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Adsorption behaviour and surfactant elution of cationic salivary proteins at solid/liquid interfaces, studied by *in situ* ellipsometry

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Abstract

Adsorption of the cationic salivary proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5 to pure (hydrophilic) and methylated (hydrophobized) silica surfaces was investigated by *in situ* ellipsometry. Effects of concentration ($\leq$10 μg ml^{-1}, for lysozyme $\leq$200 μg ml^{-1}) and dependence of surface wettability, as well as adsorption kinetics and elutability of adsorbed films by buffer and sodium dodecyl sulphate (SDS) solutions were investigated. Results showed that the amounts adsorbed decreased in the order lactoferrin $\geq$ lactoperoxidase > lysozyme $\geq$ histatin 5. On hydrophilic silica, the adsorption was most likely driven by electrostatic interactions, which resulted in adsorbed amounts of lactoferrin that indicated the formation of a monolayer with both side-on and end-on adsorbed molecules. For lactoperoxidase the adsorbed amounts were somewhat higher than an end-on monolayer, lysozyme adsorption showed amounts corresponding to a side-on monolayer, and histatin 5 displayed adsorbed amounts in the range of a side-on monolayer. On hydrophobized substrata, the adsorption was also mediated by hydrophobic interactions, which resulted in lower adsorbed amounts of lactoferrin and lactoperoxidase; closer to side-on monolayer coverage. For both lysozyme and histatin 5 the adsorbed amounts were the same as on the hydrophilic silica. The investigated proteins exhibited fast adsorption kinetics, and the initial kinetics indicated mass transport controlled behaviour at low concentrations on both types of substrates. Buffer rinsing and SDS elution indicated that the proteins in general were more tightly bound to the hydrophobized surface compared to hydrophilic silica. Overall, the surface activity of the investigated proteins implicates their importance in the salivary film formation.

Keywords: Lactoferrin; Lactoperoxidase; Lysozyme; Histatin 5; Pellicle

1. Introduction

When a clean surface is introduced in the oral environment it will quickly be covered by a thin film, composed mostly of constituents from saliva. This film, denoted pellicle [1], is formed by selective adsorption of mainly salivary proteins, among these proline-rich proteins, statherin, lysozyme, histatins and different types of glycoproteins. It is well accepted that the composition of this film influences the subsequent colonization of oral microbes on the oral surfaces (see e.g. [2] and references therein), which leads to the formation of dental plaque and, in some cases, further evolves into plaque related diseases such as caries and periodontitis. Lactoferrin, lactoperoxidase and lysozyme are examples of salivary proteins that are known to have protective functions, e.g. by decreasing the adherence and growth of hazardous microbes and thereby upholding a healthy ecosystem where pathogenic bacteria are suppressed (see e.g. review [3]). Lactoferrin and lactoperoxidase are both cationic glycoproteins with molecular weights of approximately 80 kDa, and isoelectric points (p*I*) around 9 (for reviews see [4–7]). The main antimicrobial function of lactoferrin is its iron-binding capacity which results in an indirect bacteriostatic effect, but it is also known to have bactericidal abilities not related to iron-binding [5,6]. It has been shown that the adsorption of lactoferrin to bacteria is associated with its bactericidal activities [5,6], indicating that the antimicrobial function is retained upon biological surfaces. Lactoperoxidase catalyses the oxidation of specific molecules by the aid of H_2O_2 to produce highly reactive products which have direct antimicrobial activity [4,6]. Lactoperoxidase has a high surface affinity and binds to different types of oral surfaces, upon which it retains its activity [6,7]. Lysozyme is a small protein (14.5 kDa) with a pl of 11 and acts by e.g. aggregating

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bacteria, lysis of the peptidoglycan layer of bacteria cell walls and inhibiting bacterial adherence [6]. Studies have shown that lysozyme retains its activity when adsorbed in the pellicle [8].

Salivary peptides have also been shown to have antimicrobial activities. Histatins are a large family of small peptides with a high content of histidine and they are known to be both antibacterial and antifungal, inhibiting the growth of e.g. *Candida albicans* (see [9] and references therein). The most candidacidal peptide of this family has shown to be the cationic histatin 5 (3 kDa, pI = 10) [9]. It has been suggested as a potential drug against oral candidiasis due to its potent antifungal activity against drug-resistant Candida strains and lack of toxicity to humans [9]. *In vitro* studies have shown that the candidacidal activity of histatin 5 is impaired when adsorbed to surfaces [10,11], but upon desorption it is reactivated. Histatins have been identified in both *in vitro* and *in vivo* formed pellicles [12–14].

The overall character of the pellicle has been reported to be anionic [15]. Since cationic proteins like lactoferrin, lactoperoxidase, lysozyme and histatins have been identified in the pellicle it can be hypothesized that these antimicrobial proteins may enhance the protective functions of the pellicle. Furthermore, effects on the cohesiveness and thickness of the salivary film by electrostatic interactions with anionic pellicle proteins may also be anticipated. The formation of complexes between cationic salivary proteins and the large anionic mucins have been reported in several studies [16–18].

As a first step in understanding the role of cationic proteins in the pellicle build-up, and also their possible importance in complex formations with anionic components on surfaces, it was considered relevant to investigate the adsorption behaviour of the single proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5. Due to availability reasons we chose to work with lactoferrin from human milk, lactoperoxidase from bovine milk, lysozyme from hen egg and synthetic histatin 5. The experiments in the present study were carried out to investigate and compare the adsorption behaviour of these four proteins, under identical conditions, within the concentration ranges present in saliva (for lactoferrin and lactoperoxidase $\leq$10 μg ml^{-1} [6,7], lysozyme $\leq$200 μg ml^{-1} [6], and histatin 5 $\leq$45 μg ml^{-1} [19]). Previously, studies have been performed on the adsorption of lactoferrin [20,21], lactoperoxidase [22] and lysozyme [20,23–27], however these publications were carried out using different techniques to some extent, and additionally they were not performed using the same concentration ranges, buffer solutions (or, to some extent, substrates) as the present investigation. The adsorption behaviour of the four chosen proteins was investigated on two types of silica surfaces with different characters. The influence of surface properties was included in this context. To the authors' knowledge, this is the first investigation on the adsorption of histatin 5 to silica surfaces.

Further, this study includes the investigation of elutability of adsorbed films by buffer and surfactant solutions, to characterize the interactions and the stability of the films. Surfactants such as SDS are common constituents in many oral care products (at concentrations up to 0.07 M [28]) and it was therefore considered relevant to investigate the effect of SDS on the adsorbed protein films. In addition, as several of the proteins in the present investigation are known constituents of e.g. milk, protein–surfactant interactions are of importance in applications such as cleaning of food processing equipment.

The technique of null ellipsometry was employed for these studies, and it has previously been applied successfully for studies on protein adsorption ([29] and references therein). The general analysis of the experimental results obtained in the present investigation was primarily dealt with from a physico-chemical viewpoint, but also with input on the physiological context of the results (see section on significance).

2. Experimental

2.1. Proteins

Lactoferrin (L0520, 100% pure), lactoperoxidase (L8257, 88% pure), and lysozyme (L6876, 95% pure) were purchased from Sigma–Aldrich Sweden AB, (Stockholm, Sweden). Histatin 5 (72-2-25, >97% pure) was obtained from American Peptide Company Inc. (Sunnyvale, CA, USA). All proteins were used as received. Values of the molecular dimensions of the proteins are shown in Table 1, including sited references. As different values of the dimensions of lactoferrin were found in recent literature, more than one dimension is given in Table 1. The results for lactoferrin were therefore analyzed considering the different dimensions given. The dimensions of histatin 5 are, to the authors' knowledge unknown. Studies have shown that it adopts random coil conformation in water and phosphate buffers [30]. The radius of gyration (R_g) was calculated (assuming a theta solvent), using the formula:

$$R_g^2 = \frac{nl^2}{6} \tag{1}$$

where n is the number of amino acids (24 [9]), and l is the length of each amino acid (approximated to 3.5 Å [31]). A completely stretched molecule would then have a length of 84 Å.

Also presented in Table 1 are calculated amounts adsorbed corresponding to side-on and end-on monolayer, calculated from the formula:

$$\Gamma = \frac{M_w}{AN_a} \tag{2}$$

where Γ is the amount protein per unit area (mg m^{-2}), M_w the molecular weight (mg mol^{-1}), A the area per molecule (m^2), and N_a Avogadro's constant (6.022×10^{23} mol^{-1}). The proteins were considered to have a rectangular cross section, for histatin 5 the situation of hexagonally packed spheres with a cross section of πR_g^2 was also considered.

2.2. General

The buffer solution (denoted PBS) was a 10 mM phosphate buffer supplemented with 50 mM NaCl, and adjusted to pH 7.0. SDS (L6026, >99% pure) used for the elution studies, was purchased from Sigma–Aldrich Sweden AB (Stockholm Sweden), and used as received. All water used was of ultra high quality