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Lactoperoxidase and Histatin 5 – their Adsorption Behaviour on Silica and Hydrophobized Silica Surfaces, and Implications on their Role in the Initial Salivary Film Formation

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The acquired pellicle is the thin salivary film that covers all oral surfaces, formed by selective adsorption of primarily salivary proteins. Several cationic proteins, such as lactoferrin, lactoperoxidase, lysozyme and histatin 5 have been identified in the pellicle. This study focused on the adsorption of lactoperoxidase and histatin 5, to investigate their possible importance in the initial salivary film formation. The adsorption was investigated by means of *in situ* ellipsometry, to both pure (hydrophilic) and methylated (hydrophobized) silica substrates, at concentrations relevant in saliva. The adsorption was investigated in terms of surface area per molecule and influence of surface wettability. Mass transport controlled adsorption in relation to the initial adsorption from human whole saliva and glandular saliva was also investigated. Results showed that lactoperoxidase adsorbed in larger amounts on pure silica compared to methylated surfaces. Histatin 5 adsorbed to the same extent on the two types of surfaces, but to a lesser extent compared to lactoperoxidase. The mass transport calculated adsorption rates of lactoperoxidase and histatin 5 showed that histatin 5 might potentially have a significant role in the initial adsorption from saliva, whereas lactoperoxidase may also adsorb but is not a dominating component.

1. Introduction

Human whole saliva (HWS) is an essential body fluid. It contains many different types of proteins, which rapidly cover surfaces exposed in the mouth,

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thereby creating a thin film denoted pellicle [1]. Many of these proteins, for instance glycoproteins, proline-rich proteins, lysozyme, histatins, amylase and cystatins, are known to be multi-functional (for a review see *e.g.* [2]), *e.g.* to be involved in the calcium mineral homeostasis and/or to have tissue-coating, antimicrobial and lubricating abilities. The pellicle formation, which is a process of selective protein adsorption [3] is of great interest since it influences the subsequent adherence of microorganisms (see *e.g.* [4] and references therein) and thus the formation of dental plaque. Plaque may further develop into caries, periodontitis and/or other oral diseases. The cationic proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5 are examples of proteins that are involved in the antimicrobial action of saliva (for reviews see [5, 6]), and these proteins have also been identified in the pellicle [7, 8]. As several of these proteins have shown to retain their antimicrobial activity when adsorbed to surfaces [5, 9, 10], they are believed to contribute to the protective functions of the pellicle. Further, the overall character of the pellicle has been reported to be anionic [11], and the presence of these cationic components may increase the cohesiveness and thickness of the film by complex formations with anionic pellicle proteins. This study is a complement to a preceding, more detailed study performed on the adsorption behaviour of lactoferrin, lactoperoxidase, lysozyme and histatin 5, that investigated the adsorption in terms of concentration dependence, influence of surface wettability, kinetics and elutability by buffer and surfactant solutions [12]. The present investigation focused on the adsorption of lactoperoxidase and histatin 5. These two proteins were chosen as they have very different characteristics; lactoperoxidase is a globular glycoprotein with a molecular weight of approximately 80 kDa, whereas histatin 5 is a small flexible peptide of 3 kDa. Lactoperoxidase catalyses reactions that produce *e.g.* oxidized thiocyanate ion derivatives that are toxic to microorganisms. Histatin 5 has shown to be a potent inhibitor of *Candida albicans*, a fungus known to cause stomatitis. Histatin 5 has been proposed as a potential drug against oral candidiasis due to its antifungal activity against drug-resistant Candida strains and lack of toxicity to humans [6].

The present study focused on the adsorption behaviour of these two proteins at 10 $\mu g\,ml^{-1}$, which is within the concentration ranges of these proteins in saliva [10, 13]. The adsorption was monitored using *in situ* ellipsometry which has been used quite extensively for protein adsorption studies (for a review see [14]). To investigate the influence of surface wettability on the adsorption process, two different substrates were used for the adsorption experiments, pure (hydrophilic) and methylated (hydrophobized) silica surfaces. Further, the theoretical mass transport controlled adsorption of lactoperoxidase and histatin 5 were compared to the initial adsorption kinetics of human whole saliva (HWS) [15] and glandular saliva (human parotid saliva (HPS) [16], human submandibular/sublingual saliva (HSMSLS) [16] and human palatal saliva (HPalS) [17]), to investigate the possible importance of these proteins in the initial adsorption from these secretions. HPS is known to be a watery secretion,

containing high amounts of small salivary proteins such as proline-rich proteins (PRPs) and amylase. HSMSLS consists mainly of large glycoproteins which makes the secretion more viscous than HPS. HPalS, which is secreted from the minor glands in the palate, are also known for its high content of glycoproteins and its viscous nature. As the protein composition of the secretions from the different salivary glands is known to be different, it is likely that the influence of lactoperoxidase and histatin 5 on the adsorption process would also be different.

2. Experimental details

2.1 Materials

Lactoperoxidase (from bovine milk, L8257, 88%) was purchased from Sigma-Aldrich Sweden AB, (Stockholm, Sweden). Synthetic histatin 5 (72-2-25, $>$ 97%) was obtained from American Peptide Company Inc. (Sunnyvale, CA, USA). Dimensions of the proteins are given in Table 1, together with the calculated surface area per molecule for a close packed monolayer. The dimensions of histatin 5 are, to the author's knowledge unknown. Studies have shown that it adopts random coil conformation in water and phosphate buffers [18]. The radius of gyration (R_g) was approximated (assuming a theta solvent), using the formula:

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

where n is the number of amino acids (24 [6]), and l is the length of each amino acid (approximated to 3.5 Å [19]). A completely stretched histatin 5 (*i.e.* the contour length) would then attain a length of 84 Å. A more correct value would be the hydrodynamic radius, which would be expected to be slightly larger than the estimated R_g.

Mass transport controlled adsorption rates of lactoperoxidase and histatin 5 were calculated and compared to the initial adsorption of HWS [15], HPS [16], HSMSLS [16], and HPalS [17]. The model of Trurnit was applied for the mass transport controlled adsorption calculations [20]. This model applies to a stirred system, where the mass transport from the bulk to the solid/liquid interface is determined by diffusion over an unstirred layer closest to the solid surface. In the present investigation, the thickness of the unstirred layer was approximated to 30 μm [20]. The model further relies on the assumption that the adsorption is considered irreversible and that the time to create a constant concentration gradient in the unstirred layer is negligible. Under these conditions, the initial adsorption rates for a diffusion-controlled system are:

$$\mathrm{d}\Gamma/\mathrm{d}t = DC_0/\delta\,, \tag{2}$$

Table 1. Properties of the investigated proteins.

	Lactoperoxidase	Histatin 5
Dimensions (Å)	$55 \times 81 \times 78$[a]	$R_g = 7$ Å[e] stretched: $7 \times 7 \times 84$[e]
Monolayer coverage (Å^2/molecule)	6300[b] – 4300[c]	154[f] stretched: 590[b] – 49[c]
Diffusion coefficient (10^{-11} m^2 s^{-1})	5.9[d]	16[e]

[a] data from X-ray diffraction (RCSB Protein Data Bank 2GJ1). [b] side-on, [c] end-on (where the proteins were considered to have a rectangular cross section). [d] [30], [e] estimated as described in the experimental section, [f] monolayer coverage where histatin 5 was considered as hexagonally packed spheres with a cross section of $\pi \times R_g^2$.

integration of this formula gives the amount adsorbed *versus* time:

$$\Gamma = DC_0 t/\delta\,, \tag{3}$$

where Γ is the adsorbed amount per unit area (mg m^{-2}), D the diffusion coefficient (m^2 s^{-1}), C_0 the bulk concentration (mg m^{-3}), δ the thickness of the unstirred layer (m) and t is the time (s). The diffusion coefficients for the proteins are given in Table 1. As D for histatin 5 is unknown, it was approximated to 16×10^{-11} m^2 s^{-1} from a graph constructed of known diffusion coefficients *vs.* molecular weights of a series of well defined proteins as described in Lindh *et al.* [21]. As most of the proteins used for this plot are globular, the diffusion coefficient of histatin 5 might be overestimated.

The buffer solution used was a 10 mM phosphate buffer supplemented with 50 mM NaCl, and adjusted to pH 7.0. All water used was of ultra high quality (UHQ), processed in Elgastat UHQ II Model UHQ-PS-MK3 (Elga Ltd, High Wycombe, Bucks, England), and all chemicals used were of at least analytical grade (VWR International, Stockholm, Sweden).

The silicon wafers (P-type, boron doped, resistivity 10–20 Ω cm), (Okmetic, Espoo, Finland) were oxidized to an oxide thickness of approximately 30 nm [22], and cut into pieces measuring 10×30 mm. To obtain hydrophilic and methylated (hydrophobized) silica surfaces, the surfaces were treated as previously described [15, 17, 23]. Before use, substrates were rinsed with water, ethanol and water and the hydrophilic surfaces were also plasma cleaned (Harrick Plasma Cleaner PDC-32G, Harrick Scientific Corporation, Ossining, NY, USA). Surfaces prepared as described above, have been thoroughly characterized with respect to surface charge (ζ-potential of −45 mV for both pure and methylated silica) and water

contact angle ($< 10^\circ$ for hydrophilic, and $\geq 90^\circ$ for hydrophobized substrates) [23].

2.2 Technique

In situ null ellipsometry was used for studies of adsorption at the solid/liquid interface. The technique has been described in detail previously [24]. In short, the principle of this method is the changes in the polarisation of light upon reflection at an interface. The instrument used was a Rudolph thin film ellipsometer type 43603-200E (Rudolph Research, Fairfield, N.J., USA), with a xenon arc lamp as a light source, filtered to $\lambda = 442.9$ nm. Using a fixed angle of incidence (67.8°) and wavelength, the changes in polarization depends on the optical properties of the interface (derived from the ellipsometric angles Δ and Ψ). The silica surface was mounted vertically in a thermostated (37 °C) quartz cuvette. Each measurement was preceded by a two-zone surface calibration, carried out in air and buffer solution to determine the refractive index ($N = n - ik$) of the substrate as well as the refractive index (n_0) and thickness (d_0) of the oxide layer. When a constant baseline was obtained, protein stock solutions were added to the cuvette to a final concentration of 10 μg ml^{-1}. When the optical properties of the substrate and the ambient media are known and assuming a homogeneous film, the mean thickness (d_f) and refractive index (n_f) of the growing film can be solved numerically from the changes in the optical angles [25]. From the thickness and the refractive index the adsorbed amount can be calculated according to Cuypers model [26]. The adsorption was measured for 120 min, in order to reach adsorption plateau. Each experiment was carried out in duplicate.

3. Results and discussion

The present findings show that lactoperoxidase and histatin 5 formed adsorbed films on both hydrophilic and hydrophobized silica substrates. These two model surfaces represent extremes in surface free energy, and do thus span over the surface free energies of both natural and artificial surfaces present in the oral cavity. The phosphate buffer solution used was a simplified model chosen to mimic the pH and ionic strength of saliva.

The adsorbed amounts after 120 min of adsorption of lactoperoxidase and histatin 5, respectively, in terms of surface area per molecule are given in Table 2. As can be seen, the surface area per molecule of lactoperoxidase was larger on hydrophobized silica substrates compared to hydrophilic substrates, revealing that a larger number of molecules were adsorbed to the pure silica substrate. These results are in agreement with those in previous studies [12, 27]. The main driving force for adsorption on the hydrophilic substrate is most likely electrostatic attraction between the cationic protein and the an-

Table 2. Average surface area/molecule after 120 min of adsorption.

		Lactoperoxidase	Histatin 5
Average surface coverage ($Å^2$/molecule)	Hydrophilic silica	3537	656
	Hydrophobized silica	4752	630

Variations between measurements were < 10%.

ionic hydrophilic substrate. On hydrophobized substrates however, hydrophobic interactions are also expected to contribute to the adsorption process. Comparing the experimentally obtained surface coverage with the theoretical values for a close packed monolayer (Table 1) showed that the surface area per molecule on the hydrophilic silica was lower than those expected for a end-on monolayer, indicating that either the proteins have an altered structure on the surface, resulting in a smaller surface area per molecule, or that a bilayer may have started to form. On hydrophobized substrates, the surface coverage was between those assumed for a side-on and an end-on monolayer (Table 1), indicating that the layer formed was a mixture of molecules adsorbed in these two conformations.

The surface coverage of histatin 5 (Table 2) was approximately the same on the two types of substrates investigated. The amino acid sequence of histatin 5 has only 2 hydrophobic amino acids, whereas 6 are polar, 2 are negatively charged and 14 are positively charged. Due to this dominance of positively charged amino acids and the low amount of hydrophobic ones, histatin 5 may be considered to be a small polyelectrolyte. As both types of substrates investigated have a negative charge (ζ-potential of -45 mV), the adsorption seemed to be driven by electrostatic interactions on both types of substrates.

The surface coverage obtained from the ellipsometric experiments compared to the theoretical values for a close packed monolayer (Tables 1 and 2), indicates that either there is a high degree of coverage of highly stretched, side-on adsorbed molecules on the surfaces (which would be in line with the model of histatin 5 as a polyelectrolyte) or the surfaces are incompletely covered by histatin 5 in random coil conformation.

Figure 1a–d show typical initial kinetics of adsorption from HWS (obtained from a previous report [15]), HPS and HSMSLS (both obtained from a preceding study [16]) and HPalS (data from [17]). The calculated mass transport controlled adsorption rates of lactoperoxidase and histatin 5, calculated from Eq. (3), are inserted to investigate whether these two proteins (or similarly sized proteins) could take part in the initial adsorption from these secretions. The adsorption rates based on mass transport limited adsorption were calculated based on the notion that the respective protein constituted the total amount of protein in each of the salivary secretions, thereby showing the maximum possible adsorption rates.

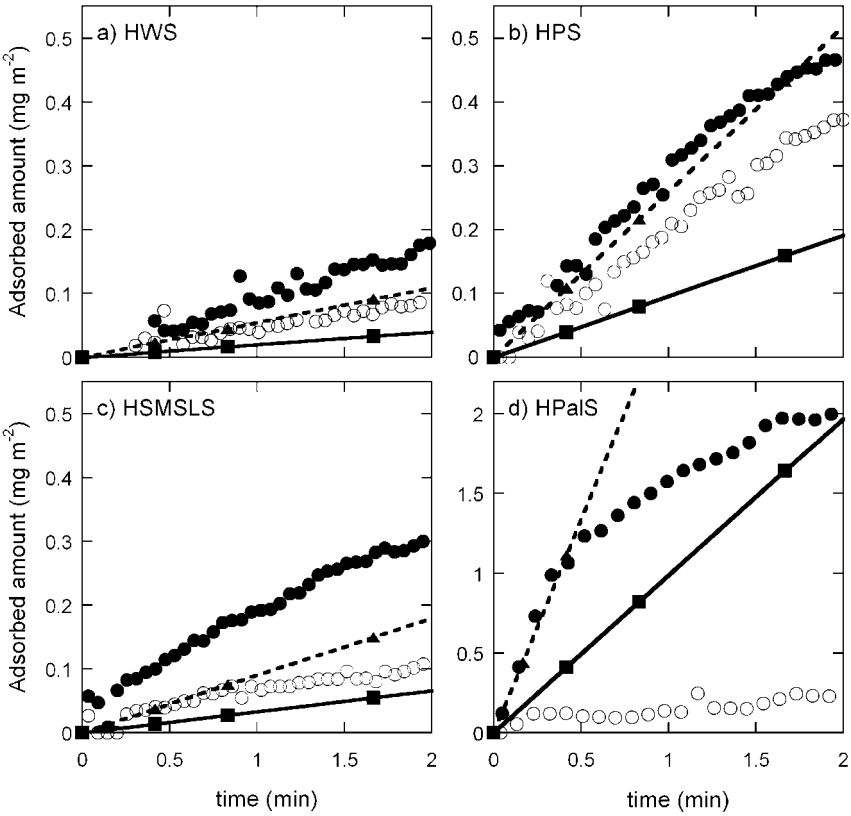

Fig. 1. Initial adsorbed amounts (mg m^{-2}) *vs.* time (min) from low concentrations of HWS (a) (data obtained from [15]), HPS (b) and HSMSLS (c) (data obtained from [16]), and HPalS (d) (data from [17]) on hydrophilic (○) and hydrophobized (●) silica substrates. The adsorption rates for lactoperoxidase (■, solid line) and histatin 5 (▲, dotted line), calculated from Eq. (3) for mass transport-limited adsorption are also inserted for comparison. Note the different scale in amounts adsorbed in Fig. 1d.

As can be seen in Fig. 1, the initial adsorption rates from proteins in HWS (Fig. 1a), HPS (Fig. 1b) and HSMSLS (Fig. 1c) to hydrophilic silica were faster than the diffusion rate of lactoperoxidase, indicating that the protein (or proteins with similar size) may be involved in the initial phase of saliva adsorption but is not the dominating component. However, the adsorption rates for these secretions correlated well with the diffusion rate of histatin 5, indicating that histatin 5 (or similarly sized peptides) may be involved in the initial adsorption. The results for the different secretions indicated that during the first phase of adsorption from HWS, HPS and HSMSLS (Fig. 1a–c) the process was dominated by molecules of the size of histatin 5. Overall, the influence of small proteins/peptides in this initial stage of adsorption is in accordance with previ-

ously published data [16]. For HPalS (Fig. 1d) on the other hand, the adsorption to hydrophilic silica was much slower than the calculated diffusion controlled adsorption rates of lactoperoxidase or histatin 5, indicating that larger proteins are involved in the initial adsorption on this surface type.

On hydrophobized silica, the initial kinetics for HWS and HSMSLS were to different extents faster than the calculated mass transport controlled adsorption of either lactoperoxidase or histatin 5, indicating that peptides smaller than histatin 5 are involved in the initial adsorption. This was particularly evident for HSMSLS (Fig. 1c). Previous studies support these findings [16]. The initial adsorption of HPS and HPalS (Fig. 1d) on hydrophobized silica showed good agreement with the diffusion controlled adsorption of histatin 5, indicating the involvement of this peptide or similarly sized ones in the adsorption at this stage.

Overall the proteins in this study have been shown to be able to form films on the surfaces investigated. The adsorption of lactoperoxidase to hydrophilic silica is indicated to be driven by electrostatic interaction. On hydrophobized substrates, hydrophobic interactions are also likely to contribute. For histatin 5 the adsorption indicated to be driven by electrostatic interactions on both types of substrates. The high adsorption affinities of these two components indicate that cationic proteins may be involved in the initial adsorption from saliva, as described in previous studies [28, 29]. As could be seen in the comparisons with the salivary secretions, the adsorption of particularly histatin 5 could be important in the initial adsorption from saliva, predominantly on hydrophilic silica substrates, where likely electrostatic interactions dominate. Lactoperoxidase may have a role in the initial adsorption phase but is not a dominating component. These results do indeed indicate the involvement of especially histatin 5 (or similarly sized peptides) during the initial salivary film formation.

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