HYDROXYAPATITE-REACTIVE SALIVARY PROTEIN REVEALED BY ISO-ELECTROFOCUSING ELECTROPHORESIS

BY

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ABSTRACT

Salivary protein involvement in the formation of acquired enamel pellicle, so far, has been discussed in terms of hydroxyapatite (HA)-reactive salivary proteins only from the parotid gland. This study was undertaken to seek this type of protein in the human whole (mixed) saliva and to investigate its normal and pathological variations. Several kinds of hydroxyapatite, either biogenous or synthesized by solid phase reaction, were used as a powder (250 mesh). HA was incubated with concentrated whole saliva at 25° for 30 min. After centrifugation and filtration, salivary proteins were analysed on a Multiphor isoelectrofocusing gel electrophoresis.

The control salivary proteins were separated into three major groups: acidic (A1-A8), neutral (N1-N4), and basic (B1-B5) isoelectric point (pI). In the HA incubated sample, one of the major neutral bands (N1) preferentially disappeared at about pI 7.5. This N1 band was missing or scarce in the parotid saliva and had an amino acid composition rich in glycine, lysine, serine, glutamic acid, aspartic acid, and histidine.

This protein was considered to be one of the major HA-reactive proteins in human whole saliva.

INTRODUCTION

In the last decade, a number of investigators*2-13 have identified various salivary proteins as being possible precursors for the dental integument which was designated as the acquired enamel pellicle by Dawes, Jenkins and Tonge.1) The pellicle is an organic membrane, about 1 μm thick, which surrounds the surface of enamel. It may be related to the initial formation of dental plaque. Although the origin of this structure is generally assumed to be salivary proteins, the precise components involved have not yet been determined.

Armstrong*2,3) was the first to suggest that a salivary glycoprotein fraction might form a significant proportion of the acquired enamel pellicle. Subsequently, various salivary proteins, including sialoprotein,4) glycoproteins,5,6,11-19) and a high molecular weight or highly aggregated salivary protein

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fraction\textsuperscript{7} were proposed as a possible precursor of this structure.

Recently, comparatively pure protein fractions have been discussed with regard to their hydroxyapatite (HA)-reactivity. Hay\textsuperscript{5,8,9} noted that several proteins in parotid saliva were selectively adsorbed on the surfaces of HA and dental enamel minerals. These proteins were later identified as tyrosine-rich acidic peptides,\textsuperscript{15} proline-rich proteins,\textsuperscript{17} and histidine-rich proteins.\textsuperscript{22} Also, Armstrong\textsuperscript{14} has shown that a certain basic protein is selectively adsorbed on HA.

The above works involve parotid saliva proteins, but it is evident that the actual sequence of events in the enamel pellicle formation occurs in the oral cavity which is circumfered with a mixed saliva. Nevertheless, so far, none of the protein components from mixed saliva have been directly demonstrated to form the acquired enamel pellicle.

The purpose of this work was to identify this kind of HA-reactive protein in the mixed saliva and to investigate its normal and pathological variations.

**Materials and Methods**

*Collection of saliva*

The non-stimulated whole (mixed) saliva was collected in a polyethylene centrifuge tube cooled in ice. Polyethylene was used throughout to avoid exposure of the salivary protein to polar surfaces and to prevent adsorption or denaturation. Whole saliva was lyophilized immediately after collection and dissolved in 0.05 M Tris-HCl buffer (pH 7.4) at a concentration 5 times higher than the original saliva. The solution was then centrifuged at 20,000×g at 4°C.

Parotid secretion was collected by a Teflon cup without using any particular stimulant. These were lyophilized and concentrated as described above.

*Hydroxyapatite*

According to the method previously reported,\textsuperscript{20} hydroxyapatite was synthesized by the solid-phase reaction of CaHPO\textsubscript{4}·2H\textsubscript{2}O and CaCO\textsubscript{3} (3:2) in air at 1,000–1,200°C, and used as a powder (250 mesh). The Ca/P ratio was 1.67 and the surface area was approximately 30 m\textsuperscript{2}/g.

*Enamel powder*

The surface of the extracted human teeth was thoroughly polished with pumice, and the teeth were completely dried in a desiccator over P\textsubscript{2}O\textsubscript{5}. The enamel layer was removed mechanically with a chisel and a dental diamond bar. The removed enamel was powdered in a stainless mortar to 250 mesh.

*Adsorption of salivary proteins by hydroxyapatite of human enamel powder*

One-ml portions of concentrated saliva, prepared as described above, were placed in small polyethylene tubes containing various amounts of HA or from 5 to 100 mg of enamel powder. The bottles were set horizontally, rotated about the horizontal axis, and incubated for 30 min at 37°C. This technique avoids turbulence and formation which could denature the salivary proteins. Generally, the solid phase dispersed rapidly in the saliva, suggesting adequate surface-liquid contact. The saliva remained in contact with the solid phase for 30 min. It was then centrifuged at 20,000×g for 15 min at 4°C and filtered through a 0.22 mn Millipore. Aliquots of the filtrate were analyzed on the Multiphor system.

*Isoelectrophoresis of saliva*

According to the technique described by Karlsson et al.,\textsuperscript{1,3} protein components of saliva before and after the reaction with hydroxyapatite or enamel powder were analyzed on an LKB Multiphor isoelectro-
focusing system. Polyacrylamide gels containing Ampholites of isoelectric point (pI) ranging between 3.5 and 9.5 (Ampholine PAG plate) were used in the electrofocusing. Isoelectrofocusing was performed at 900 V, 50 mA, 20 W. After electrophoresis, the gel was fixed for 0.5 to 1 hr in a solution containing 17.25 g sulfosalicylic acid, 57.5 g trichloroacetic acid in 150 ml of methanol, and 350 ml of distilled water. The gel was then stained for 10 min at 60° in a solution containing 0.115 g Coomassie Brilliant Blue R250 in 100 ml of a destaining solution (500 ml ethanol and 160 ml acetic acid diluted to 2 liters with distilled water). Excess stain was removed by several applications of the destaining solution.

Amino acid analysis

The portions in the isoelectrofocusing gel plates containing N1, N2, and N3 bands were each cut out, minced, and extracted with 70% formic acid overnight at 0°. Formic acid extracts were passed through an anion-exchange column of AG 1×2, and the effluents were collected by lyophilization after dilution with 6 vol. of distilled water. Samples were hydrolysed at 110° for 24 hr in 2 ml of 6 N hydrochloric acid in evacuated, sealed tubes. The acid was removed by 3 to 4 evaporations with distilled water. Amino acid analysis was performed using a standard Jeol 6AH system.

RESULTS

Fig. 1 shows the electrophoretic profile of whole saliva and the properties of individual band. Human saliva was separated into clearly distinguishable bands. The upper end corresponded to pI 3.5 and lower end to pI 9.5. The darkest band in the center corresponded to pI 6.6. To the side of it, there are bands at pI 6.2 and 7.5. These three bands at neutral pI region were designated as N1, N2 and N3. At the acidic pI region between pI 4 and 5, there are a total of 8 fine bands which are designated as group A and numbered A1 to A8. At the alkaline pI, 2 or 3 bands were detected around pI 9.5. Thus, we can clearly distinguish a total of 15 bands which can be grouped into acidic, neutral, and basic pI.

Fig. 2 shows the pattern from nine individuals. Although there is a slight variation in the N1 and B groups, the patterns of mixed saliva are fundamentally the same.

![Fig. 1. Electrophoretic profile of whole saliva and some of the characteristics of each band.](image)
Fig. 2. Isoelectric patterns of whole saliva obtained from nine individuals. A pH gradient in the gel from pH 3.5 (top) to 9.5 (bottom) was employed. The N2 and N3 bands were invariably present, while considerable variation was observed in bands of N1 and B group.

Fig. 3. Adsorption of salivary proteins on hydroxyapatite. One-ml portions of concentrated saliva were incubated with various amounts of hydroxyapatite. N1 band is preferentially adsorbed. Numbers indicate the amount of hydroxyapatite in mg.

Fig. 5 shows the adsorption on HA of salivas with 15 bands. A suspension of 10 mg of hydroxyapatite in 1 ml of concentrated saliva was incubated for 30 min at 25°. The mixture was then centrifuged and filtered through a 0.22 μm Millipore. Aliquots of the filtrate were analyzed on the Multiphor system. The N1 band in the incubated saliva disappeared, but other bands were almost unchanged.

The identical adsorption test was performed varying the amount of hydroxyapatite and its result is also shown in Fig. 3. In the salivas incubated with 10, 20, 30 mg,
Fig. 4. Adsorption of salivary proteins on enamel. One-ml portions of concentrated saliva were incubated with various amounts of enamel powder. Numbers indicate the amount of enamel powder in mg.

Fig. 5. Difference in isoelectrophoretic patterns between whole saliva and parotid saliva.

N1 band is not found in parotid saliva. W.S. refers to the pattern of whole saliva, P.S. that of parotid saliva from the same (middle) and different (right) donor.

the N1 band gradually faded and finally disappeared. In the salivas incubated with more than 40 mg, the N2 and N3 bands also disappeared. Almost all the bands disappeared in the sample incubated with 50 mg. It is clearly shown by these results, that the N1 band preferentially disappears from the N group. This suggests that this band has a higher affinity to hydroxyapatite. Fig. 4 shows the results of identical adsorption test with various amounts of human enamel powder. In the saliva incubated with more than 10 mg of enamel powder, the N1 band disappeared.

Characterization of the N1 band was done by comparing the patterns of whole and parotid salivas. As shown in Fig. 5, it was found that the N1 band was detected only in the whole saliva. No detectable band corresponding to N1 has been observed in the parotid saliva from normal individuals so far examined. In Fig. 5, clear difference was observed in the distribution of N1 fraction in the parotid and whole saliva obtained from the same donor. It is interesting that the N1 band was not detected in the parotid saliva, since most investigators have used parotid saliva for their studies on HA-reactive salivary proteins.

As shown in Table 1, the amino acid composition of the N1 band is characterized by high levels of glycine (137 residues/
Table 1. Amino acid composition of N1, N2, and N3 bands and human whole saliva

<table>
<thead>
<tr>
<th>Residues/1,000 residues</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>Whole saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>76</td>
<td>119</td>
<td>108</td>
<td>66</td>
</tr>
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<td>Thr</td>
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<td>Ser</td>
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<tr>
<td>Gly</td>
<td>137</td>
<td>158</td>
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<td>Ala</td>
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<td>Ile</td>
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<td>30</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Leu</td>
<td>52</td>
<td>53</td>
<td>65</td>
<td>47</td>
</tr>
<tr>
<td>Tyr</td>
<td>19</td>
<td>18</td>
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<td>Phe</td>
<td>31</td>
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<td>Lys</td>
<td>109</td>
<td>43</td>
<td>47</td>
<td>51</td>
</tr>
<tr>
<td>Arg</td>
<td>72</td>
<td>18</td>
<td>50</td>
<td>42</td>
</tr>
</tbody>
</table>

Values of whole saliva are the average of these analyses of different dhnors.

1,000 residues), acidic amino acids (total 184 residues), lysine (199 residues), serine (106 residues), and histidine (75 residues). These characteristics of amino acid composition of the N1 fraction clearly differ from that of whole saliva as presented in Table 1. Amino acid composition of whole saliva is unique in being rich in proline as the most abundant amino acid, and glycine as the successors (Table 1). The amino acid composition of the N1 band is similar to that of the N2 and N3 bands in terms of high glycine, acidic amino acid, and serine contents, but there were significant variations in the proteins of the N group in terms of alanine, histidine, and lysine contents. The N1 band showed comparatively higher content of histidine, lysine, and arginine. The apparent molecular size of the N1 band was estimated from the elution position on a Sephadex G75 chromatograph to be about 10,000.

**Discussion**

The objective of this study was to identify salivary proteins which possess a high affinity for dental enamel and synthetic hydroxyapatite *in vitro*. Isoelectrofocusing electrophoresis enables distinctly reproducible separation of salivary proteins between pI 3.5 and 9.5 without chromatographic pre-separation. The data described above clearly demonstrate that one specific component of whole saliva shows a relatively high affinity for the above mentioned mineral surfaces. This protein, which had an isoelectric point of about 7.5, contained a slightly higher proportion of basic amino acid residue (256 residues/1,000 residues) than acidic amino acid residue (184 residues/1,000 residues). Remarkable difference in the amino acid compositions between the N1 fraction and whole saliva resides in the content of proline (Table 1). This indicates that the N1 fraction may be a rather minor component, not giving the proline-rich characteristics to the amino acid composition of the whole saliva. It is interesting that the serine content was notably high in the N1 band. This amino acid can be phosphorylated to phosphoserine which is known to possess a strong affinity to HA and calcium. Sufficient amount of the N1 fraction to determine phosphorus or phosphoserine content was not available, though these could be useful in detecting the possible binding sites with HA.

Another important characteristic of the N1 band is its absence in parotid saliva. This fraction was detected only in whole saliva, so that sublingual and/or submandibular saliva. This finding is important, since most of the previously reported HA-reactive salivary proteins are of parotid origin. Thus, the N1 fraction is a novel
HA-reactive proteins which clearly differs from those previously reported.

Hay\textsuperscript{(15)} has demonstrated seven specific parotid salivary components which show a relative high affinity for HA surfaces. The nature of four of these components has been determined.\textsuperscript{(17)} They have been termed proline-rich proteins (PRP) and are characterized by high levels of proline (22–27\%) of total residues), glycine (20–22\%), dicarboxylic acids (26–36\%), particularly glutamate. They have molecular weight of either 12,000 (PRP-I) or 6,000 (PRP-III), appear to possess a high degree of molecular asymmetry, and are eluted from gel filtration columns close to human serum albumin (mol. wt. ca. 65,000). Interestingly, these proteins are not easily detected on gel patterns obtained from whole saliva\textsuperscript{(17)} and are unlikely to be the N1 fraction demonstrated in the present work. The two proteins showing the greatest affinity for HA surface are an acidic tyrosine-rich peptide with pI 4.22\textsuperscript{(16)} and a histidine-rich (190 residues/1,000 residues) peptide with pI 7.04.\textsuperscript{(22)} Neither can be identified as N1 because of the difference in pI and amino acid compositions. The seventh component showed a marked resemblance to PRP and appeared to be a subgroup of related proteins. Conclusively, the N1 fraction is not identical with any of the seven HA-reactive proteins reported by Hay.

Bennick and Connell\textsuperscript{(19)} have isolated and purified four proteins from human parotid saliva, designated as A, B, C, and D. Proteins A and C were examined for their homogeneity by isoelectrofocusing electrophoresis in the pH range of 3–10. The isoelectric points of proteins A, B, and C lie between 4 and 5.10,\textsuperscript{(23)} which is far more acidic than N1 with pI 7.5. The pI of protein D is about 7,\textsuperscript{(19)} which is comparatively closer to the pI value of N1. Protein D is characterized by high content of glutamic acid (15.3\% of total residues), glycine (12.3\%), tyrosine, histidine (both 8.3\%), aspartic acid (8.3\%), and serine (7.3\%), but a low content of lysine (5.7\%). This latter factor suggests non-identity to N1 fraction. Further, the invariable presence of protein D in all parotid secretions\textsuperscript{(19)} is not consistent with the characteristics of N1 fraction. Protein D was not tested for its affinity to HA or calcium.

Belcourt\textsuperscript{(25)} recently described a calcium precipitable salivary protein in whole saliva and suggested that this protein fraction could be related to the formation of the acquired pellicle. The molecular weight was 62,000 and the isoelectric pI was determined by electrofocusing as 4.75±0.05.\textsuperscript{(29)} This protein is too acidic and heavy to be the N1 fraction. He compared the amino acid composition of this protein fraction with that of the protein extracted from dental plaque\textsuperscript{(28)} but it is not easy to find a close similarity between the amino acid compositions of these fractions. Rather, the amino acid composition of the dental plaque which he described seems to be closer to that of the N1 band when compared on a star diagram of their amino acid composition.\textsuperscript{(27)}

From the above considerations, we conclude that the N1 fraction is a major and novel HA-reactive salivary protein which may be secreted by the sublingual or submandibular glands. To the best of our knowledge, no protein with identical or at least similar properties have been reported.

With respect to the biological significance, the high affinity of N1 fraction to HA clearly suggests that the exposure of HA surfaces to saliva may result in the occurrence of specific processes and it is not unreasonable to speculate that the effect may occur \textit{in vivo} on the tooth surface. The simple one-
step procedure of the isoelectrofocusing methods, as used in the detection of the N1 fraction, will permit further studies of the normal and pathological variations in this salivary component. Preliminary studies on the parotid saliva from a patient with Sjögren syndrome revealed an unexpected band very close to the position of N1 fraction. Further studies on the characterization and significance of the N1 fraction are being made in this clinical laboratory.

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References


