Fascinating families of proteins: electrophoresis of human saliva
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Introduction
The proteins in saliva are synthesized mainly in the acinar cells of the major salivary glands, namely the parotid, submandibular and sublingual. Minor components derived from serum are also present in the secretions of these three pairs of glands which, together with the secretions of the minor glands, gingival exudate, micro-organisms and exfoliated buccal epithelial cells, constitute whole or mixed saliva, sometimes known as oral fluid.

The proteins present in human salivary duct secretions are shown in Table 1. The major ones are proline-rich proteins, histatins (histidine-rich proteins), \( \alpha \)-amylase and mucins. The first three occur as polymorphic families of proteins with proline-rich proteins comprising about two thirds of the total protein in duct secretion [1]. Contemporary electrophoretic techniques have facilitated extensive studies on these families of proteins and have yielded fascinating new information about them.

Electrophoresis of salivary proteins
One-dimensional electrophoresis
Early studies on salivary proteins involved disc gel electrophoresis in non-denaturing gels, but in order to obtain the optimum amount of information, both anionic and cationic gels were necessary [2–5]. With the exception of studies on polymorphic species, this procedure has now been largely superseded by SDS/PAGE. A major problem in electrophoretic studies on saliva is the fact that proline-rich proteins stain poorly with conventional staining procedures such as Coomassie Blue or silver and are therefore frequently overlooked. However, it is now known that after SDS/PAGE, if the gels are stained with Coomassie Blue R-250 and organic solvent is omitted from the destain, proline-rich proteins stain pink-violet and other proteins stain blue [6]. A modified silver-staining procedure results in proline-rich proteins staining yellow-brown [6]. Because of their unusual amino acid compositions, these proteins migrate atypically on SDS/PAGE.

Isoelectric focusing
Whilst carrier ampholyte (CA)/PAGE isoelectric focusing (IEF) of salivary proteins yields sharp bands and gives superficially good results, especially in terms of resolution of the isoenzymes of \( \alpha \)-amylase, it is necessary to concentrate and desalt samples prior to analysis in order to prevent band distortion; this procedure is time consuming and results in the loss of protein. In addition, there is the problem of cathode drift with the consequent loss of basic proteins. These difficulties have been overcome largely by the use of immobilized pH gradients (IPGs), with optimum results being obtained with an IPG hybrid CA/PAGE-based system containing urea followed by Coomassie Blue G-250 or silver staining [12]. The technique enables direct application of duct saliva to gels and the preparation of stable narrow pH range gels which facilitate the study of polymorphisms which differ in charge. Staining of proline-rich proteins in this system can still be difficult, however.

Two-dimensional analysis
Two-dimensional analysis of human salivary proteins has presented special problems in that much of the protein being proline-rich is difficult to stain, and many of these proteins are sufficiently basic to migrate off the end of the gel when one of the dimensions involves a conventional CA system. Accordingly, two-dimensional analysis based on O’Farrell [13] systems has produced poor results. Reports of large numbers of small protein-staining spots using this system with whole saliva (e.g. [14])

Abbreviations used: CA, carrier ampholytes; IEF, isoelectric focusing; IPG, immobilized pH gradient; MG, mucin glycoprotein; PRP, proline-rich protein.
<table>
<thead>
<tr>
<th>Protein</th>
<th>$M_r$</th>
<th>$p_I$</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Proline-rich proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic</td>
<td>16 300, 9000</td>
<td>3.5–4.5</td>
<td>Binding to hydroxylapatite, Ca$^{2+}$ binding, Adhesion of microorganisms, Protection against dietary tannins</td>
</tr>
<tr>
<td>basic</td>
<td>6 000–12 000</td>
<td>$&gt; 8.0$</td>
<td></td>
</tr>
<tr>
<td>glycosylated</td>
<td>38 900</td>
<td>$&gt; 8.0$</td>
<td></td>
</tr>
<tr>
<td>*α-Amylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glycosylated</td>
<td>63 000</td>
<td></td>
<td>Digestion, Antibacterial</td>
</tr>
<tr>
<td>non-glycosylated</td>
<td>59 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Histatins</td>
<td>4500–7000</td>
<td>7.0, $&gt; 9.5$</td>
<td>Anti-fungal (non-immune), Pellicle formation, Regulation of mineralization</td>
</tr>
<tr>
<td>Statherin</td>
<td>5 380</td>
<td>4.2</td>
<td>Inhibition of CaPO$_4$ precipitation, Regulation of mineralization, Binding to hydroxyapatite</td>
</tr>
<tr>
<td>*Cystatins</td>
<td></td>
<td></td>
<td>Cystatin protease inhibition, inhibition of CaPO$_4$ precipitation, Binding to hydroxyapatite</td>
</tr>
<tr>
<td>*Acid phosphatase</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mucins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1</td>
<td>$&gt; 1000 000$</td>
<td></td>
<td>Lubrication, Bacterial agglutination, Pellicle formation</td>
</tr>
<tr>
<td>MG2</td>
<td>200 000–250 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulphated</td>
<td>15 000–30 000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kallikrein</td>
<td>9 600</td>
<td>3.8–4.5</td>
<td>Protease, ?Activation of polypeptide hormones</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>405 000</td>
<td></td>
<td>Antibacterial</td>
</tr>
<tr>
<td>(slg A, etc.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>77 000</td>
<td></td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>15 000</td>
<td></td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>37 000</td>
<td></td>
<td>Taste sensation, ?</td>
</tr>
<tr>
<td>Gustin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secretory component</td>
<td>67 000</td>
<td></td>
<td>Maintenance of bicarbonate buffering</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>71 000</td>
<td></td>
<td>Maintenance of oral and gastrointestinal mucosa</td>
</tr>
<tr>
<td>Polypeptide hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(epidermal growth</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>factor</td>
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</table>

are almost certainly the result of bacterial contamination. By means of the two-dimensional procedure of Görg et al. involving CA/IPG hybrids in the first dimension and thin-layer gradient horizontal SDS/PAGE in the second [15, 16], together with modified staining procedures [6], a substantial improvement has been achieved [17]. Because IPGs prevent loss of proteins of $p_I > 8$, basic components,
including basic proline-rich proteins, can now be resolved. A two-dimensional map showing the location and identities of most of the major proteins in parotid saliva has been constructed [17].

**Polymorphic proteins**

**α-Amylase**

Salivary α-amylases are the product of the salivary amylase (Amyl) gene (S-type) and are distinct from the pancreatic (Amy2) gene (P-type). The isozymes of salivary α-amylase arising from post-translational modifications of the products of this gene have been characterized by PAGE [18]; they result from the partial glycosylation of the primary gene product followed by deamidation. IEF and PAGE have been used to study the inheritance of polymorphisms in salivary α-amylase [7, 19] and to separate the salivary and pancreatic isozymes in serum [7]. Although the major role of salivary α-amylase is almost certainly one of digestion of starch, it also binds to streptococci [20] and may, in addition, be involved with bacterial metabolism.

**Proline-rich proteins**

Proline-rich proteins (PRPs) have a proline content of 28-40% and this, together with glutamine and glycine, constitutes 75-80% of all amino acid residues. They are the major proteins in duct saliva and can be divided into acidic, basic and glycosylated PRPs which constitute 30%, 23% and 17% of total parotid salivary protein [1]. Although a large number of polymorphisms have been reported [1, 21], they are encoded by only six genes with two PRH genes encoding the acidic proteins and four PRB genes the basic and glycosylated types. The genes encoding the basic proteins are not expressed in the submandibular gland. There are two major acidic groups, proteins A and C, protein A being formed by the cleavage of a C-terminal peptide from protein C by kallikrein [22]. Within these groups there are several polymorphic forms. Nine basic PRPs, IB1-IB9, have been characterized [23] (which are probably similar to those referred to
Two other distinctive basic non-glycosylated PRPs, G1, is basic and contains 30% carbohydrate [25]. PRP 'size polymorphisms', Ps-1 and Ps-2 in parotid saliva [6] appear to correspond to basic PRPs from the antidigestive and potentially oral and oesophageal carcinogenic effects of these constituents of our diet [29, 30]. However, the precise roles of PRPs are still uncertain.

**Histidine-rich proteins**

Histidine-rich proteins (histatins) occur in parotid and submandibular secretions and are comprised of a neutral molecule (pI = 7.0) and a distinct group of basic polypeptides (pI > 9.5) with anti-microbial activity. The latter are more basic than lysozyme on cationic gel electrophoresis and are of lower molecular mass than PRPs on SDS/PAGE. There are several polymorphic forms which are the products of more than one gene and, together with the products of highly specific proteolytic processing of products of these genes, result in six major forms and several others of related structure [1, 31, 32]. As a result of a study of these proteins using capillary electrophoresis, it now appears that there are six major histidine-rich polypeptides, referred to as histatins 1–6, with other molecules of this type being derived from them [33]. Histatin 1 is absorbed selectively on to hydroxyapatite and is probably involved in pellicle formation on the enamel surface; it also inhibits hydroxyapatite crystal formation and may be responsible for maintenance of the surface integrity of enamel [31]. Histatins also have anti-fungal properties and appear to constitute a non-immune protection system for the oral surfaces. They are fungicidal to *Candida albicans* [34] with histatin 5 being the most active [35] whilst histatin 4 inhibits blastospore germination [36, 37]. Saliva has also been reported to inhibit the infectivity of human immunodeficiency virus (HIV-1) [37] and, whilst histatins do not exhibit broad spectrum anti-viral activity, a possible involvement cannot be excluded [31].

Interestingly, the tyrosine-rich salivary-specific polypeptide statherin (pI 4.2), which also inhibits hydroxyapatite crystal growth, appears to be the product of a gene of common ancestry with those coding for histatins [38]. There have been no reports of polymorphisms in this protein.

**Cystatins**

Cystatins are a family of cysteine protease inhibitors produced by a seven-member multigene family, and are widely distributed in mammalian tissues and fluids. The three major cystatins in saliva, S, SA and SN, are synthesized in salivary glands and can easily be separated by SDS/PAGE. Cystatin C (a minor component) probably originates from crevicular fluid [39]. Salivary cystatins bind to hydroxyapatite and are stronger inhibitors of crystallization than statherin [40]; accordingly, they may be involved in regulation of mineralization at the tooth surface. However, they do not appear to be involved in the modulation of tissue destruction in periodontal disease [41].

**Acid phosphatase**

Acid phosphatase in human parotid saliva, although only a minor component, also exists in polymorphic forms. There are three phenotypes determined by two autosomal alleles which can be separated electrophoretically [42].

**Other proteins**

In addition to complex families of polymorphic proteins, some simple proteins are also present. The level of viscosity of saliva is related to the presence of mucins, substantial amounts of which are secreted by the submandibular glands. They are glycoproteins which contain 70–80% carbohydrate and there have been no reports of genetic variations. Two types, mucin glycoprotein (MG) 1 and 2, can readily be separated by SDS/PAGE and have been studied extensively. MG1 (Mr > 1000000) has good lubricating properties, binds to hydroxyapatite and is a constituent of pellicle, whilst MG2 (Mr 200000–250000) agglutinates oral streptococci [43]. Sulphated glycoproteins are also present. Mucin glycoproteins also contain blood group substance activity.

In addition, saliva contains a group of antibacterial proteins including lysozyme, lactoferrin, lactoperoxidase and immunoglobulins (mainly slgA). Other proteins include albumin, tissue kallikrein, polypeptide hormones (epidermal growth factor, nerve growth factor, etc.), gustin (required for taste bud sensation) and carbonic anhydrase.
The proteins in whole saliva

Electrophoretic analysis of parotid saliva clearly indicates the presence of large amounts of proline-rich proteins, α-amylase and histatin-like components, whilst submandibular saliva contains mucins in addition. In contrast, in mixed saliva, whilst containing α-amylase and mucins as major components, the levels of proline-rich proteins and material corresponding to histatins are either substantially reduced or absent (Figure 1) [6]. Although some proline-rich proteins and histatins may bind to the enamel surface and contribute to pellicle formation, this is unlikely to account for a loss of protein on this scale.

One possibility is that some proteins, including acidic PRPs, are absorbed onto the surface of buccal epithelial cells and then covalently linked to them by transglutaminases to form a mucosal pellicle [44, 45]. Bacterial degradation may also be involved and a large number of phosphopeptides derived by cleavage of acidic PRPs have been identified by electrophoresis on 35% gels and shown to retain their ability to bind to hydroxylapatite [46].

More recent studies have involved a detailed analysis of the effects of incubation of various oral micro-organisms on the parotid saliva protein pattern on SDS/PAGE. Whilst some organisms have little effect, some specifically remove certain proline-rich proteins and histatin-like components [47]. Although the mechanism involved is still being investigated, basic proteins are removed more readily than acidic ones, the process is species-specific and there are strain to strain variations. Particularly effective are Streptococcus parasanguis (NCTC15912) and S. gordonii (NCTC10231), both of which remove all histatin-like components and basic PRPs, leaving only reduced levels of acidic PRP-C (Figure 2). S. gordonii (NCTC7868) is slightly less effective and two new PRPs, probably degradation products, are formed. Incubation with hydroxylapatite had little effect on parotid salivary protein patterns unless very high apatite: saliva ratios were used.

Conclusions

Electrophoretic analysis has yielded a substantial amount of information about the proteins in saliva, especially the major components and their polymorphisms. Currently, the technique is being used to yield new information about the functions of these proteins. Although clinical applications of the electrophoresis of human salivary proteins have been limited by the complexity of the poly-morphisms of the major proteins, now that these have been characterized, the findings together with new electrophoretic analytical procedures should facilitate the development of this area. Electrophoretic analysis of polymorphisms also appears attractive as a future basis for forensic and anthropological studies.

The contribution of Mrs F. Newman to Figure 2 is gratefully acknowledged.


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