) as well as at different KCl concentrations (Fig. 2(b)). In the negative surface potential range, an increase in KCl concentration and the presence of 20 mM MgCl₂ or K₂SO₄ instead of KCl resulted in a decrease in the final amount of adsorbed LSZ, as shown in Fig. 2(b). This can be attributed to the increase in ionic strength, which is consistent with the contribution of an external surface electric field on LSZ adsorption. On the other hand, a slight increase in the amount of LSZ that was adsorbed was detected at the positive surface potentials in the presence of 20 mM K₂SO₄. This can be attributed to the surface-bound divalent anion (SO₄²⁻) functioning as a bridge between the positively charged LSZ molecules and the positively polarized surface [44,45].
Fig. 3 Possible mechanism of the dependences of LSZ adsorption on pH and applied surface potential. The cases surrounded by dotted lines allow the LSZ adsorption on a base metal surface.
2.3.4 Influence of applied electric potential on LSZ adsorption onto different types of base metal surfaces

The LSZ adsorption processes were measured for different six base metals in the applied potential ranges where the generated electric currents were ignorable (< several µA/cm²). In Fig. 4, the amount of adsorbed LSZ on different base metal surfaces are plotted as a function of the applied surface potential, as well as the amount of LSZ adsorbed at 7,000 s for the case of the consecutive adsorption (closed keys in Fig. 4). As shown in Fig. 4, increasing the surface potential generally tends to decrease the LSZ adsorption, which is similar to the tendency observed for the St substrate (Figs. 2). Interestingly, the Ta surface shows a noticeable increase in the amount adsorbed at around + 0.2 V vs Ag/AgCl, and a possible mechanism for this is discussed below.

The tested base metals not including Cr show the consecutive LSZ adsorption when the amount of adsorbed LSZ exceeds of 3 mg/m². The threshold for the amount adsorbed coincides with the adsorption of a monolayer of protein [3]. This suggests that the surface area occupied by the adsorbed LSZ molecule may be the same for the tested base metal surfaces and, furthermore, supports the above interpretation that origin of the consecutive adsorption may be due to the screening of the surface potential as the result of the adsorbed LSZ.

It is naturally expected that the surface roughness of sample plate could affect the adsorbed amount of a LSZ monolayer. However, the adsorbed amount thresholds for the consecutive adsorption for different base metal surfaces appear within the same range (Fig. 4), indicating that the base metal plates used in this study did not have any significant difference in the surface roughness.
Fig. 4. Amounts of LSZ adsorbed on different base metal surfaces as a function of applied surface potential. The electrolyte concentration was 20 mM (pH 5.8). LSZ concentration and temperature were 0.01 mg/mL and 25±2°C, respectively. Closed keys denote the amount adsorbed at 7000 s for the cases of consecutive adsorption. Error bars represent the highest and lowest values obtained for each condition.
As shown in Fig. 4, the potential ranges where the LSZ adsorption is minimal are different among the examined base metals. Namely, (when compared at pH 5.8,) the adsorption of LSZ on St and Ti surfaces is minimized at potentials at around and above 0 V vs Ag/AgCl, and, for Ta and Zr, LSZ adsorption is minimized in the potential ranges >-0.4 and >-0.6 V vs Ag/AgCl, respectively. The threshold potentials for Cr and Ni appear slightly (±0.2 V) more negative and positive than that for St and Ti, respectively. Regarding the minimum values of the amount of LSZ adsorbed, the extent of adsorption was approximately 1 mg/m² for Ta and Ti, even in the potential range where LSZ adsorption is minimized. The amounts of LSZ adsorbed on the other surfaces are largely decreased below 0.3 mg/m² in the potential ranges for the minimal LSZ adsorption (Fig. 4).

Based on the above interpretation of the dependences of LSZ adsorption on pH and applied surface potential (Fig. 3), the potential range for allowing LSZ adsorption (Fig. 4) may indicate the magnitude of the electrostatic repulsion between LSZ and surface cationized hydroxyl groups: In the cases of Ta and Zr, a more negative surface potential is required to overcome the repulsion between LSZ and surface –OH₂⁺ groups and to allow the LSZ adsorption to proceed than in the cases for the others. However, there found no correlation of the adsorption threshold potential with the isoelectric point of base metal [24,43,46] although the ionization state of surface hydroxyl groups is determined by the surface isoelectric point. Such a conflict may occur because the surface treatment history can alter the isoelectric point [47,48] while further investigations would be needed to confirm this.

As shown in Figs. 2 and 4, the adsorption of LSZ on St, Ti, Zr, Cr, and Ni surfaces occurs only in potential ranges below certain values. Similarly, a carbon-based
electrode was also reported to support protein adsorption only in the positive or negative potential range [28,30]. On the other hand, previous studies indicated that the adsorption of a protein on Au [28,30] and Pt [26] was significant both in the negative and positive potential ranges. Accordingly, the electroconductive surfaces can be categorized into two types, according to the dependence of protein adsorption on the surface potential. Considering the contribution of a divalent anion (SO$_4^{2-}$) on the adsorption of LSZ on the St surface at positive potentials (Fig. 2(b)), the difference in the surface potential dependences could have originated from the binding of counterions on the surfaces. Namely, counterions, including monovalent ones, could be bound on a noble metal surface so tightly as to bridge protein molecules with the repulsive charges to the surface. This bridging effect of surface-bound counterions may also be responsible for the adsorption of LSZ on the Ta surface at potentials $>0.2$ V vs Ag/AgCl (Fig. 4). In contrast, the high hydrophobicity of the carbon-based electrode surface and the hydroxyl groups on the base metal surface may preclude any tight surface binding of monovalent counterions, resulting in minimizing protein adsorption at potentials that are repulsive to protein molecules.

Our previous studies demonstrated that electrostatic interactions between ionized groups of a protein and surface hydroxyl groups largely contribute to the adsorption of a protein on base metal surfaces [13,35,42,49]. On the other hand, it has also been suggested that hydrophobic interactions [50] and complexation with oxide species [51,52] could be related to the adsorption of a protein on a solid surface. These, as well as counterion-bridging, might explain the persistence of the LSZ adsorption on Ti and Ta surfaces in the positive potential range (Fig. 4). However, the contact angles of the base metal surfaces that were examined (Table 1) indicate that the base metal surfaces are generally hydrophilic, and the contribution of hydrophobic interactions may
Fig. 5. Initial LSZ adsorption rate under different conditions as a function of applied surface potential. The LSZ concentration and temperature were 0.01 mg/mL and 25±2°C, respectively. The adsorption rate constants were determined by dividing the initial slopes of the LSZ adsorption process by the amount of adsorbed LSZ. The adsorption rate constants for consecutive adsorption were not determined because of the absence of the amount adsorbed. Error bars represent the highest and lowest values obtained for each condition.
thus be quite limited for the adsorption of LSZ to the base metal surfaces, including the persistent ones to the positively polarized Ta and Ti surface (Fig. 4).

2.3.5 Initial adsorption rate of LSZ adsorption onto different types of based metal surface

The first order adsorption rate constants were determined from the adsorption process by dividing the initial increasing slope by the thickness of the attained adsorption layer (Fig. 5). As shown in Fig. 5, the rate constants for the adsorption of LSZ are mostly in the same order of ~0.01 s\(^{-1}\) and appear to be independent of the applied surface potential and the surface material as well as the type of or the concentration of the electrolyte used. On the other hand, previous studies dealing with the adsorption of protein on electrode surfaces [2,26,28-32] indicated that the initial adsorption rate was increased in the presence of an external electric field. In this study, the protein concentration used (10 µg/mL) was one tenth or lower than those in the reported literature (100~500 µg/mL) [2,26,28-32], and thus the diffusion of LSZ molecules on the sample plate surface can be assumed to be the rate-limiting step in the adsorption in the testing conditions. Accordingly, the initial rate of adsorption may not be increased, even in the case of an additional mechanism (electric field-based one) for LSZ adsorption, as shown in Fig. 5.

According to these findings, the adsorption of protein on an electrically-polarized base metal surface can be considered to be closely related to the two modes of interactions, that is, electrostatic interactions between the protein and ionized surface hydroxyl groups and electric field-based interactions. This study suggests that the nature of the ionization of surface hydroxyl groups may vary with the type of surface material, and the external electric potential clearly determines the direction (attraction or repulsion)
and strength of the electric field-based interaction. Considering these factors, it may be possible to control the adsorption of a protein on a base metal surface appropriately adjusting the applied surface potential, as has been indicated for the protein adsorption on the electrode materials [2,30].

However, while lysozyme is a small rigid molecule, a large and/or structurally flexible protein would undergo a significant conformational change upon its adsorption [3, 11, 12], which may affect adsorption behavior in the presence of an external electric field. Hence, further studies using different types of proteins are planned as the next step of this study to examine the general validity of the methodology for controlling protein adsorption on a base metal surface by applying an electric potential to the surface.

2.4. Conclusion

The adsorption of hen egg white lysozyme (LSZ) on base metal surfaces (stainless steel SUS316L (St), Ti, Ta, Zr, Cr, or Ni) in the presence of an external electric field was investigated, focusing on the impacts of surface electric potential, pH, electrolyte type and concentration, and base metal type. The adsorption of LSZ for different conditions was characterized by the amount of LSZ that was adsorbed and the initial adsorption rate at a given LSZ concentration (10 µg/mL). The amount of adsorbed LSZ was increased with more negative potentials in the potential range below a certain threshold and was minimized at potentials above the threshold. The threshold potential for the adsorption of LSZ was higher at higher pH, and when the amount of LSZ adsorbed increased above that of a monolayer, the adsorption turned from a finite mode into a consecutive one. Considering these results and the knowledge of the
ionization states of hydroxyl groups on a base metal surface, the adsorption of LSZ was deduced to depend on (i) the electric force by an external electric field and (ii) electrostatic interactions between the positively charged LSZ and ionized surface hydroxyl groups. Namely, the adsorption of LSZ on a base metal surface may occur when the (i) electric field-based interaction surpasses the (ii) electrostatic repulsion between positively charged LSZ and surface $-$OH$_2^+$ groups (or when LSZ molecules form stronger (ii) electrostatic interactions with surface $-$O$^-$ groups than (i) electric repulsion to the surface). The presence of a divalent anion (SO$_4^{2-}$) instead of a monovalent one (Cl$^-$) resulted in an increase in LSZ adsorption in the positive potential range, possibly because the surface-bound divalent anion (SO$_4^{2-}$) serves as a connection between both the positively charged (polarized) LSZ and the surface. Among the different base metal types, the Ta and Zr surfaces exhibited a markedly lower threshold potential for LSZ adsorption than the others, implying the existence of more cationized surface hydroxyl groups at the tested pH (5.8). All these findings support the conclusion that the adsorption of protein on a base metal surface can be controlled via the use of an external electric potential, which has been suggested also for the protein adsorption on the electrode materials. On the other hand, protein adsorption often causes a conformational change, which is more significant for larger and/or softer proteins and would likely have an effect on the adsorption of a protein to an electrically polarized surface. The surface electric field-based influence on the adsorption of a protein to a base metal surface should therefore investigated using different types of proteins and findings in the area will be reported in the near future.
2.5. References


1747–1754.


CHAPTER 3

Adsorption characteristics of various proteins onto metal surface in the presence of an external electric field

3.1. Introduction

Adsorption of protein onto solid surfaces is a topic that has extensively and continuously intrigued researchers in the fields of surface science and bioengineering [1]. In the adsorption of protein onto solid surfaces, various adsorption conditions including pH [2,3], surface material [4-8], temperature [9,10], protein type [11-14], type and concentration (ionic strength) of electrolyte [15,16] and so on [17] have been examined. As for the mechanism of the protein adsorption onto a metal (oxide) surface, some researches have proved that the electrostatic interaction between a solid surface and protein molecule is essential [18,19]. Since the net charge of adsorbate protein molecules and the charge state of metal (oxide) surface markedly vary depending on the pH, the pH dependence of protein adsorption may be explained by the net charges of protein and solid surface.

Control of the adsorption of proteins onto solid surfaces are interested in a wide varieties of fields [20]. For example, the reduction of the amount of adsorbed protein onto the manufacturing facility wall in food and drug industries leads to the facilitation of the cleaning of them, which have been actually carried out using considerable water, detergent, energy, and time [1]. On the other hand, the immobilization of proteins on solid surface in an intended ordination is a key technology for developing and
improving nano-devices such as protein chip [1,20].

Based on the findings on the crucial contribution of the protein-surface electrostatic interaction to the adsorption, as described above, one may think that the protein adsorption can be controlled by electrochemically controlling the electric potential of the adsorbent surface. Actually, to date, several trials to control (reduce or enhance) the protein adsorption have been conducted with the aid of applying electric potential to the adsorbent metal surface [21-25]. Also in our previous study [26], the influence of an external electric field on the protein adsorption onto a base metal oxide surface was investigated, using representative protein (lysozyme, LSZ) and six types of base metals (St, Ti, Ta, Zr, Cr, and Ni). Results demonstrated the feasibility of the electrochemical control of the protein adsorption: The negative polarization of metal (oxide) surface tended to suppress the protein whereas the protein adsorption was strongly enhanced in the surface potential range above certain value.

As the next step, adsorption characteristics of protein on base metal (oxide) surface in the presence of an external electric field were investigated, focusing on the impact of protein types, herein. Different electric potentials (−1.5~+0.4 V vs. Ag/AgCl) were applied to the metal (oxide) surface, which was in contact with the protein solution. The amounts of adsorbed protein are monitored by an in-situ ellipsometry. Eighteen types of protein and six types of base metals were used as adsorbate and adsorbent, respectively. The dependence of the protein adsorption on the applied surface potential was measured and compared among different types of proteins as well as different base metal surfaces. The relationship of protein characteristics with the surface potential dependence of the protein adsorption was discussed.

3.2. Materials and Methods

3.2.1. Materials

Fifteen types of proteins as adsorbate were purchased from Sigma-Aldrich Co. (St. Louis, MO). All types of proteins were of highest purity (>99 %) and used without
further purification. Information on the proteins are listed in Table 1. Six types of metal plates (30 x 50 x 1 or 2 mm) having mechanically polished mirror surfaces were the products of Furuchi Chemical Co. (Tokyo, Japan). Potassium chloride powder and hydrogen peroxide solution (ca. 12 M) were obtained from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The sample plates that had been used for the adsorption experiment were regenerated by an H₂O₂-electrolysis treatment [27] and repeatedly used in this study.

3.2.2. Protein adsorption onto base metal surfaces with applied electric potential

The adsorption of protein onto the sample metal surface with applied electric potential was conducted using the same experimental setup as was used in our previous study [26]. In brief, a sample metal plate was immersed in 20 mM KCl, and a prescribed electric potential was applied to the sample plate, using a potentiostat. The protein adsorption was initiated by adding the protein stock solution so as to give a final protein concentration of 10 µg/mL. The thickness of adsorption layer of the adsorbed protein was monitored by using an ellipsometer, in which a He-Ne laser (633 nm, wavelength) was irradiated to the sample plate surface, and a nitrogen gas was purged into the glass cell solution at the flow rate of 35 mL/min to prevent gas dissolution and constantly stir the solution [26]. The refractive indexes (RI) and extinction coefficients of the tested metal surfaces as well as the RI value of the adsorbed protein, used for the calculation of the adsorption layer thickness, had been determined in our previous studies [26,28,29].
<table>
<thead>
<tr>
<th>Protein (Cat. No.)</th>
<th>Source</th>
<th>pI</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin (P-7012)</td>
<td>Porcine gastric mucosa</td>
<td>&lt;1</td>
<td>34</td>
</tr>
<tr>
<td>α-Lactalbumin (L-6010)</td>
<td>Bovine milk</td>
<td>4.3</td>
<td>14.2</td>
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<td>Ovalbumin (A-5503)</td>
<td>Chicken egg white</td>
<td>4.6</td>
<td>42.7</td>
</tr>
<tr>
<td>BSA (A-7638)</td>
<td>Bovine serum</td>
<td>4.8</td>
<td>69</td>
</tr>
<tr>
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<td>5.1</td>
<td>18.4</td>
</tr>
<tr>
<td>Carbonic anhydrase (C-3934)</td>
<td>Bovine erythrocytes</td>
<td>5.3-5.</td>
<td>30</td>
</tr>
<tr>
<td>Conalbumin (C-7786)</td>
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<td>77</td>
</tr>
<tr>
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<td>Sheep serum</td>
<td>5.8-7.</td>
<td>153</td>
</tr>
<tr>
<td>Lactoferrin (L-9507)</td>
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<td>70</td>
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<tr>
<td>Trypsinogen (T-1143)</td>
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<td>24</td>
</tr>
<tr>
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<td>Bovine pancreas</td>
<td>9.5</td>
<td>25.7</td>
</tr>
<tr>
<td>RNase A (R-5500)</td>
<td>Bovine pancreas</td>
<td>9.6</td>
<td>13.7</td>
</tr>
<tr>
<td>Trypsin (T-1426)</td>
<td>Bovine pancreas</td>
<td>10.1-</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Lysozyme (L-6876)</td>
<td>Chicken egg white</td>
<td>11.2</td>
<td>14.5</td>
</tr>
<tr>
<td>Protamine (P-4005)</td>
<td>Salmon</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>
3.2.3. Fourier transform Infrared spectroscopy of adsorbed protein on a base metal surface

The relationship between the measured thickness of protein adsorption layer and the amount of adsorbed protein were determined for all the tested proteins in the same way as was used in our previous studies [26,28,30]. Namely, the known amounts (mg) of protein were dry-fixed on the sample plates and analyzed for the IR absorption areas due to protein amide I band (~1650 cm\(^{-1}\)) by a Fourier transform infrared reflection-absorption (FTIR-RA) spectroscopy. The amide I band area of the fixed protein was plotted against the surface protein density (mg-protein/m\(^2\)-surface). Next, the IR spectra of the sample plates, that had been measured for the protein adsorption layer thickness, were measured. The amide I band area of the adsorbed protein was determined and then converted into the amount adsorbed, using the preliminarily obtained relationship between amide I band area and the fixed protein amount. Finally, the conversion factors from the protein adsorption layer thickness to the amount of adsorbed protein were determined for all the tested proteins, as shown in Table 1.

3.3. Results and discussion

Representative time courses of thickness of adsorbed on metal surface are shown in Fig.1. As exemplified by the results for -0.2 V vs Ag/AgCl in Fig. 1, many of the tested proteins exhibited the potential range where the adsorption was minimized. On the other hand, in the applied potential range where the protein adsorption occurs, the adsorption process drastically proceeded at the early stage of the adsorption experiment, and the increasing rate of the adsorption layer thickness was then slowed down. The attained value of the adsorption layer thickness (namely, amount adsorbed) as well as the initial adsorption rate are varied depending on the applied surface potential while the adsorption process turned into a continuous one at the extremely negative and positive applied surface potentials. These findings are qualitatively consistent with those previously observed for the adsorption of lysozyme onto base metal surfaces [26]. In the following, the maximum amount of adsorbed protein and the initial adsorption rate
were compared among different conditions.

The relationships between the protein adsorbed amount and the applied surface potential for different pHs (4.0, 5.8 and 7.0) were compared, using β-lactoglobulin (β-Lg, pI 5.1) and lysozyme (LSZ, pI 11.2) as typical acidic and basic proteins (Fig. 2). As shown in Figs. 2(a) and (b), the adsorbed amounts for β-Lg and LSZ tend to increase with more positive and negative surface potential, respectively. It should be noticed that β-Lg and LSZ exhibit contrasting dependences on pH. Namely, the applied surface potential ranges of the minimum adsorption for β-Lg and LSZ appear to be shifted toward a more negative and positive potential as the pH becomes higher while the adsorbed amount of β-Lg for pH 4 is almost constant in the tested potential range.

In our previous study [26], the pH dependence of the relationship between the amount adsorbed and the surface potential for LSZ were explained based on the ionization state of the surface hydroxyl groups, the (sign of) applied surface potential, and the net charge of LSZ molecule. Similarly, the pH dependence of the relationship between surface potential and the amount adsorbed, observed for acidic β-Lg, also can be interpreted as follows: In the acidic pH range (pH ~4.0), the surface hydroxyl groups exist as cationized –OH$_2^+$, which is known to induce the dissociation (anionization) of carboxylic groups of β-Lg molecule [31,32] although the β-Lg molecule has a positive net charge in an acidic aqueous solution. Consequently, the electrostatic interaction of β-Lg and –OH$_2^+$, responsible for the adsorption, may be formed. The adsorptive interactions may be maintained against the electric repulsion to the negatively polarized surface in the tested surface potential range, as shown in Fig. 2(a). On the other hand, when the pH increases from 4.0 to around the pI value (~5.8) and then to 7.0, the cationized –OH$_2^+$ group is considered to turn into neutral –OH and subsequently anionized –O$^-$ [33,34]. Accordingly, the increasing pH may decrease the electrostatic interactions between β-Lg carboxylic group and surface –OH$_2^+$ group, resulting in the positive shift of the potential range of the minimum protein adsorption, as shown in Fig. 2(a).
Fig. 1. Courses for the thickness of adsorbed proteins layer under different conditions. (a) Protein: β-lactoglobulin, lysozyme, α-albumin, Applied surface potential: +0.4 V vs Ag/AgCl; (b) Protein: β-lactoglobulin, lysozyme, α-albumin, Applied surface potential: -0.3 V vs Ag/AgCl; (c) Protein: lactoferrin, Applied surface potential: -0.4, -0.2, 0.0 V vs Ag/AgCl; (d) Protein: BSA, Applied surface potential: -0.4, -0.2, 0.0, +0.2, +0.4 V vs Ag/AgCl. Stainless steel SUS316L plate was used as a metal oxide surface, and the pH and temperature in the adsorption experiment were 5.8 and 25 ± 2°C, respectively.
Fig. 2. Amount of protein adsorbed on St pate surface and initial protein adsorption rate constant as a function of applied surface potential under different pH conditions (pH 4, 5.6 and 7, at 25 ± 2°C). As an adsorbate protein, (a) β-lactoglobulin (β-Lg) and (b) lysozyme (LSZ) were used. The protein concentration was 10 μg/ml (in 20 mM KCl). Closed keys represent the amount of adsorbed protein at 7000 s for the cases of the consecutive adsorption. Error bar indicates the maximum and minimum values of each condition.
Next, the dependence of the adsorbed amount of protein on the applied surface potential was compared among six types of (base) metal. Figures 3(a) and (b) show the amount of adsorbed β-Lg and LSZ on different base metal surfaces as a function of the applied surface potential, as well as the amounts of protein adsorbed at 7,000 s for the cases when the amount adsorbed continued to increase (closed keys in Fig. 3). As shown in Fig. 3, the Ta and Zr surfaces allow the adsorption of β-Lg in a considerably wide range of applied surface potential, including -1.0 V vs Ag/AgCl, whereas the threshold potentials of the β-Lg adsorption onto the other tested metals are more positive (~1.0 V vs Ag/AgCl). In contrast, in the case for basic adsorbate protein, LSZ (Fig. 3(b)) [26], the potential ranges allowing significant adsorption onto Ta and Zr are limited below considerably negative values (> ~1.2 V vs Ag/AgCl) whereas the LSZ adsorption onto the other metals were allowed at much higher applied potentials (> ~0.2 V vs Ag/AgCl).

Such contrast relationships for β-Lg and LSZ between the threshold surface potential of the adsorption and the metal type may be deduced to represent different ionization states of surface hydroxyl groups for the individual metals in the adsorption experiment (at pH 5.8): The hydroxyl groups on Ta and Zr surfaces may exist mostly as –OH₂⁺ at pH 5.8, which naturally is considered to avoid the adsorption of LSZ, positively charged, and allow the adsorption of β-Lg, negatively charged, over a wide potential range down to -1.0 V vs Ag/AgCl. Whereas the other tested metals may display less surface –OH₂⁺ groups at pH 5.8 than Ta and Zr, resulting in the more positive threshold surface potential for allowing the β-Lg adsorption and minimizing the LSZ adsorption, as shown in Figs. 3.
Fig. 3. Attained amounts of protein adsorbed on different types of metal (oxide) surfaces as a function of applied surface potential. As an adsorbate protein, β-lactoglobulin (β-Lg) (a) and lysozyme (LSZ) (b) were used. Protein concentration and pH were 10 µg/mL and 5.8 (in 20 mM KCl, 25 °C), respectively. Closed keys represent the amount of adsorbed protein at 7000 s for the cases of the consecutive adsorption. Error bar indicates the maximum and minimum values of each condition.
Fifteen types of proteins were compared for the maximum amount adsorbed onto a stainless steel surface at different applied surface potentials (Fig. 4). Figures 4 suggest that, except lactoferrin, the tested proteins can be classified into three groups, according to the applied potential dependence of the amount adsorbed. The “first group” of proteins show upward-sloping curve for the surface potential dependence of the amount adsorbed, and the “second group” of proteins do not exhibit significant change in the amounts adsorbed over the tested applied surface potential range. Downward-sloping relationship of the amount adsorbed with the applied surface potential is observed for the “third group” of proteins.

This classification of the tested proteins may be determined by the pI of the protein. Namely, proteins, the pI values of which are up to 9.3 (trypsinogen), show upward dependence of the amount adsorbed on the surface potential (first group), and the basic proteins with the pI values above 10 (trypsin) do not adsorb at positive potentials (third group). The pI values for α-chymotrypsinogen and RNaseA (second group) are between those for the first and third groups.

The protein molecules, containing more than fair number of acidic amino acid residues, (first group) is considered to form multiple electrostatic interactions between the surface –OH₂⁺ groups and the protein –COO⁻ groups at pH 5.8 while the protein –COO⁻ groups would be repelled against the negative surface potential. Consequently, the adsorption of the first group proteins may tend to be minimized at negative surface potentials and increased with more positive surface potentials, as shown in Figs. 4(a~j). In contrast, a strongly basic protein, containing large number of basic amino acid residues, (third group) is considered to electrically repulse against the surface –OH₂⁺ groups and attract to the negative electric field on the surface, resulting in the downward-sloping dependence of the adsorption on the surface potential, as shown in Figs. 4(m–o). α-Chymotrypsin A and ribonuclease A (second group) having the intermediate pI values may receive the adsorptive force both in the negative and positive surface potential ranges (Figs. 4(k and l)).
Fig. 4. Attained amount adsorbed of different types of proteins onto a stainless steel surface as a function of applied surface potential. As an adsorbate protein, (a) pepsin, (b) a-lactoalbumin, (c) ovalbumin, (d) BSA, (e) β-lactoglobulin (β-Lg), (f) carbonic anhydrase, (g) conalbumin, (h) immunoglobulin G, (i) lactoferrin, (j) trypsinogen, (k) α-chymotrypsinogen A, (l) ribonulcease A, (m) trypsin, (n) lysozyme (LSZ), and (o) protamin were used. Protein concentration and pH were 10 µg/mL and 5.8 (in 20 mM KCl, 25°C), respectively. Closed keys represent the amount of adsorbed protein at 7000 s for the cases of the consecutive adsorption. Error bar indicates the maximum and minimum values of each condition.
One might think that neutral and weakly basic proteins, that is, conalbumin (pI 6.6), immunoglobulin (pI 5.8-7.3) and trypsinogen (pI 9.3), should have shown the constant or the downward-sloping dependence of the adsorption on the surface potential, as indicated by the second or third groups of proteins, respectively. However, it has been indicated that the attraction force between protein –COO⁻ and surface –OH₂⁺ groups is stronger than that between cationized basic amino acid reside of the protein and surface –O⁻ group [31]. Considering this, the protein –COO⁻ groups may be more strongly attracted by surface positive electric field than the protein –NH₃ or -C(NH₂)₂=NH₂⁺ group is attracted by negative electric field. Consequently, the positive threshold of pI for the first group proteins may be biased up to 9.3 (trypsinogen), as suggested by Figs. 4.

As shown in Fig. 4(i), lactoferrin exhibits extraordinarily large amount adsorbed in the negative surface potential range (-0.2 V vs Ag/AgCl) and the downward-sloping dependence on the surface potential, irrespective of the neutral pI (7.8), suggesting that the adsorption mechanism for lactoferrin is different from that for the other proteins. This may be possibly related to the fact that lactoferrin molecule binds plural ferric ions [35].

The first order adsorption rate constants for different adsorption conditions were determined by dividing the initial slope of the adsorption process by the attained value of the adsorption layer thickness. As shown in Figs. 5, the rate constants of the adsorption of different proteins onto a stainless steel surface as well as those onto different base metal surfaces are all in the order of ~0.001 s⁻¹. This suggests that the protein adsorption under this experimental condition (protein concentration: 10 µg/mL) is a diffusion-limiting process, irrespective of the protein types as well as the type of base metal, and is well consistent with the findings obtained in our previous study [26].
Fig. 5. Initial protein adsorption rate under different conditions as a function of applied surface potential. The protein concentration and temperature were 10 µg/mL and 25±2°C, respectively. The adsorption rate constants were determined by dividing the initial slopes of the protein adsorption process by the amount of adsorbed protein. The adsorption rate constants for consecutive adsorption were not determined because of the absence of the amount adsorbed. Error bars represent the highest and lowest values obtained for each condition.
3.4. Conclusion

The adsorption processes of different types of protein on a metal (oxide) surface in the presence of a external electric field were measured by using situ ellipsometry and compared, focusing on the impact of the acidity/basicity of protein on the adsorption characteristics. Fifteen types of proteins and six types of base metals were used. Results indicated that the pI value of a protein determined the dependence of the protein adsorption on the surface electric potential and pH. Namely, in the adsorption onto a stainless steel surface at pH 5.8, the amount adsorbed for the proteins with the pI value ≤9.3 tend to show the minimum amount adsorbed in the negative surface potential range and significantly adsorb at positive surface potentials; The amount adsorbed of basic protein, of which pI value is above ~10, oppositely increased with decreasing surface electric potential; α-Chymotrypsinogen and RNaseA, having the intermediate pI values, show roughly constant amount adsorbed in the tested surface potential range. On the other hand, the increasing pH positively and negatively shifted the threshold surface potential that allows the adsorption of acidic (β-lactoglobulin, pI 5.1) or basic protein (lysozyme, pI 11.2), respectively. These findings were explained based on the electrostatic interactions among protein ionized groups, the surface ionized hydroxyl groups, and the applied external electric field. Different surface potential dependences of the amount adsorbed onto different base metal surfaces were interpreted to represent the difference in the different ionization states of hydroxyl groups on the base metal surfaces.

3.5. References


23. Song, Y.-Y., Li, Y., Yang, C., and Xia, X.-H. 2008. Surface electric field manipulation of the adsorption kinetics and biocatalytic properties of cytochrome c on a


CHAPTER 4

Influence of an external electric field on removal of protein fouling on a stainless steel surface by hydrolytic enzymes

4.1. Introduction

The adsorption of protein to a solid surface often plays a vital role in various industrial fields, including the food and pharmaceutical industries [1-3]. Protein adsorption has been a subject of extensive and continuous interest [3,4]. The surface area occupied by a protein molecule has been reported for various proteins of different sizes, net charges, fragility, and related properties [5,6]; The characteristics of the adsorptive surface (materials, surface functional groups) strongly affect the nature of the adsorption mode, i.e., the interaction mechanism and the irreversibility of such systems [7,8]; Processing conditions, including pH, ion strength, electrolyte type, and related properties, which are closely relate to the adsorption isotherm, determine the strength of the interaction and the occupied surface area as well as the adsorption mode [9-11]. The application of an external electric field on the adsorptive surface also was examined regarding its influence on protein adsorption behavior [12-15]. The results revealed that protein adsorption could be both enhanced or suppressed when an external electric potential is applied to the (electro-conductive) adsorption surface [16-18].

Food and pharmaceutical industries often suffer from the fouling of the metal surfaces of manufacturing equipment, in which the protein adsorption has been known to play an important role [19,20]. The protein layer that is adsorbed on the equipment wall surface can be the scaffold for the accumulation of additional fouling and often
results in severe problems in terms of maintaining sanitary conditions [21]. The removal of a solid surface fouled with proteinaceous material (cleaning) is thus extremely important in these industrial fields. However, it is often difficult to remove adsorbed protein from the metal surface without using high temperatures, extreme pH, or oxidizing agents [22], and the methodologies available for cleaning protein-fouled surface under mild conditions are limited [23,24]. As a result, the cleaning under severe conditions is generally conducted for the removal of proteinaceous fouling, in which a large amount of energy is consumed and the after-treatment of the cleaning wastewater is required.

One of the cleaning techniques for proteinaceous soilings involves the use of proteolysis protein(s), a process that is referred to as enzymatic cleaning [25,26]. In enzymatic cleaning, the proteolytic enzyme initially binds to the surface-adhering protein, thus initiating the hydrolysis of proteinaceous soiling into soluble fragments. It is also the case that the proteolytic enzyme molecules can be localized on the protein-fouled surface by applying an external electric potential, which may possibly increase the effectiveness of the enzymatic cleaning.

Hence, in this study, the effect of an external electric field on the enzymatic cleaning of the protein-fouled metal surface was investigated. A model protein was allowed to foul a metal surface and the resulting protein that was adsorbed on the metal surface was treated with a proteolytic enzyme in the presence of an external electric potential. The enzymatic cleaning processes under different conditions were measured and the effectiveness of the process compared. In order to explore how the external electric field affects enzymatic cleaning behavior, the layers of adsorbed protein remaining on the metal surfaces at different cleaning stages in the presence and absence of the external electric field were analyzed by atomic force microscopy (AFM).

4.2. Materials and Methods

4.2.1. Materials

Stainless steel SUS316L plate (30 x 50 x 2 mm) (Furuuchi Chemical Co., Tokyo,
Japan) was used as a model surface. The stainless steel SUS316L sample plate was thoroughly cleaned by H$_2$O$_2$ electrolysis [23,27] before use. Two types of model fouling proteins, bovine serum albumin (BSA, A4503) and lysozyme (LSZ, L6876) were obtained from the Sigma-Aldrich Co. (St Louis, MO). The proteolytic enzymes and their characteristics are listed in Table 1. Trypsin (from porcine pancreas, T4799) and α-chymotrypsin (from bovine pancreas, C0342) were obtained from Sigma-Aldrich Co., and thermolysin (from Geobacillus stearothermophilus, 207-08333) was from Tokyo Chemical Industries Co., Ltd (Tokyo, Japan). Na$_2$HPO$_4$•12H$_2$O, NaH$_2$PO$_4$•2H$_2$O, KCl and H$_2$O$_2$ (30-35% solution) were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). All of these proteins and chemicals were analytical grade and were used as received without further purification.

4.2.2. Enzymatic Cleaning Experiment

A 50 µL droplet of an aqueous solution containing a 100 µg/ml solution of the model fouling protein (BSA or lysozyme) was placed onto the center of a sample plate, followed drying at 37 °C for 90 min. The dried proteins on the sample plate were rinsed with 50 mM of phosphate buffer (pH-8) solution for 60 min to remove weakly bound protein molecules. The sample plate with the model proteinaceous fouling material was then immersed in 200 mL of 50 mM sodium phosphate buffer (25°C), containing a 10 µg/mL solution of the protease, to initiate the enzymatic cleaning. The pH for the enzymatic cleaning was adjusted to 8, the pH ranges for maximum activity of the proteases used herein. During the enzymatic cleaning test, the protease solution was stirred vigorously at ~300 rpm with a Teflon-coated magnetic stirrer bar (1.5 cm), and an electric potential (+0.3 ~ -0.6V vs Ag/AgCl) was applied to the sample plate using a potentiostat (HSV-100, Hokuto-Denko Co., Tokyo, Japan) with a three electrode system. Platinum sheet (20 x 20 x 0.1 mm) and Ag/AgCl electrodes were used as
counter and reference, respectively.

Table 1  Characteristics of the model fouling proteins and proteolytic enzymes used in this study

<table>
<thead>
<tr>
<th>Proteins/Enzymes</th>
<th>MW (kDa)</th>
<th>Size (nm)</th>
<th>Isoelectric point (-)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>68.6</td>
<td>4 x 4 x 14(^{a})</td>
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<td>-</td>
</tr>
<tr>
<td>Lysozyme (LSZ)</td>
<td>14.3</td>
<td>3 x 3 x 5(^{a})</td>
<td>11(^{e})</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin</td>
<td>23.4</td>
<td>4 x 4 x 5(^{b})</td>
<td>10.7(^{e})</td>
<td>~1,500(^{g})</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>25.1</td>
<td>4 x 4 x 4(^{c})</td>
<td>8.4(^{e})</td>
<td>10,000(^{h})</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>34.6</td>
<td>4 x 4 x 6(^{d})</td>
<td>4.5(^{f})</td>
<td>9,000(^{i})</td>
</tr>
</tbody>
</table>

\(^{a}\)[26]; \(^{b}\)[27]; \(^{c}\)[28]; \(^{d}\)[29]; \(^{e}\)[30]; \(^{f}\)[31]; \(^{g}\) One unit hydrolyzes 1.0 µmol of \(\text{N-\[\text{\text{\text{-benzoyl-L-Arginine ester}}\}}\) per minute at pH 7.8 at 25 °C.

\(^{h}\) One unit hydrolyzes 1.0 µmole of \(\text{N-benzoyl-L-tyrosine ethyl ester}\) per minute at pH 7.8 at 25 °C.

\(^{i}\) One unit hydrolyzes casein to produce color equivalent to 1.0 µmole of tyrosine per minute at pH 7.5 at 37°C.
In preliminary tests, the enzymatic cleaning was conducted at different concentrations of hydrolytic enzyme (in the absence of an external electric potential). The results indicated that the rate of removal of protein (BSA) on the sample plate was increased with increasing protease concentration up to 10 µg/mL, as would be expected. However, further increases in the protease concentration resulted in a larger amount of residual protein on the sample plate probably because the hydrolytic enzyme molecules were also being adsorbed to the plate. Hence, in this study, the concentration of protease was set to 10 µg/mL for all of the tested proteases, as described above.

4.2.3. Measurement of the amount of protein remaining on the sample plate

At 10-min intervals, the sample plate was removed from the protease solution, and the remaining liquid on the sample plate was blown off with a stream of nitrogen gas. After further drying at 37°C for 10 min, the IR spectra of the protein remaining on the sample plate (Fig. 1) was measured by Fourier transform infrared spectrometry (FTIR) using a reflection absorption (RA) technique, as described previously [23,34]. Nicolet 4700 FTIR (Thermo Electron Co., Madison, WI) with FT-80 RA-IR attachment (SpectraTech, Shelton, CT) was used in this study. From the IR absorbance due to protein amide I band (~1650 cm⁻¹), the relative amounts of protein remaining after the enzymatic cleaning were determined [23,34].
Fig. 1. IR spectra of model protein fouling on the St plate before (a) and after the enzymatic cleaning in the presence of external electric potential (-0.1 V vs Ag/AgCl) for (b) 10 min and (c) 50 min. Trypsin was used as a hydrolytic enzyme (10 µg/ml).
4.2.4. Atomic force microscopy observation

Nano-scale images of the stainless steel surfaces, fouled with proteins and treated with proteolytic enzymes, were obtained by an atomic force microscopy analysis, using a Nanoscope E (Digital Instruments, Santa Barbara, CA) and a Pivo SPM (Molecular Imaging, Phoenix, AZ) to analyze the microstructure of the layer of protein that was fixed on the stainless steel surface [35]. A Pyrex-nitride probe that had a triangular silicon nitride cantilever (PNP-TR-50, Nano World AG, Neuchâtel, Switzerland) with spring constant of 0.33 N/m and resonance frequency of 67 Hz was used. The AFM scanning was conducted at six locations on each plate and the images acquired in the tapping mode.

4.3. Results and Discussions

4.3.1. Removal Characteristics of BSA Fouling in Enzymatic Cleaning with an Applied External Electric Field

Figure 2 show the courses for the decrease in the amount of model fouling protein (BSA) on the sample plate during the cleaning tests under different conditions, in which the initial slopes and attained residual amount are different. Hence, the initial rates of removal in the cleaning test and amounts of residual material after a 50-min cleaning were determined from the removal processes under different conditions and the findings compared.
Fig. 2. Time courses of relative residual amounts of model fouling protein (%) during enzymatic cleaning in the absence or presence of external electric fields on the St sample plate surface. The results for buffer rinsing in the absence of protease are shown in (a). Trypsin (b), α-chymotrypsin (c), and thermolysin (d) were used as hydrolytic enzyme, and the hydrolytic enzyme concentration was 10 μg/mL in 50 mM phosphate buffer solution (pH 8). The solution temperature was 25±1°C.
In Fig. 3, the initial removal rates are shown as a function of the applied surface potential. The values for buffer rinsing in the presence and absence of an external potential as well as enzymatic cleaning without applying an electric potential are also shown as controls in Fig. 3. As shown in Fig. 3, the initial rate of removal was found to be approximately 2 %/min\(^{-1}\) in the case for rinsing with a buffer solution and then increased up to 3~3.5 %/min\(^{-1}\) when an external electric potential of less than -0.1 V vs Ag/AgCl was applied. This indicates that a negative surface potential below a certain value facilitates the detachment of the BSA from the sample plate surface. On the other hand, the use of a 10 µg/mL solution of trypsin increased the initial removal rate by approximately 150% from that for buffer rinsing, which can be attributed to the hydrolytic enzyme. \(\alpha\)-Chymotrypsin (10 µg/mL) also showed a slight increase in the initial removal rate but no significant increase in the initial removal rate was observed in the case of thermolysin.

As shown in Fig. 3, the effect of the external electric field on the initial rate of removal appears to be limited. An external potential below -0.2 V vs Ag/AgCl increased the initial removal rate for trypsin by only approximately 20% (Fig. 3(a)). However, the initial removal rates for \(\alpha\)-chymotrypsin and thermolysin appeared to be unchanged in the usual range of the applied surface potential, while the application of 0.1 V and -0.6 V vs Ag/AgCl, respectively, resulted in a marked suppression in removal for \(\alpha\)-chymotrypsin, as shown in Fig. 3(b) and (c).

Figures 4(a-c) show the amount of BSA remaining on the sample plate after a 50-min cleaning under different conditions against surface electric potential, as well as the values for rinsing with a buffer (with and without the application of an external electric potential) and cleaning only by a hydrolytic enzyme. The amount of BSA that remained in the case of buffer rinsing reached approximately 65% at 50 min. The application of a negative electric potential, less than -0.1 V vs Ag/AgCl, to the sample plate surface served to decrease the amount of protein remaining after 50 min, the extent of which becomes more significant for a more negative applied surface potential (Fig. 4). The presence of hydrolytic enzymes (in the absence of any external electric field) also
reduces the amount of residual protein (Fig. 4) while the attained residual amount of BSA varies significantly, depending on the type of protease used. According to these findings, both applying a surface negative potential and the presence of a hydrolytic enzyme both appear to be effective for the removal of protein fouling from the stainless steel surface and however, individually, these 2 processes are not as effective in thoroughly removing protein fouling within 50 min. It should be noted that, as shown in Fig. 4, the combination of trypsin with an applied negative applied potential below -0.2 V vs Ag/AgCl removes most of the protein fouling on the St sample surface. Considering that solely applying -0.2 V vs Ag/AgCl does not result in any significant removal of the protein fouling (Fig. 4), it can be concluded that the presence of a negative electric field functions to enhance the cleaning effect of hydrolytic enzyme.

In the RA-FTIR measurement, the sample surface to be analyzed must be dried, as was typically conducted in this study. However, one might think that the drying of model protein fouling could affect the removal behavior in the cleaning test. Hence, the sample plate surface having model protein (BSA) fouling was subjected to the trypsin solution (in the absence of external electric field) continuously for 50 min without being interrupted at 10-min interval and then dried; The remaining amount of BSA after the continuous 50-min cleaning was compared with that for the tryptic cleaning for 10 min x 5 times (Fig. 2). As shown in Fig. 2, the continuous 50-min tryptic cleaning shows approximately 20% larger remaining amount of BSA than the intermittent 50 min cleaning. This may happen because the repetition of drying of the model protein fouling causes further unfolding of the fouling protein molecules and thus facilitates the digestion by trypsin.

On the other hand, in the case of α-chymotrypsin, an extremely high negative potential (-0.6 V vs Ag/AgCl) was required for the complete removal of BSA fouling, and the cleaning effect of thermolysin was not enhanced in the applied potential range (Fig. 4). These findings indicate that the efficiency of combining hydrolytic enzyme with an external electric field varies markedly and is dependent on the characteristics of the specific enzyme being used.
Fig. 3. The initial rates for the removal of BSA fouling from the St sample plate surface in the cleaning tests under different conditions (opened circles) as a function of applied electric surface potential. As a hydrolytic enzyme, trypsin (a), α-chymotrypsin (b), and thermolysin (c) were used as a final concentration of 10 μg/mL, and the pH and temperature in the cleaning test were 8±0.01 and 25±1°C. The values for enzymatic cleaning and buffer rinsing at rest potential are also shown.
Fig. 4. The residual amount of BSA fouling on the St sample plate surface after 50-min cleaning under different conditions (open circles) as a function of applied surface potential. As a hydrolytic enzyme, trypsin (a), α-chymotrypsin (b), and thermolysin (c) were used at the final concentration of 10 μg/mL, and the pH and temperature in the cleaning test were 8.0±0.01 and 25±1°C. The values for buffer rinsing in the present and absent of an external electric field as well as the enzymatic cleaning without applying electric potential are also shown.
To date, many studies of the influence of an external electric field on the adsorption of a protein to a metal surface have appeared [12-18]. Most of them indicate that the protein adsorption tends to be increased by electric polarization of an opposite sign to that of the net charge of the protein whereas it is suppressed by the same sign polarization [13, 15, 17, 18]. Considering this and according to the pI value for trypsin (10.7), trypsin molecules would have a net positive charge in the cleaning test at pH 8 and thus may be concentrated on the negatively polarized surface. The higher trypsin concentration on the sample plate surface than in the bulk solution would be expected to enhance the efficiency of digestion frequency by trypsin and consequently result in a more extensive removal in the presence of a comparatively weak negative potential (-0.1 V vs Ag/AgCl), as shown in Fig. 4. On the other hand, the net charges of α-chymotrypsin (pI 8.4) and thermolysin (pI 4.5) in the cleaning test would be less positive than that of trypsin and negative, respectively. Therefore, the complete removal of BSA fouling would be expected to require a much greater negative potential (-0.6 V vs Ag/AgCl) in the case for α-chymotrypsin than for trypsin and would be very incomplete in the applied range in the case for thermolysin (Fig. 4).

Another mechanism of the higher attained cleaning at more negative potential (Figs. 4) may be related to the net charge of the fouling protein, BSA. Namely, since BSA is an acidic protein, the digestion fragments from BSA may also be negatively charged. The negatively charged fragment (peptide) molecules would naturally be repelled to the negatively polarized surface, which also may seemingly enhance the removal effectiveness of the hydrolytic enzymes, as shown in Figs. 4. In contrast, a positively polarized surface would attract the BSA-originated fragments and thus suppress their detachment. Consequently, the effectiveness of the hydrolytic enzymes may be
lowered at a positive applied potential than without any external electric field, as shown in Figs. 4.

When compared with the impact magnitudes of the external electric field on the cleanliness level obtained after 50-min (Fig. 4), the initial rate of removal in the enzymatic cleaning (Figs. 3) appeared to be less affected as the result of applying an external electric field. This suggests that the detachment of digested fragments of BSA is the rate-limiting stage for removal, rather than the rate of digestion of the fouling protein.

4.3.2 AFM analysis of Protein fouling on the St Surface in Enzymatic Cleaning with External Electric Field

Figures 5 show AFM images of the surface of the sample plate at different steps in the cleaning test. A bare stainless steel surface typically contains numerous linear scratches, which are produced during mechanical polishing (Fig. 5(a)). As indicated by a micro-roughness in the order of several tens of nm in Figs. 5(b), the BSA fouling layer forms a different rugged pattern from that for a bare unscratched one (Fig. 5(a)). When buffer rinsing decreased the amount of adsorbed BSA, a linear hump with a ~100 nm width as well as hump spots with 10~100 nm diameters are significant (Fig. 5(c)). The AFM images after a 10-min trypsin cleaning (Figs. 5(d-i and ii)) show no clear rugged pattern, indicating that trypsin molecules acted on and consequently softened the BSA adsorption layer, irrespective of the presence and absence of an applied surface potential (-0.1 V vs Ag/AgCl). This can be attributed to the digestion of the BSA fouling layer by trypsin molecules.
Fig. 5. AFM images of (a) bare stainless steel surface and (b) the surface fouled with BSA. The fouled stainless steel surface was rinsed for 60 min with 50 mM sodium phosphate buffer (c) and then treated with 10 μg/mL trypsin in the presence of applied surface potential (-0.1 V vs Ag/AgCl) for 10 min (d-i), and 40 min (e-i). The cleaning solely with 10 μg/mL trypsin was also conducted for 10 min (d-ii), and 40 min (e-ii).
As shown in Fig. 5(e-i), the combination of trypsin cleaning in conjunction with applying a surface potential for 40 min uncovers the linear scratches on the bare stainless steel surface. In addition, in the absence of an applied surface negative potential, the linear scratches become visible after a 40-min cleaning (Fig. 5(e-ii)), which is less significant in the presence of an applied surface potential (Fig. 5(e-i)). According to these findings, the surface negative potential appears to facilitate the trypsin molecules to reach the inner surface of the scratches and thus digest the BSA fouling inside the scratch. Consequently, the stainless steel surface can be more efficiently and completely cleaned by trypsin cleaning in conjunction with an applied negative surface potential than by trypsin cleaning in the absence of a potential, as shown in Fig. 4.

4.3.3. Removal Behavior of LSZ Fouling in Enzymatic Cleaning with an External Electric Field

Model protein fouling was prepared on the St sample plate surface, using LSZ, and subjected to the cleaning tests in order to investigate the influence of the fouling protein characteristics on the removal behavior in the cleaning test: LSZ is well known as hard and basic protein while BSA is soft and acidic [5,36]. The initial rates of removal and the residual amounts of LSZ on the sample plate after 50 min-cleaning were determined from the time courses for the amount of LSZ remaining under different conditions and are shown in Figs. 6. Tryptic cleaning without applying an electric potential showed a much smaller initial rate of removal and greater amount of LSZ remaining on the sample plate after the cleaning test when compared to the values for BSA fouling (Figs. 3 and 4). This may be related to the hard structure of LSZ molecule, relative to BSA, soft protein [36].
Fig. 6. Initial rates (a) and residual amount of LSZ fouling after 50 min (b) for the removal of LSZ fouling on the St sample plate surface under different conditions (opened circles) as a function of applied surface electric potential. As a hydrolytic enzyme, trypsin was used as at the final concentration of 10 μg/mL, and the pH and temperature in the cleaning test were 8.0±0.01 and 25±1°C. The values for buffer rinsing in the present and absent of an external electric field as well as the enzymatic cleaning without applying electric potential are also shown.
The highest initial rate (Fig. 6(a)) and the greatest removal of LSZ fouling (Fig. 6(b)) were both achieved at around -0.1 V vs Ag/AgCl. Similarly to the removal behavior of BSA fouling in the enzymatic cleaning, this can be explained by two interactions of the St surface with trypsin and digested LSZ fragments. The digested LSZ fragments would likely be positively charged and be thus be attracted, along with trypsin molecules, by the negative polarization of the St sample plate whereas both of them would be repelled by the positively polarized sample surface. Consequently, the highest removal efficiency for LSZ fouling would be predicted to be at around neutral applied potentials and be lowered both with increasing and decreasing applied potential, as shown in Fig. 6.

As shown in Fig. 6(b), the amount of LSZ remaining after 50-min is decreased only by the negative electric field, which is similar to the cases for the BSA fouling (Fig. 4(a)). Judging from the fact that the net charges for LSZ and BSA have opposite signs, the mechanism for protein removal solely by negative applied potential cannot be explained by a simple electrostatic repulsion between the fouling protein and the St surface, which is different from that for the facilitated detachment of the digested protein fragments.

4.4. Conclusions

Enzymatic cleaning represents a promising technique for removing protein fouling on a solid surface under moderate conditions. This study investigated the influence of an external electric field on the enzymatic cleaning of a stainless steel surface fouled with protein. Three types of hydrolytic enzymes (trypsin, α-chymotrypsin, and
thermolysin) were used. An aqueous droplet, containing bovine serum albumin or lysozyme, was dried on a stainless steel (St) sample plate to fix the model protein fouling, followed by rinsing with a buffer. The model protein foulings on the St surface were subjected to enzymatic cleaning in the presence and absence of an external electric field (-0.6~+0.2 V vs Ag/AgCl). The efficiency of the enzymatic cleaning for protein fouling was markedly increased in the presence of certain applied electric potentials, the range of which varied depending on the type of hydrolytic enzyme being used and the nature of the model fouling protein. An atomic force microscopy analysis demonstrated that the fouling protein molecules that were located inside scratches on the metal surface well as the outer surface of the sample plate were effectively removed by a protease in the presence of an adequate electric potential. The enhancement in enzymatic cleaning by an external electric field can be explained by two electrostatic interactions of the external electric field with protease molecules and digested fragments derived from the fouling protein.

4.5. References


Protein adsorption to a solid surface often plays a vital role in various industrial fields, and has been extensively and continuously investigated with regard to various factors, including protein nature, surface characteristics and medium conditions. Beside them, the electric potential of a solid surface is also naturally expected to affect the protein adsorption behavior. For those reasons, the adsorption behavior of various proteins on a metal (oxide) surface at various potentials was measured and compared. Fifteen types of proteins and six types of metal surface were used for model protein and metal oxide surface. The \(-0.8 \sim +0.4\) V vs. Ag/AgCl of potential was applied to the stainless steel surface, which was in contact with protein solution. The amount of adsorbed protein is monitored by an \textit{in-situ} ellipsometry. The influence of protein types on the relationship between the protein adsorption and surface electric potential was examined. On the other hand, the removal of a solid surface fouled with proteinaceous soilings (cleaning) is extremely important in food and pharmaceutical industries. One of the removal techniques for proteinaceous
soilings is enzymatic cleaning. Since the binding of hydrolytic enzyme with surface-adhering protein initiates the hydrolysis of proteinaceous soilings into soluble fragments, the control of adsorption of hydrolytic enzyme by an external electric potential may possibly increase the effectiveness of the enzymatic cleaning. Hence, the enzymatic cleaning of adsorbed protein on metal surface was herein conducted in the presence of an external electric potential. The cleaning processes under different conditions were compared.

In Chapter 2, the author studied the impact of external electric potential on the adsorption of a protein to base metal surfaces. For this purpose, hen egg white lysozyme (LSZ) and six types of base metal plates (stainless steel SUS316L (St), Ti, Ta, Zr, Cr, or Ni) were used as the protein and adsorption surface, respectively. LSZ was allowed to adsorb on the surface under different conditions (surface potential, pH, electrolyte type and concentration, surface material), which was monitored using an ellipsometer. LSZ adsorption was minimized in the potential range above a certain threshold and, in the surface potential range below the threshold, decreasing the surface potential increased the amount of protein adsorbed. The threshold potential for LSZ adsorption was shifted toward a positive value with increasing pH and was lower for Ta and Zr than for the others. A divalent anion salt (K₂SO₄) as an electrolyte exhibited the adsorption of LSZ in the positive potential range while a monovalent salt (KCl) did not. A comprehensive
consideration of the obtained results suggests that two modes of interactions, namely
the electric force by an external electric field and electrostatic interactions with
ionized surface hydroxyl groups, act on the LSZ molecules and determine the extent
of suppression of LSZ adsorption. All these findings appear to support the view
that a base metal surface can be controlled for the affinity to a protein by
manipulating the surface electric potential as has been reported on some electrode
materials.

In Chapter 3, the author examined the influence of protein characteristics and
structure on the protein adsorption onto base metal surfaces in the presence of external
electric potential. Fifteen types of protein and six types of base metal surfaces were
used as adsorbate protein and metal surfaces. The attained amounts adsorbed and the
initial adsorption rates in the protein adsorption at different applied surface potentials
were measured by using an in-situ ellipsometry. As a result, it was indicated that the
relationship among the protein adsorption, the surface electric potential, and pH
strongly depends on the balance of acidic and basic amino acid residues, namely, the pI
value of a protein: In the adsorption onto a stainless steel surface at pH 5.6, the proteins
with the pI value ≤9.3 exhibited the minimum adsorptive affinity at the negative surface
potentials below a certain value and more highly absorb at more positive surface
potential; Basic proteins having the pI value >~10 significantly adsorbed at the negative
potentials; α-Chymotrypsinogen and RNaseA, with the intermediate pI values, show
roughly constant amount adsorbed in the tested surface potential range. On the other hand, as the pH increases, the threshold surface potential for the adsorption of acidic and basic proteins shifted positively and negatively, respectively. These results coincide with the protein adsorption mechanism based on the electrostatic interactions among protein ionized groups, the surface ionized hydroxyl groups, and the applied surface potential, as described in Chapter 2. Furthermore, from the differences among the surface potential dependences of the amount adsorbed onto different base metal surfaces, it was deduced that the ionization states of hydroxyl groups varied by the type of base metal.

In Chapter 4, the author focused for removing proteinaceous fouling from solid surfaces by enzymatic cleaning that is a potentially useful method under mild conditions. Herein, the influence of an external electric field on the enzymatic cleaning of a metal surface fouled with a protein was investigated. The model fouling protein (bovine serum albumin (BSA) or lysozyme (LSZ)) was prepared on a stainless steel (St) surface, and the resulting surface subjected to enzymatic cleaning with an electric potential being applied to the St sample plate. Trypsin, α-chymotrypsin, and thermolysin were used as model proteases. The amounts of protein fouling that remained on the plate before and during the enzymatic cleaning process were measured by means of a reflection absorption technique using Fourier transform infrared spectroscopy. In the case for BSA fouling, the cleaning efficacy of the protease tended to increase at more
negative applied potentials. On the other hand, there was an optimum applied potential for removing of the LSZ fouling. Atomic force microscopy analyses indicated that applying an adequate range of electric potential enhanced the enzymatic removal of protein fouling inside scratches on the sample plate surface. These findings suggest the existence of two modes of electrostatic interactions for the external electric field, one with protease molecules and the other with digested fragments of the fouling protein.
List of Publications

1) Ei Ei Htwe, Yuhi Nakama, Hiroshi Tanaka, Hiroyuki Imanaka, Naoyuki Ishida, and Koreyoshi Imamura

“Adsorption of lysozyme on base metal surfaces in the presence of an external electric potential.”


2) Ei Ei Htwe, Yuhi Nakama, Hiroshi Tanaka, Yuko Yamamoto, Hiroyuki Imanaka, Naoyuki Ishida, and Koreyoshi Imamura

“Adsorption characteristics of various proteins onto metal surface in the presence of an external electric field.”

*under preparation.*

3) Ei Ei Htwe, Yuhi Nakama, Hiroyuki Imanaka, Naoyuki Ishida and Koreyoshi Imamura

“Influence of an external electric field on removal of protein fouling on a stainless steel surface by proteolytic enzymes.”