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TWO-DIMENSIONAL ELECTROPHORESIS OF HUMAN SALIVA

C. S. Giometti and N. G. Anderson

Molecular Anatomy Program, Division of Biological and Medical Research,
Argonne National Laboratory
Argonne, Illinois 60439, USA

Introduction

Saliva is essential to the maintenance of the teeth, tongue, and mucous membranes of the oral and oral-pharyngeal areas, as demonstrated by the deleterious effects of salivary gland malfunction due to irradiation, disease, or drug effects (1). The saliva performs this maintenance function partially through lubrication and mechanical cleansing of the oral structures and partially by providing certain protein components such as lysozyme, IgA, lactoferrin, and lactoperoxidase which have antibacterial activities (2). In addition to the maintenance of the oral cavity, saliva contains nerve growth factor (3), epithelial growth factor (4), and factors thought to be capable of accelerating the wound healing process (5). Salivary glands may also provide as yet unidentified factors important to the normal function of the alimentary tract, and may be to that tract what endocrine glands are to other organs (6).

The number of salivary protein components appears to increase as the resolving power of electrophoretic techniques improves and more specific and sensitive protein stains are developed (7). One-dimensional polyacrylamide gel electrophoresis techniques used thus far have demonstrated the presence of approximately 30 protein components in parotid saliva samples (7-10). Numerous genetic polymorphisms have been found including the parotid proline-rich proteins, double-band protein, acidic protein, salivary peroxidase, acid phosphatase, and parotid basic proteins (11). Using polyacrylamide gel electrophoresis and isoelectric focusing, salivary amylase has been resolved into 7 or more protein species which differ in both charge and molecular weight (7, 12). The resolution possible using these

one-dimensional techniques is severely limited, however, and such techniques cannot completely separate the complex mixture of proteins present in saliva (8, 9). Two-dimensional electrophoresis on the other hand, separates proteins by both differences in charge and molecular weight by combining isoelectric focusing in the first dimension with sodium dodecyl sulfate (SDS) electrophoresis in the second dimension. The two-dimensional technique, with modifications to permit the analysis of multiple samples (13, 14), offers the opportunity for studies of genetic and environmental factors influencing the salivary protein composition. Using this approach, polymorphisms not previously observed using one-dimensional separations may be discovered and those seen with previous methods can be further elucidated.

In a previous report, two-dimensional electrophoresis of whole saliva yielded a pattern of approximately 50 spots by Coomassie Brilliant Blue staining (6). In this paper we describe the resolution of over 100 spots when samples of whole saliva are analyzed. In addition, the two-dimensional patterns of whole and parotid saliva samples from one individual are shown to be different, and some interesting polymorphisms are found when whole saliva from different individuals is compared. This work lays the foundation for the investigation of salivary proteins in connection with oral pathology and systemic disease, and opens the way for detailed genetic studies using a sample that can be easily obtained using noninvasive collection techniques.

Materials and Methods

Sample preparation. Whole saliva was collected from both male and female adult subjects using lemon drops as a stimulus. The samples were centrifuged for 45 minutes at 35,000 x g to remove food debris and cells, and the resulting supernatants were dialyzed against 100 volumes of distilled water for 16-20 hours at 4°C. The dialyzed samples were lyophilized, reconstituted to 5% of the original sample volume using SDS-CHES [0.05 M cyclohexylaminoethane sulfonic acid, pH 9.5, 2% SDS, 1% dithiothreitol, and 10% glycerol (15)], and heated for 5 minutes at 95°C.

Parotid saliva was obtained from an adult female using lemon drops as a stimulus and a Curby cup (16) for collection from Stensen's duct. The samples were centrifuged, dialyzed, and solubilized as described for whole saliva.

Two-dimensional electrophoresis. The ISO-DALT system (6, 13, 14) was used for all two-dimensional electrophoresis. The isoelectric focusing gels were cast and run as previously described (13) using 2-11 ampholytes (Servalyt). After equilibration with a buffer containing SDS (13), the first-dimension gels were placed on polyacrylamide linear gradient gels (10-20%) also containing SDS and electrophoresed (14). Staining with Coomassie Brilliant Blue R-250, destaining, and photography were as described previously (17, 18).

Protein identification. The positions of human serum albumin and immunoglobulin light and heavy chains have been identified in the two-dimensional electrophoresis patterns of human serum (17). Therefore, identification of those serum proteins in the two-dimensional pattern of human saliva was possible by the co-electrophoresis of whole saliva and serum (1 part serum to 2 parts SDS-CHES) from the same individual. Purified salivary amylase, provided by D. D. Bayse, U. S. Department of Health, Education, and Welfare (Atlanta, Georgia), was used to identify amylase in both whole saliva and parotid saliva patterns.

Results and Discussion

Figure 1 shows the two-dimensional electrophoresis pattern of human whole saliva. After the initial centrifugation of all samples, the supernatants obtained for further analysis contained no detectable bacteria. The spots in these two-dimensional patterns, therefore, represent salivary proteins. The proteins range in molecular weight from approximately 10,000 up to 90,000 daltons, with about 40% found below 20,000 daltons. Dialysis and lyophilization were used for desalting and concentration because a loss of proteins smaller than 15,000 daltons was observed when ultrafiltration methods were used. Approximately 100 spots can be seen when Coomassie

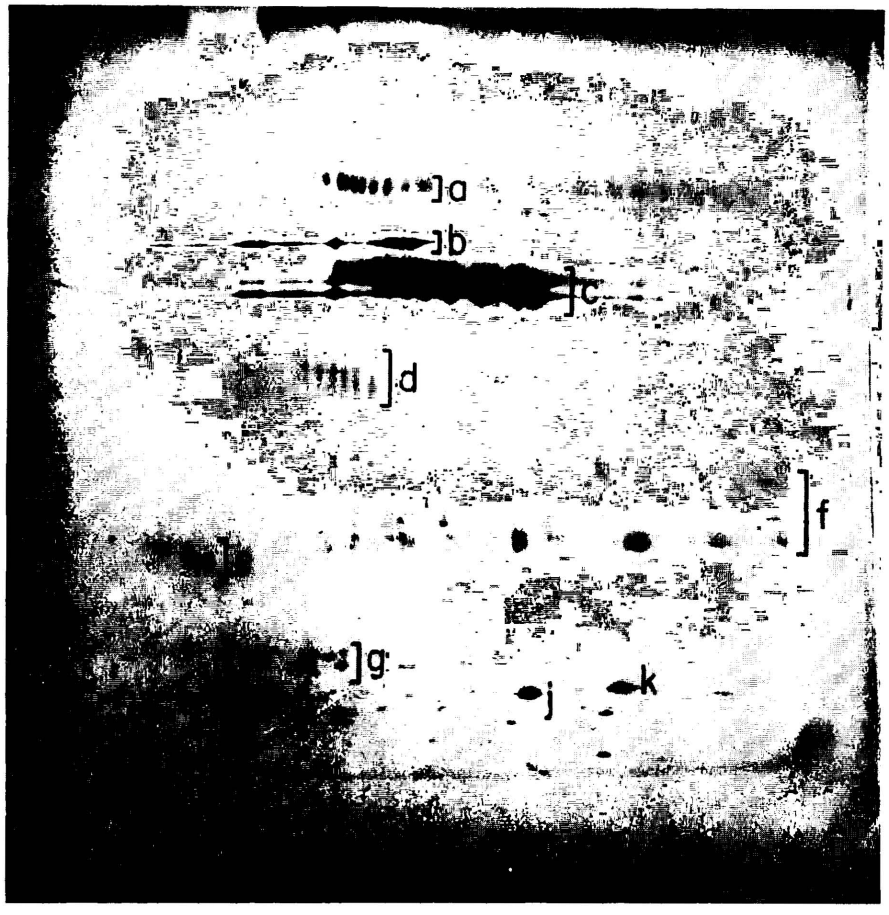

Fig. 1. Two-dimensional electrophoresis pattern of human whole saliva. The gel is oriented with the acid side to the left and basic side to the right. The letters designate the eleven "landmarks" which are characteristic of whole saliva: a, d, e, g, h, i, j, k, unidentified; b, albumin; c, amylase; f, immunoglobulin light chains. Trains of spots are bracketed to the right. The sample was obtained from a female, age 29.

Brilliant Blue staining is used, as in Figure 1, and over 120 spots have been visualized using silver staining as described by Switzer and co-workers (19). The resolution of minor protein components is limited by the high concentration of amylase in saliva samples. In addition, since the isoelectric focusing conditions used do not allow basic proteins

(pI above 8.0) to enter the first-dimension gel (15, 20), the pattern shown here does not include the basic salivary proteins. Therefore, the total number of proteins or protein subunits present in whole saliva is greater than 120. The major spots in the whole saliva pattern can be divided into eleven "landmarks" which have been found in all whole saliva samples analyed thus far. Of these spots (illustrated in Figure 1), albumin (b), salivary amylase (c), and immunoglobulin light chains (f) have been identified by co-electrophoresis with either purified proteins or human serum. In addition to these identified spots, preliminary results suggest that one group of spots (a) may be the components of IgM heavy chains. The identities of the remaining spots are as yet unknown.

In order to determine which protein components of whole saliva are products of the parotid glands, a saliva sample was collected directly from Stensen's duct and analyzed by two-dimensional electrophoresis (Figure 2). Comparison of the parotid and whole saliva patterns shows many similarities between the two as one might expect. Amylase is in greater abundance relative to other proteins (e.g. albumin), and is overloaded in the parotid saliva pattern shown in order to see some of the minor proteins. In addition to amylase and albumin, the spots thought to represent the heavy chains of IgM (a) and other "landmarks" [d, e, g, i] in the whole saliva pattern are also present in the parotid saliva pattern. On the other hand, four of the unidentified proteins (h, j, k) are conspicuously missing from the parotid saliva pattern. Three obvious "parotid" spots are present in Figure 2 but absent from Figure 1. Other similarities and differences will be found as sample preparation methods and analysis are refined. The spots found thus far that are common to both the whole and parotid saliva patterns represent proteins that may be derived from the parotid gland without further modification. Those spots present in the parotid pattern but missing from the whole saliva pattern, on the other hand, could be proteins that are modified once secreted into the mouth (11). The whole saliva spots that are missing from the parotid saliva pattern are probably products from the other salivary glands (e.g. submandibular, sublingual). One-dimensional electrophoresis has shown that each of the glands produces some unique proteins (10, 21).

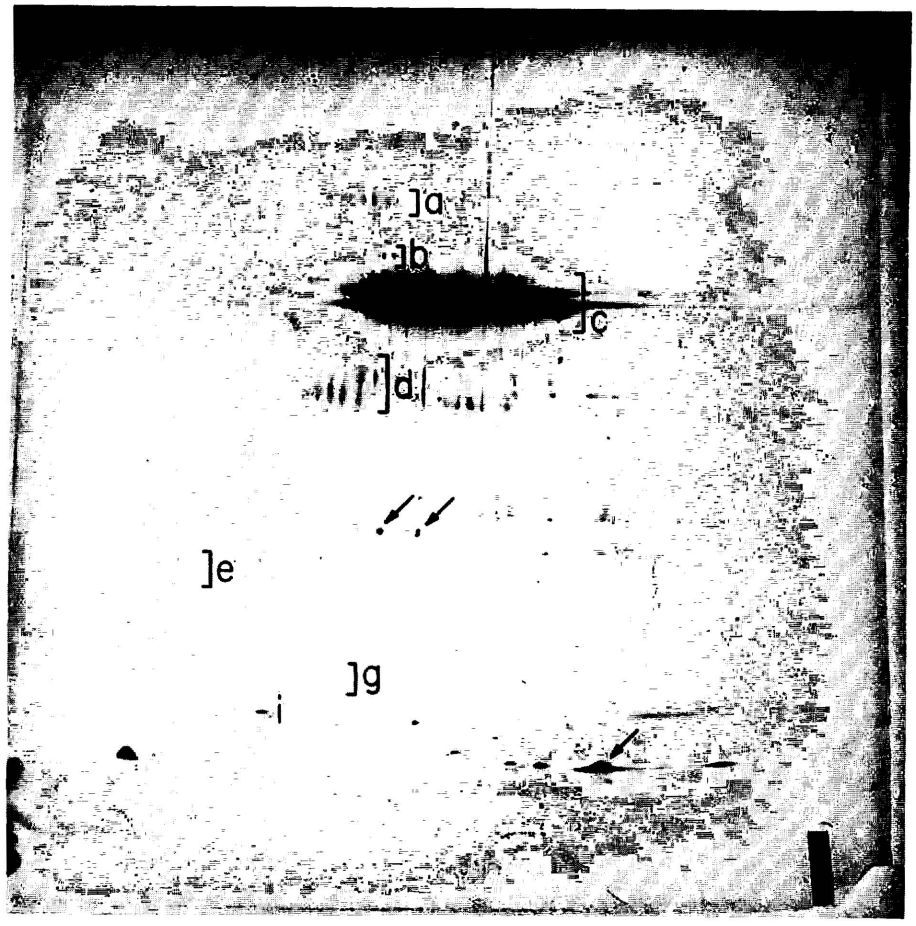

Fig. 2. Two-dimensional electrophoresis pattern of human parotid saliva. The gel orientation and labels are as noted for Figure 1. The arrows show proteins that are characteristic of the parotid sample. The sample was obtained from the same individual who donated whole saliva for the gel in Figure 1.

Amylase accounts for approximately 40% of salivary protein (22). As seen in both Figure 1 and 2, two-dimensional electrophoresis resolves amylase into a series of spots which differ in both size and charge. To confirm the identity of these spots as amylase and to show that the observed heterogeneity is a property of that protein, purified salivary amylase was run alone and together with a parotid saliva sample (Figure 3). The

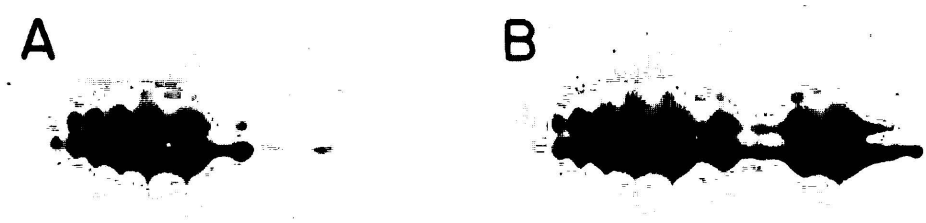

Fig. 3. Two-dimensional electrophoresis pattern of purified salivary amylase and amylase in human parotid saliva. A, Purified human salivary amylase; B, Purified human salivary amylase co-electrophoresed with parotid saliva (only amylase portion of the pattern is shown).

purified amylase was resolved into two lines of 4 major spots and 2-4 minor spots (depending on the amount of protein loaded). These spots co-electrophoresed with all but the four most basic spots assumed to be amylase in the parotid sample. There are minor spots in the purified amylase pattern which correspond to those four spots in the parotid saliva pattern, a fact that may indicate the selective loss of some protein during the amylase purification. The double row of spots represents the two salivary amylase families, the so-called "odds and evens" (23). In parotid saliva patterns, the number of spots in each row ranges from 6-10, depending on the protein loading. Karn and co-workers have described a scheme for the post-translational modification of a single amylase gene product involving both glycosylation and deamidation which may explain the electrophoretic heterogeneity characteristic of amylase (24). Evidence of *in vitro* deamidation generating new forms of amylase has also been documented (23). We investigated the possibility that *in vitro* deamidation produces some of the more acid amylase spots, but comparison of the two-dimensional patterns of samples heated or not heated were found to be identical. Samples prepared in 2% Nonidet P40 with 9 M urea and 2% β-mercaptoethanol also produced the same pattern. Therefore, we favor the model for *in vivo*

modification of the amylase molecule as an explanation of the electrophoretic heterogeneity observed. The high resolution of two-dimensional electrophoresis relative to the one-dimensional techniques used in the past confirms that amylase is a very complex protein with unique biochemical properties still to be elucidated.

Since several interesting genetic polymorphisms have been found using one-dimensional electrophoresis for salivary protein analysis (11), we were interested to find that the two-dimensional electrophoresis patterns of whole saliva from different individuals also contain polymorphisms. One example of such a polymorphism is shown in Figure 4. This group of spots (designated (g) in Figure 1) varied within a sampling of nine individuals. However, all nine samples do not have totally different patterns. Two major types of group g patterns have been observed, one consisting of three major spots (Figure 4, A-G) and the other five major spots (H-I). The five spot pattern is actually the three spot pattern with two additional more basic spots situated to the right. These differences are not sex-linked since representative individuals of both sexes were found having the three or five spot patterns. The identification of these spots and the significance of their presence or absence remains to be determined.

Conclusions

This paper shows that two-dimensional electrophoresis can resolve a minimum of 100 protein components from whole saliva and approximately 70 components from parotid saliva compared to the 30-40 proteins resolved by one-dimensional electrophoresis techniques (7-10). The high resolution of the two-dimensional electrophoresis technique is invaluable for further investigation of salivary proteins such as amylase and for the overall study of salivary proteins as related to oral health and pathology. In addition, the ISO-DALT system (6, 13, 14) opens the way for use of two-dimensional electrophoresis in genetic screening projects and even clinical diagnosis. Saliva is valuable in both efforts as a source of material requiring noninvasive collection methods which contains genetic markers and possibly useful indicators of systematic disorders.

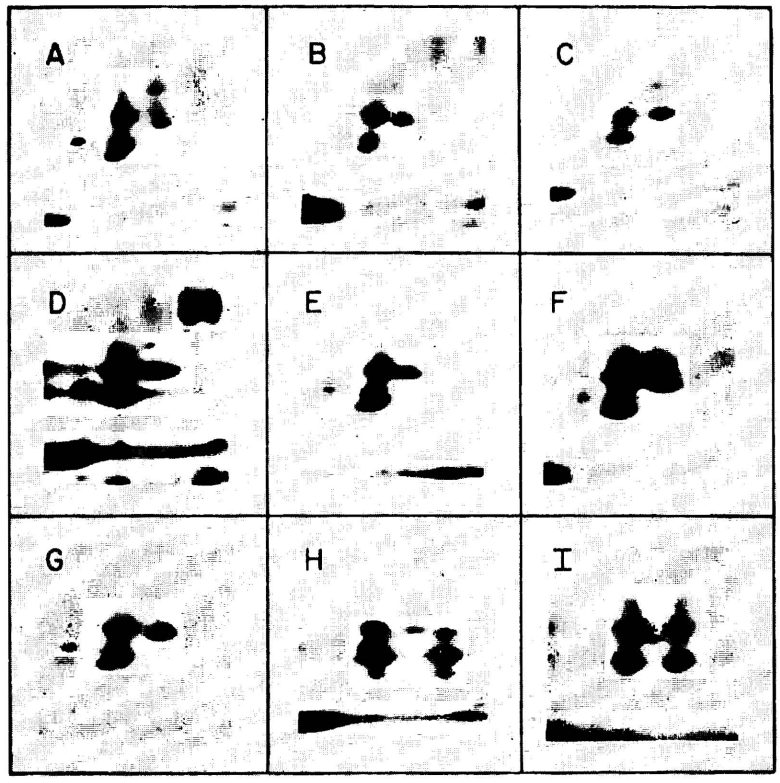

Fig. 4. Comparison of "Landmark g" in two-dimensional electrophoresis patterns of whole saliva from nine individuals. A, male, age 30; B, male, age 60; C, male, age 36; D, female, age 39; E, female, age 39; F, female, age 26; G, female, age 25; H, male, age 52; I, female, age 29.

Acknowledgments

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