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Salivary Proteins: Protective and Diagnostic Value in Cariology?

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Agglutinin · Antimicrobial peptides · Cathelicidin · Histatins · Lactoferrin · Mucins · Saliva · Salivary proteins

Abstract

Saliva is essential for a lifelong conservation of the dentition. Various functions of saliva are implicated in the maintenance of oral health and the protection of our teeth: (i) The tooth surface is continuously protected against wear by a film of salivary mucins and proline-rich glycoprotein. (ii) The early pellicle proteins, proline-rich proteins and statherin, promote remineralization of the enamel by attracting calcium ions. (iii) Demineralization is retarded by the pellicle proteins, in concert with calcium and phosphate ions in saliva and in the plaque fluid. (iv) Several salivary (glyco)proteins prevent the adherence of oral microorganisms to the enamel pellicle and inhibit their growth. (v) The salivary bicarbonate/carbonate buffer system is responsible for rapid neutralization of acids. An overview is presented on the major antimicrobial systems in human saliva. Not only the well-known major salivary glycoproteins, including mucins, proline-rich glycoprotein and immunoglobulins, but also a number of minor salivary (glyco)proteins, including agglutinin, lactoferrin, cystatins and lysozyme, are involved in the first line of defense in the oral cavity. Besides, small cationic antimicrobial peptides, e.g. defensins, cathelicidin and the histatins, have come into focus. These are potentially suited as templates for the design of a new generation of antibiotics, since they kill a broad spectrum of microorganisms, while hardly evoking resistance, in contrast to the classical antibiotics.

Saliva contains a large number of proteins that participate in the protection of the oral tissues, for instance lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, agglutinin and mucins [e.g. Nieuw Amerongen and Veerman, 2002]. In addition, several peptides with bacteria-killing activity have been identified. These include histatins, defensins and the only human cathelicidin, LL-37 (table 1). Because all these proteins and peptides have a broad spectrum of antimicrobial activity there seems to be a considerable overlap in functionality. This may account for the observation that susceptibility to oral diseases can apparently not be related to the concentration of a single component [Rudney et al., 1999]. The exact reason for this 'redundancy' is not really understood but different features may play a role.

The oral cavity is the home of numerous different microorganisms, of which many still await identification

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Table 1. Antimicrobial proteins in glandular salivas

Salivary (glyco)protein	Tissue of origin	Relative %
MUC5B (mucin MG1)	all mucous salivary glands	5–20
MUC7 (mucin MG2)	all mucous salivary glands	5–20
Immunoglobulins	B lymphocytes: in all salivary glands	5–15
Proline-rich glycoprotein	parotid	1–10
Cystatins	submandibular > sublingual	10
Histatins	parotid and submandibular	5
EP-GP (= GCDFP15, SABP, PIP)	submandibular, sublingual	1–2
Agglutinin (= DMBT1, gp340)	parotid > submandibular > sublingual	1–2
Lysozyme	sublingual > submandibular, parotid	1–2
Lactoferrin	all salivary glands: mucous > serous	1–2
Lactoperoxidase	parotid > submandibular	<1
Cathelicidin (hCAP18, LL37)	salivary glands, neutrophils	<1
Defensins	salivary glands, epithelial cells, neutrophils	<1

and characterization. In addition, an unknown number of microorganisms are temporary guests that are transiently present. To cope with such a wide variety of potential invaders, the oral defense should be equipped with a diverse armament to prevent uncontrolled colonization by microorganisms. In this context it has to be noted that the conditions in the oral cavity for some defense systems are suboptimal. For instance, the microbicidal activity of cationic antimicrobial peptides like defensins, histatins and LL37 is known to be sensitive to the ionic environment as evidenced by a reduction in the presence of elevated salt concentrations or low concentrations of divalent cations.

Each type of salivary gland secretes a characteristic spectrum of proteins. The complete arsenal of antimicrobial proteins present in whole saliva is thus the sum of contributions from different glands. As a consequence, the concentration of a single antimicrobial protein will vary over the day in accordance with the activity of its glandular source.

Functional overlap in defensive systems means that no single component is necessary for the overall antimicrobial capacity of the salivary defense system.

The salivary armory contains defensive components/systems protecting specifically the dentition. Examples of such 'tooth-specific' systems are the carbonate/bicarbonate buffer system (for rapid neutralization of acids) and specific proteins that form a protective coating on the enamel surface, which serves as a barrier to prevent free diffusion of acid. In addition, generic protective systems are present, comprising antimicrobial proteins and peptides, that afford protection against microbial infections and are found in other protective secretions as well. With the exception of the immunoglobulins, antimicrobial components in saliva are not focused on elimination of specific (cariogenic) species, such as *Streptococcus mutans* (table 2). Rather they prevent massive overgrowth of microorganisms, and govern the establishment and maintenance of a stable ecosystem in which harmless species outnumber potentially dangerous species, thus forming a protection in its own right. In this paper different aspects of the antimicrobial action of a number of salivary protective systems will be discussed.

Protective Properties of the Major Salivary Proteins

The most important antimicrobial proteins in saliva, and their glandular source, are summarized in table 1.

The salivary immunoglobulins belong primarily (>85%) to the IgA subclass and to a lesser extent to the IgG subclass. Together they make up about 5–15% of total salivary proteins. Salivary IgA is synthesized by B lymphocytes located in the vicinity of secretory epithelia. After secretion in the interstitial fluid, it is taken up by acinar and ductal cells of the salivary gland and subsequently secreted into saliva. IgG in saliva mainly derives from crevicular fluid leaked into the oral cavity. Because of its highly specific binding characteristics, a single immunoglobulin idiotype binds and agglutinates just one or at best a few cross-reactive microbial species. However, the entire population of salivary immunoglobulins binds the majority of microorganisms present in saliva, thus presenting a broad-spectrum defense system. In contrast to immunoglobulins in serum, IgA in saliva does not function as an opsonizing agent, since under normal condi-

Table 2. Salivary proteins: protective properties

Salivary protein	Properties
Agglutinin	aggregation of bacteria
Cathelicidin (LL37)	broad-spectrum killing of bacteria
Cystatins/VEGh	protease inhibitor
Defensins	broad-spectrum killing of bacteria
EP-GP	unknown
Histatins	broad-spectrum killing of bacteria
Immunoglobulins	inactivation and aggregation of bacteria
Lactoferrin	growth inhibition
Lactoperoxidase	growth inhibition
Lysozyme	killing
MUC5B (mucin MG1)	proton-diffusion barrier in pellicle
MUC7 (mucin MG2)	aggregation
Proline-rich glycoprotein	unknown: aggregation?
Proline-rich proteins (aPRPs)	adherence
Proline-rich proteins (bPRPs)	unknown: membrane disturbing?
Statherin	adherence

tions no cytotoxic T cells are present in saliva. Also components of the complement system, which in serum cause direct killing of bacteria, are absent in saliva. Thus, the main functions of salivary immunoglobulins tentatively will be inhibition of bacterial adherence and colonization, e.g. by blocking surface structures involved in binding.

Mucins constitute another important class of salivary glycoproteins. In unstimulated whole saliva they are the major components, making up 20–30% of the total protein. Two types of genetically different salivary mucins can be distinguished [Levine et al., 1987; Loomis et al., 1987]: MG1, high-molecular-weight mucin (M_r 10–30 MDa), encoded by the *MUC5B* gene, now designated MUC5B [Thornton et al., 1999], and the low-molecular-weight MG2 ($M_r \sim 130$ kDa), the translation product of the MUC7 gene, now designated MUC7 [Bobek et al., 1993]. Characteristic of mucins is the abundance of carbohydrate side chains which are covalently attached to the polypeptide backbones, forcing the molecule into an extended conformation. On a weight basis, the carbohydrates comprise 60% (for MUC7) to 80% (for MUC5B) of the molecule. The large dimensions and elongated form of MUC5B, in combination with the presence of a hydrophilic sugar coat, are responsible for the characteristic viscoelastic character of MUC5B-containing solutions [van der Reijden et al., 1993]. MUC5B is synthesized exclusively in mucous acinar cells of all (sero)mucous salivary glands [Nieuw Amerongen et al., 1995; Veerman et al., 2003]. MUC5B is a constituent of the protein layers that form on dental enamel after prolonged incubation with saliva, and is indispensable for the proton-barrier function of these so-called pellicles [Nieuw Amerongen et al., 1987]. Because of its hydrophilic properties, MUC5B-containing pellicles lubricate the dental surfaces, protecting them against mechanical wear. Despite its highly diverse population of oligosaccharides, which are potential receptors for bacterial adhesins, MUC5B binds to relatively few oral microorganisms, including *Haemophilus parainfluenzae* [Veerman et al., 1995] and *Helicobacter pylori* [Veerman et al., 1997a]. The low-molecular-weight mucin MUC7 differs from MUC5B in structure, localization and function. MUC7 is a single monomeric protein, decorated with short oligosaccharide side chains, which are two or three residues long. MUC7 is synthesized in serous acinar and demilune cells of the (sero)mucous glands [Veerman et al., 1997b, 2003] and is detectable in all (sero)mucous glandular salivas [Bolscher et al., 1999]. In contrast to MUC5B, MUC7 binds a wide variety of bacterial species, including *S. mutans* [Liu et al., 2000]. Both mucins have been implicated in the protection against viruses [Bergey et al., 1993a, b; Bolscher et al., 2002].

The proline-rich glycoprotein, only present in parotid saliva, makes up about 15–20% of all parotid proteins. In unstimulated saliva it is a minor component that increases with increasing stimulation of the parotid glands to about 10% in stimulated whole saliva. This small cationic glycoprotein (M_r 36 kDa) interacts particularly with *Fusobacterium nucleatum* and is involved in plaque formation [e.g. Kolenbrander and London, 1993].

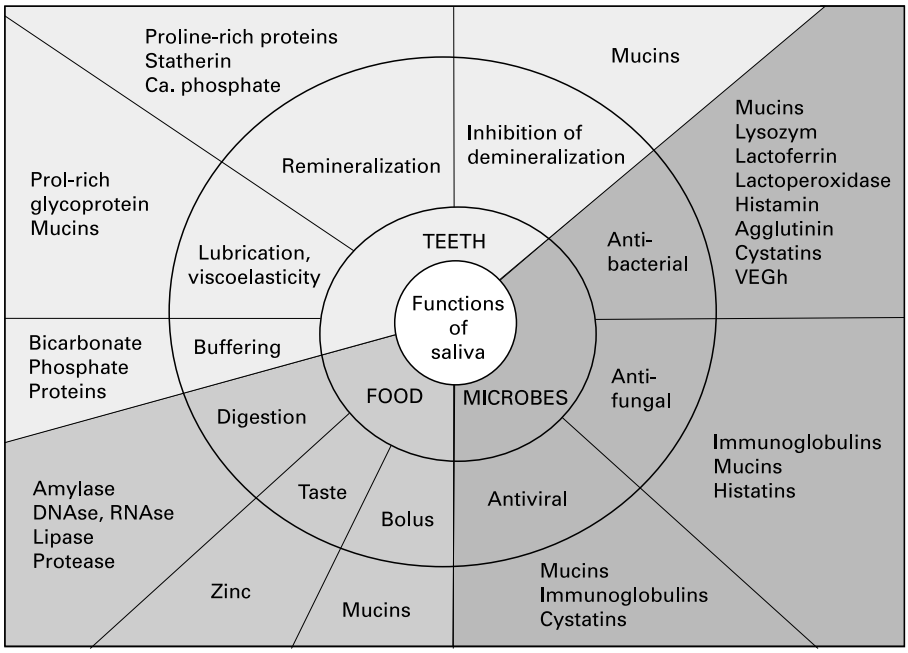

Fig. 1. Main functions of saliva in relation to its constituents.

Protective Properties of Minor Salivary Proteins

Besides the major proteins described above, which account for approximately 50% of its total protein, saliva contains a number of antimicrobial proteins that are present in lower concentrations (table 1). A number of these are enzymes which even in low concentration can exert significant biological activity. Examples of antimicrobial proteins with enzymic activity are lactoperoxidase and lysozyme.

Lactoperoxidase catalyzes the oxidation of SCN^- by hydrogen peroxide, resulting in the formation of $OSCN^-$.

Lysozyme (muramidase) is another example of an antimicrobial enzyme. By hydrolyzing cell wall polysaccharides, it makes bacteria more vulnerable to lysis due to e.g. hypo-osmotic conditions in saliva, or other antimicrobial components. Strikingly, after heat inactivation, lysozyme still exhibits bactericidal activity, probably through its cationic character. This suggests a two-step working mechanism involving initial enzymatic cleavage of the cell wall, followed by killing of the bacterium due to physical-chemical perturbation of the cell membrane by lysozyme itself, or by other antibacterial systems. Studies on the cooperative action of salivary defense systems under physiological conditions are scarce, but it is conceivable that the concerted action of proteins having different mechanisms of action enhances the power of the oral defense.

Lactoferrin is an example of a nonenzymic antimicrobial protein. Its antimicrobial action is generally attributed to its iron-chelating property, which deprives microorganisms of this essential element. In addition, lactoferrin exhibits in vitro anti-inflammatory activities. Moreover, several domains are present within its polypeptide chain that exhibit antimicrobial activities. One of these is lactoferricin, an N-terminal peptide of 40 amino acid residues that is liberated upon combined pepsin and trypsin digestion. Lactoferricin is a cationic peptide which has a broad-spectrum bactericidal activity [Groenink et al., 1999]. Another domain of lactoferrin has been implicated in the binding to salivary agglutinin, suggesting that both salivary proteins can act together [van der Kraan et al., 2004].

Salivary agglutinin was originally characterized as an *S. mutans*-agglutinating glycoprotein isolated from parotid saliva [Ericson and Rundegren, 1983; Lamont et al., 1991; Carlén and Olsson, 1995], but it is also present in submandibular and sublingual saliva [Ligtenberg et al., 2000; Bikker et al., 2002b]. It has now become clear that, besides *S. mutans,* a variety of other microbes are bound by agglutinin. The binding appears to be mediated by a relatively short peptide stretch, in the Scavenger Receptor Domains, which occur as tandemly repeating domains in agglutinin [Bikker et al., 2002a]. Besides being in saliva, agglutinin or closely related proteins have been detected in lung fluid, designated gp-340, and in brain, designated DMBT1 [Prakobphol et al., 2000; Ligtenberg et al., 2001].

Protective Properties of Salivary Peptides

In saliva at least three types of antimicrobial peptides can be distinguished: histatins, defensins and hCAP18/LL37, a human cathelicidin. Of these antimicrobial salivary peptides the histatins have attracted the most attention over the last decades. These antimicrobial peptides have a broad antimicrobial activity not only against bacteria, but also against yeasts. Such peptides can be used as templates to develop a new generation of antibiotics, because they work very rapidly and efficiently, while they are negligibly cytotoxic [Helmerhorst et al., 1999; van 't Hof et al., 2001] and do not evoke resistance. Years before the discovery of the magainins, it was reported that histidine-rich proteins in human saliva had killing activity against *Candida albicans* and *S. mutans* [MacKay et al., 1984; Pollock et al., 1984]. Since then most of the research on histatins has focussed on their fungicidal activity [Helmerhorst et al., 1997, 1999, 2001; Edgerton et al., 2000; Gyurko et al., 2001; Ruissen et al., 2001, 2003; Faber et al., 2003]. The histatins are synthesized in the parotid and submandibular glands, meaning that under both stimulated and nonstimulated saliva flow conditions, histatins will be secreted into saliva. The fungicidal, and to a lesser extent the bactericidal activity, of histatins is sensitive to ionic strength, diminishing with increasing salt concentrations [Helmerhorst et al., 1997].

The salivary glands contribute relatively little to the defensin population in saliva, which mostly derives from epithelial cells and neutrophils [Mathews et al., 1999]. Particularly during oral inflammations the expression of e.g. β-defensin-2 is up-regulated [Abiko et al., 2002; Sawaki et al., 2002]. The same holds true for hCAP18/LL-37, derived from both neutrophils and the salivary glands [Murakami et al., 2002; Woo et al., 2003]. For hCAP18, the precursor of LL-37, no biological activity has been demonstrated thus far. Activation of hCAP18 results in the release of LL-37, consisting of the C-terminal 37 amino acids, which has broad-spectrum antimicrobial activity [Sörensen et al., 2001; den Hertog et al., 2004].

Future Perspectives for Research

Insight into the mechanism of action, in addition to knowledge of the structure-function relationship of antimicrobial proteins and peptides, makes it possible to design small, biologically active peptides that can be used as natural antimicrobials. In many cases it is not necessary to biosynthesize by recombinant techniques the whole polypeptide chain of biologically active proteins but instead only peptides encompassing the functional domain. This opens new perspectives for the application of peptides as instruments to fight multiresistant microorganisms, or as additives in mouthrinses, to restore functionality in patients in whom the natural protection is compromised.

Potential Impact on Clinical Practice

New formulations containing antimicrobial peptides derived from natural salivary components have been tested for their applicability in the treatment of oral inflammatory processes such as gingivitis and periodontitis. An example of a potential clinically applicable antimicrobial peptide is IB-367, a protegrin-derived synthetic peptide with in vitro and in vivo antimicrobial activity against the microflora associated with oral mucositis [Mosca et al., 2000]. Protegrin is the porcine analogue of human cathelicidin.

Another example is P113, an 11-amino acid fragment of histatin-3 [Rothstein et al., 2001]. Topical oral application of P113, or use of a mouthrinse containing P113, leads to significant reduction in experimental gingivitis [Paquette et al., 2002] without causing side effects. In addition, the nonhydrolyzable derivative P113D retains its killing activity on *Pseudomonas aeruginosa* in the presence of sputum having increased electrolyte concentrations from cystic fibrosis patients [Sajjan et al., 2001].

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