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Isolation of Milligram Quantities of a Group of Histidine-Rich Polypeptides from Human Parotid Saliva

BRUCE J. MACKAY,[1] JERRY J. POLLOCK,[1]* VINCENT J. IACONO,[2] AND BRUCE J. BAUM[3]

Department of Oral Biology and Pathology[1] and Department of Periodontics,[2] State University of New York at Stony Brook, Stony Brook, New York 11794, and Clinical Investigations and Patient Care Branch, National Institute of Dental Research, Bethesda, Maryland 20205[3]

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Freshly collected parotid saliva collected from human donors were shown by polyacrylamide gel electrophoresis to continuously secrete a group of low-molecular-weight cationic polypeptides. Up to 14 bands could be identified by Coomassie blue staining, and all bands migrated more rapidly than purified human leukemic lysozyme in cationic polyacrylamide gel electrophoresis. These peptides could be isolated as a group relatively free of other salivary components and recovered in high yields from concentrated parotid saliva by Sephadex G-25 chromatography. In sodium dodecyl sulfate gel electrophoresis, the histidine-rich polypeptide bands appeared as just two bands migrating at the tracking dye and ahead of insulin chain B. Amino acid analysis of the mixture revealed an average content of at least 48% cationic residues, of which half were histidine. When stained bands were eluted from electrophoretic gels, hydrolyzed, and subjected to amino acid analyses, they were found to be enriched in histidine. There was also a correlation of the electrophoretic mobility with the content of basic amino acids. Sephadex G-25 chromatography is a convenient, simple method for preparing milligram quantities of the histidine-rich polypeptides for chemical and biochemical studies.

The exocrine secretions of the salivary glands have been shown to be complex in both organic and inorganic composition (10, 30). Parotid saliva is known to contain several unique proteins and polypeptides which include a group of basic proline-rich glycoproteins (16, 19, 21), a group of acidic proline-rich proteins (5, 7, 24), a tyrosine-rich polypeptide called statherin (12), and a group of histidine-rich polypeptides (HRPs; 2, 4, 25, 26).

Several of these proteins and glycoproteins have been purified and characterized. For example, the three phosphoproteins, statherin, proline-rich protein A, and proline-rich protein C, have all been sequenced (29, 30, 31; H. S. Belford, F. G. Oppenheim, G. D. Offner, and R. F. Troxler, J. Dent. Res. **62**:A292, 1983) and have been shown to bind calcium (5, 6, 29). The experimental evidence also indicates that these phosphoproteins inhibit the spontaneous and seeded precipitation of calcium phosphate salts (5, 14, 29). The purification of a single basic proline-rich glycoprotein and its corresponding protein core has been described by Levine and co-workers (20, 21); however, Levine and Keller (19) reported the isolation of a family of proline-rich glycoproteins from parotid saliva which differed in amino acid composition. One of these proteins (SP9) has been purified to apparent homogeneity and partially sequenced (16). Moreover, one or more of the basic proline-rich proteins seem to be structurally related to the acidic proline-rich proteins (5, 15).

Homogeneous preparations of the individual HRPs have not been obtained. Of the seven HRPs reported to be found in human parotid saliva (3, 4), only HRP 1 has been isolated in small quantities as a highly enriched preparation (4, 13, 26; B. J. MacKay, V. J. Iacono, B. J. Baum, and J. J. Pollock, J. Dent. Res. **58**:A256, 1979). Impure preparations containing HRP 1, HRPs 3 and 4, and HRPs 5 and 6 have been partially sequenced (25, 26). As the first step in studying biological function, a method was devised for the isolation of preparative amounts of the HRP essentially free of other salivary components.

MATERIALS AND METHODS

Biochemicals. Bio-Rex 70 (200 to 400 mesh) and polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-25 (medium grade) was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. An amino acid calibration kit and W-3H resin were products of Beckman Instruments, Inc., Palo Alto, Calif. Constant boiling HCl was obtained from Pierce Chemicals, Rockford, Ill. Dialysis tubing (3,500-molecular-weight cutoff) was purchased from Spectrum Medical Industries, Los Angeles, Calif. ACS-grade reagents were products of Fisher Scientific Co., Pittsburgh, Pa. Human lysozyme was isolated and purified from leukemic urine by immunoadsorption affinity chromatography (23). Enzyme concentration was determined from the extinction coefficient of $E_{1\,cm}^{1\%} = 25.65$ at 280 nm (17).

Saliva collection. Parotid saliva from healthy donors served as the source of the HRPs. Volunteers included three female and six male subjects between 20 and 42 years of age. The saliva was obtained by using modified Carlson-Crittenden collecting devices (9) and 2% citric acid or sour lemon drops as stimulants of salivary flow. To assess the effect of prolonged stimulation on the appearance of the HRPs, 50 ml of parotid saliva was collected from the right gland of a donor in successive 1-ml volumes, using a fraction collector. The salivary fractions were then analyzed by gel electrophoresis (3). From an analysis of the appearance of the HRP on gels, 50 ml was collected during a 30- to 60-min time period from each gland per sitting into screw-cap plastic centrifuge tubes chilled on ice. For initial fractionation attempts, freshly collected parotid saliva (50 to 100 ml) was applied to either ion-exchange or gel chromatography columns. For prepara-

* Corresponding author.

tive separations, salivas were acidified to pH 4.5 by dialysis for 1 h against 0.1 M acetate buffer and centrifuged (48,000 × *g*, 30 min, 4°C), and supernatants were stored in a lyophilized form at −20°C.

Cationic PAGE. Cationic polyacrylamide gel electrophoresis (PAGE) was performed with the pH 2.75 system described by Baum and co-workers (3). To enhance resolution, a modification of the Baum technique was used (B. J. MacKay, Ph.D. thesis, State University of New York at Stony Brook, 1982). Slab gels were used instead of disc gels; however, to reduce slab gel polymerization time, the concentration of ammonium persulfate in the gel mixture was increased from 0.03 to 0.06% and riboflavin was replaced with riboflavin-5′-phosphate, with the concentration being raised from 0.0005 to 0.001%. It was also necessary to prepare slabs 12 h in advance to obtain consistent resolution of salivary components; however, the design of the slab gel form made it difficult to prevent the top of prepared slabs from drying out during this period. To overcome this problem, a 9.0-cm-high separating gel was prepared and overlaid with a 1:1 mixture of butanol-gel buffer during polymerization. Slab lanes were then formed by the use of a stacking gel which was prepared 30 min in advance of each electrophoretic run. In addition, the concentration of bisacrylamide in the separating gel was doubled.

Gels were stained with Coomassie blue R250. The slab was fixed in 20% trichloroacetic acid for 1 h and then stained for 2 h at 60°C in a solution of 7% acetic acid–8% methanol containing 0.1% Coomassie blue R250. The slab was diffusion destained in aqueous 7% acetic acid–5% methanol. Slabs were also fixed and simultaneously stained with 0.04% Coomassie blue G250 in 3.5% perchloric acid (28). Coomassie blue G250 exhibits a light orange color in this acid but turns blue when bound to protein (28).

Sodium dodecyl sulfate-PAGE. The analysis of fractionated materials by sodium dodecyl sulfate-PAGE was performed by a modification of the technique described by Laemmli (18). Fifteen percent acrylamide separating gels were used, but the ratio of acrylamide/bisacrylamide was maintained at 36:1.

Cation-exchange chromatography. Cation-exchange chromatography on Bio-Rex 70 was performed by a modification of the procedure described by Baum and co-workers (4). The resin was suspended in 0.5 M KOH for 3 h, equilibrated in 0.1 M potassium phosphate buffer (pH 6.7), and then poured as a 1.6- by 35-cm column. Approximately 5 ml of packed resin was removed from the top of the column and incubated for 2 h with stirring at 4°C with 800 ml of parotid saliva which had been pooled from eight donors and adjusted to pH 6.5 with 1.0 M HCl. The resin was collected on a Büchner funnel, washed with the starting buffer, and reapplied to the top of the column. The column was eluted successively with starting buffer, 0.25 M phosphate buffer (pH 6.7), 0.3 M phosphate buffer (pH 8.0), and 2.0 M potassium phosphate dibasic (pH 9.0). Elution was carried out at room temperature at a flow rate of 30 ml/h, and 4-ml fractions were collected and monitored for absorbance at 227 and 280 nm, conductivity, and pH. Tubes were pooled, dialyzed against 0.01 M HCl with 3,500-molecular-weight cutoff tubing, and lyophilized. Pools were analyzed by cationic PAGE as described by Baum and co-workers (3).

Gel permeation chromatography. Sephadex G-25 (medium grade) was swollen in distilled water and poured as a 2.6- by 100-cm column. The column was eluted with 0.2 M NaOH containing 1 M NaCl to remove cationic contaminants bound to the gel and then was reequilibrated with distilled water. Reconstituted lyophilized parotid saliva (50 ml; 50 mg per ml of distilled water) collected from a single donor was applied to the column, which was eluted with distilled water at a flow rate of 50 ml/h. Fractions of 10 ml were collected and assayed for absorbance at 227 and 280 nm and for conductivity. Pools were analyzed by a modification of Baum's cationic PAGE procedure. Where lysozyme levels were high, saliva was first passed through an immunoadsorbent column (23) to remove and purify the enzyme before lyophilization and fractionation on Sephadex G-25. This procedure also prevented any contamination of the HRP peak with lysozyme and is now routinely used in our laboratories for HRP purification.

Amino acid analysis. The mixture of HRPs isolated by gel permeation chromatography was rerun a second time through the Sephadex G-25 column and then (1 mg, dry weight) hydrolyzed in vacuo in 6 M constant boiling HCl at 110°C for 24 h. The HCl hydrolysates were dried under nitrogen and dissolved in 0.2 M sodium citrate buffer, pH 2.2. Samples were analyzed on a Beckman 119 CL amino acid analyzer, using a 220-mm column of W-3H resin as described in Beckman Instruments 118/119 CL Application Note 001.

Isolation of the HRPs directly from gel slabs. The HRPs were extracted from cationic PAGE gels by the method of Gibson and Gracy (11). Briefly, bands were cut from the gel and homogenized (15 to 30 strokes) in a ground-glass tissue homogenizer containing 2 to 10 ml of 60% formic acid. The suspension was sedimented by centrifugation in an IEC clinical centrifuge (setting 5, 5 min), and the blue supernatant was removed. The supernatant was dried in a Büchler Evapomix or under a stream of nitrogen. The dried material was dissolved in 1 to 2 ml of 6 N HCl and extracted with 2 ml of *n*-octanol. The mixture was vortexed and phase separation was facilitated by centrifugation. The blue organic layer was removed. The extraction procedure was repeated three times. The HCl phase was hydrolyzed as described above for amino acid analyses.

RESULTS

Appearance of HRPs in stimulated parotid saliva. By using slab gels, prepared under identical conditions described for the tube gel system of Baum and co-workers (3, 4), nine protein bands were observed to migrate ahead of lysozyme (Fig. 1), and seven of these were tentatively designated as the major HRPs (HRP 1 through 7) based on previously reported studies (3, 4). Polypeptides appeared as three pairs, each consisting of a darkly and a lightly staining component and usually a seventh component migrating with the same mobility as the tracking dye. Figure 1 demonstrates that the seven major HRPs appear to stain equally well and are continuously secreted in human parotid saliva.

Modified cationic PAGE. Figure 2 illustrates that, in addition to the seven major HRPs, seven minor bands were also observed. These salivary components in the HRP region were found to stain equally well with either amido black or Coomassie blue R250, but amido black was found to leach from the stained bands more rapidly. Compared with staining with Coomassie blue R250, the HRPs were poorly stained with Coomassie blue G250, although staining differences may be due to differences in methodology (see Materials and Methods). The identification of these additional bands was aided in some cases by observing the stained slabs while they were held obliquely to a high-intensity lamp. When different subjects' salivas were examined, variations were noted in the presence and intensity of stained HRP bands, particularly the minor bands (Fig. 2). There were also

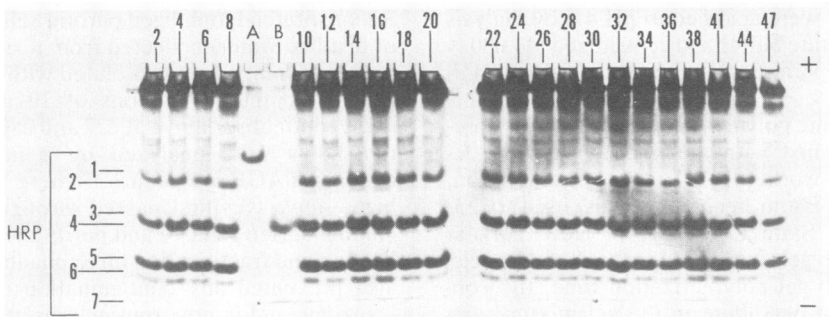

FIG. 1. Effect of prolonged stimulation on the appearance of the HRPs in human parotid saliva. Parotid saliva was collected continuously, using a fraction collector (see text). Aliquots (900 μl) of selected fractions were lyophilized and resuspended in 90 μl of distilled water. The concentrated fractions (50 μl) were subjected to cationic PAGE. The number over each lane refers to the fraction electrophoresed. A, 10 μg of purified human leukemic lysozyme; B, 10 μg of reference HRP. Cathode is at the bottom.

differences in the staining of the protein area corresponding to lysozyme (Fig. 2) which we have confirmed by catalytic assays (data not shown) to represent true differences in lysozyme concentration.

Cation-exchange chromatography fractionation of parotid saliva. The HRPs have been previously reported to bind avidly to Bio-Rex 70, a weak cation-exchange resin (2, 4). To assess the potential of this technique for the purification of preparative amounts of the HRP, as a group or as individual components, freshly collected parotid saliva was adsorbed to and fractionated on this resin. Elution of the column with buffers of different pH and ionic strength yielded six major UV-absorbing peaks (Fig. 3). The salivary components contained in the first peak, which was eluted with 0.25 M phosphate buffer (pH 6.7), were not identified, but the second peak, which was also eluted by this buffer, contained lysozyme activity (data not shown). The third and fourth peaks, containing a large amount of 227-nm-absorbing material, were eluted by 0.3 M phosphate buffer (pH 8.0), whereas two additional peaks of 227-nm-absorbing material were obtained by elution of the column with 2.0 M dibasic potassium phosphate (pH 9.0). When pooled fractions were assayed by cationic PAGE, using the system described by Baum and co-workers (3), the third peak was found to contain an enriched preparation of the slowest migrating component in the HRP region (Table 1). This component was tentatively identified as HRP 1 based on electrophoretic mobility relative to a mixture of purified reference HRP components containing mostly HRP 3. The fourth, fifth, and sixth peaks were divided into four pools which were found to contain varying amounts of different HRP components when analyzed by cationic PAGE (Table 1). A subsequent study indicated that significant amounts of 227-nm-absorbing material could also be eluted from the cation-exchange resin by replacing the last two buffers with 0.3 M phosphate buffer (pH 8.6).

Isolation of HRPs by gel filtration. In an effort to develop an alternative procedure for isolation of the HRPs from parotid saliva, gel filtration was investigated. Since the mobility of the salivary components in the HRP region of cationic polyacrylamide gels suggested that these constituents were basic and had low molecular weights, parotid saliva was fractionated on Sephadex G-25. Figure 4 shows the column profile resulting from the fractionation of 50 ml of reconstituted parotid saliva containing 50 mg of salivary solids per ml in distilled water. The fractions were divided into six pools (Fig. 4) and analyzed in the modified cationic PAGE system (Fig. 5). The majority of the HRP components were obtained in pool C, although only small amounts of HRPs 1 and 2 were present (cf. lanes 11, 12, and 13). Pool C appeared to consist mainly of HRPs 3 and 5 with some HRP 4 and 6 (see lane 14, Fig. 5). Pools D and E were also shown to contain the mixture of the HRP components (lanes 7 and 8), although pool E, interestingly, contained HRPs 6a, b, c and 7 (lane 8, Fig. 5). The Sephadex G-25 fractionation, therefore, permitted the isolation of the HRP relatively free of other salivary components (note intentional overload of gels in lanes 6 and 11, Fig. 5). This was confirmed by sodium dodecyl sulfate-PAGE of pool C material (Fig. 6) which showed two major bands (migrating more rapidly than insulin chain B) and some minor components. Refractionation on Sephadex G-25 yielded highly purified HRP components (data not shown). The HRPs contained in pool C were

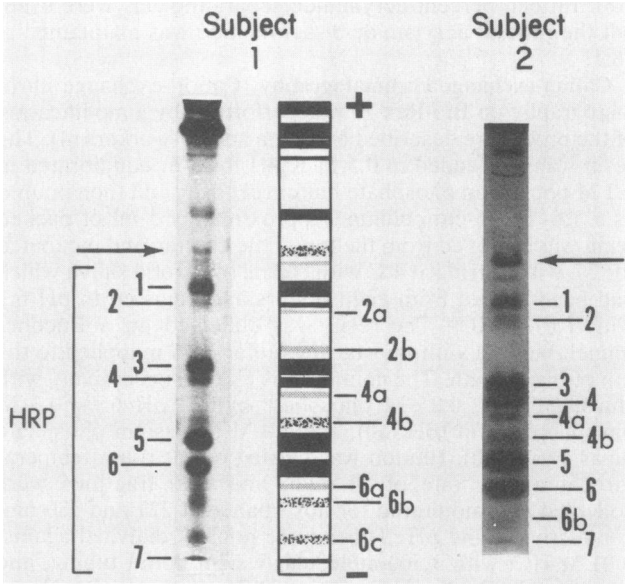

FIG. 2. Appearance of the major and minor bands of the HRPs in parotid saliva by cationic PAGE. The modified cationic PAGE system was used (see text). Subject 1, Reconstituted parotid saliva (50 μl, 10× concentrated) and corresponding schematic. Subject 2, Reconstituted parotid saliva (50 μl, 10× concentrated). Arrow indicates the migration of human leukemic lysozyme. Cathode is at the bottom.

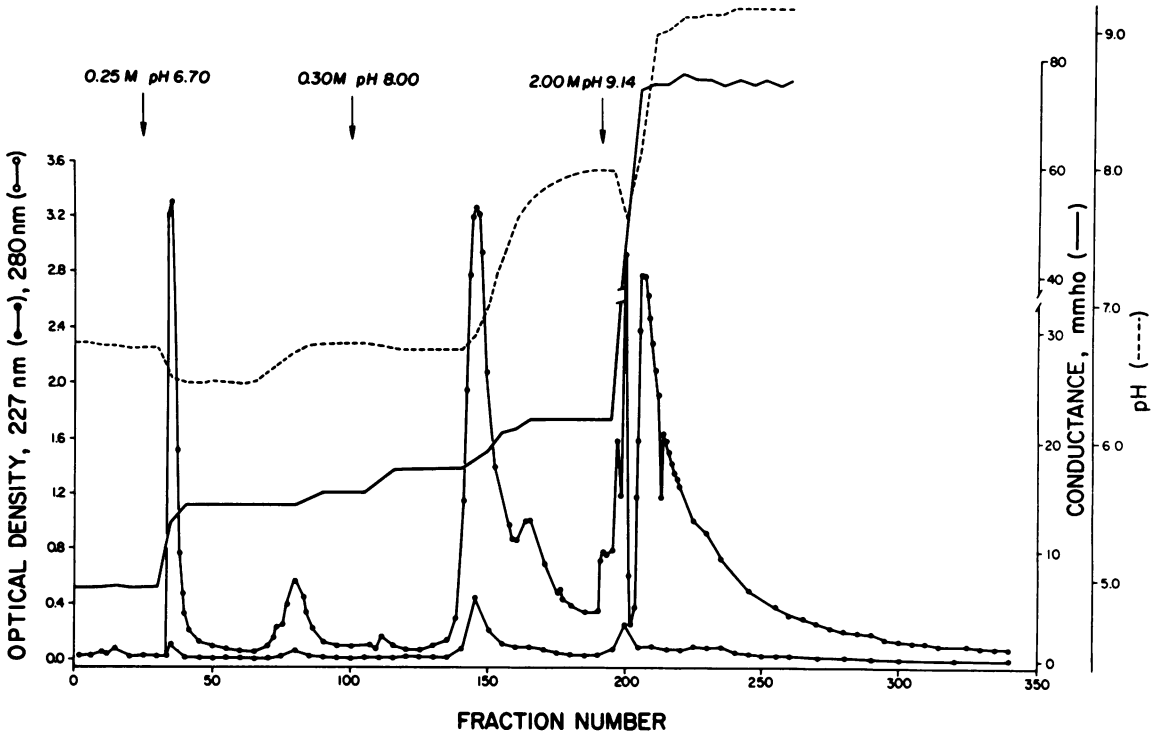

FIG. 3. Fractionation of the HRPs by cation-exchange chromatography. Freshly collected parotid saliva was adsorbed to and fractionated on Bio-Rex 70 (see text). Symbols: ●, 227-nm absorbance; ○, 280-nm absorbance; ——, conductivity; ----, pH.

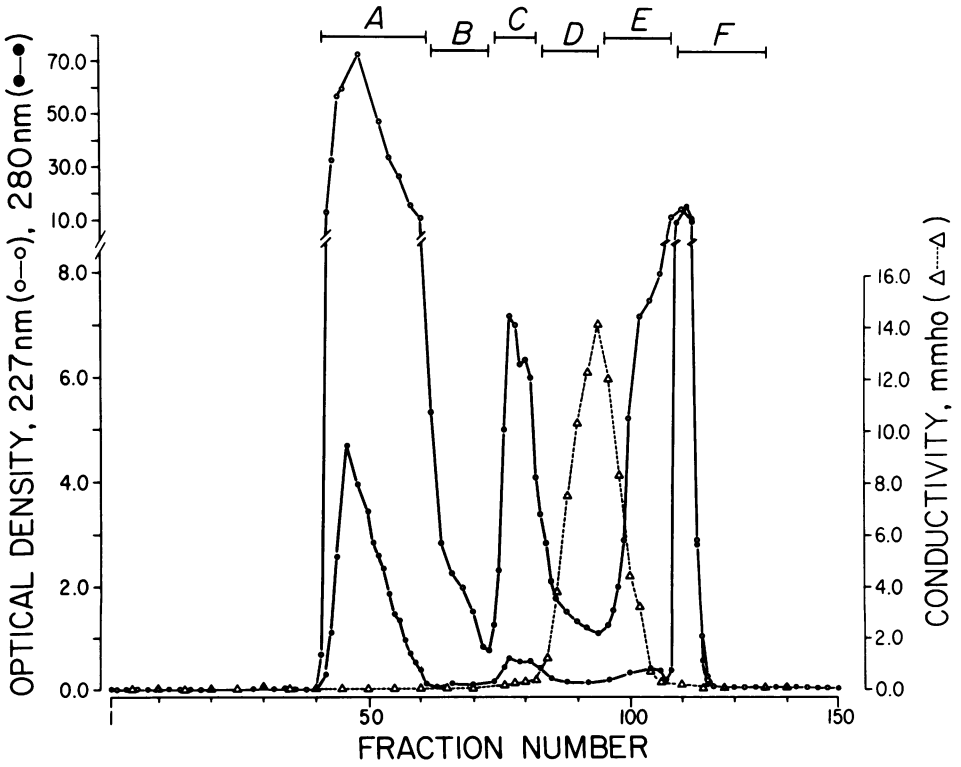

FIG. 4. Isolation of the HRPs from concentrated parotid saliva by gel filtration. Parotid saliva (50 ml; 50 mg of lyophilized solids per ml of distilled water) was fractionated in water on a column of Sephadex G-25. Symbols: ○, 227-nm absorbance; ●, 280-nm absorbance; △, conductivity.

TABLE 1. Identification of the HRP components isolated by Bio-Rex 70 chromatography

Fraction no.[a]	HRP identified[b]	
	Major[c]	Minor[c]
139–160	1	4, 7
161–170	1, 3, 4, 7	5
171–202	1, 2, 3, 4, 5, 7	
203–240	3, 5, 6	7
241–340	3, 5, 6	7

[a] See Fig. 3 for Bio-Rex 70 column profile.
[b] Identification was based on cationic PAGE according to Baum and co-workers (3).
[c] Visual assessment based on staining of bands with Coomassie blue R250.

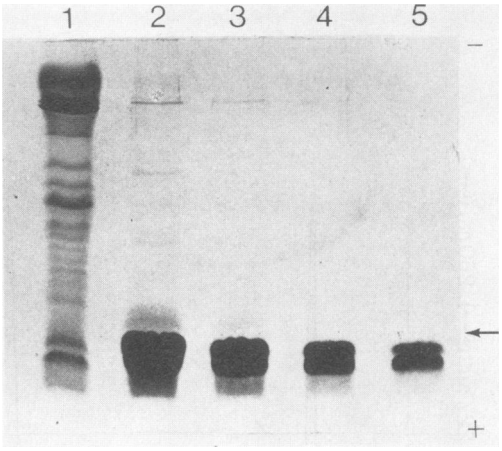

FIG. 6. Sodium dodecyl sulfate of Sephadex-PAGE G-25 fractionated HRPs. (Lane 1) Reconstituted parotid saliva (50 μl; 50 mg/ml); (lane 2) Sephadex G-25 pool C (40 μl; 5 mg/ml); (lane 3) pool C (20 μl; 5 mg/ml); (lane 4) pool C (10 μl, 5 mg/ml); (lane 5) pool C (5 μl; 5 mg/ml).

calculated to represent 6 mg (dry weight) of the total protein per 100 ml and peptides of stimulated parotid saliva (actually pools A plus B plus C). The yield from 2.5 g of lyophilized parotid saliva was approximately 50 mg of purified HRP components. Quantities of 35 to 75 mg of HRP have been recovered from the parotid salivas of different donors (data not shown).

Partial characterization of the HRP mixture isolated by Sephadex G-25 chromatography. Amino acid analysis showed that pool C or rechromatographed pool C contained 47.7% basic amino acids (excluding possible contributions by asparagine and glutamine) and 24.1% histidine, confirming that the salivary constituent mixture present in this fraction was HRPs (Table 2). Pool B was found to contain 11.2% basic residues and 1.5% histidine. However, these constituents were found to contain 41.6% proline, suggesting that they were not histidine-rich salivary components but consisted of one or more of the previously described proline-rich proteins (16) which in our fractionation system concentrated in this region of the Sephadex column (Fig. 4).

When the components of pool C were isolated directly from the cationic gels and then hydrolyzed and characterized by amino acid analysis, each analysis of the bands revealed the presence of a high percentage of histidine (Table 3). Values of histidine ranged from 14 to about 35% (Table 3), and the number of cationic residues was highest in HRP 7 and lowest in HRPs 1 and 2 (data not shown). The complete amino acid analyses together with the purification of individual components by high-performance liquid chromatography will be presented elsewhere (MacKay et al., unpublished data).

DISCUSSION

Human parotid saliva has previously been shown to contain a group of apparently small macromolecules which migrate more cathodally than lysozyme in cationic PAGE systems (2, 8). Balekjian and Longton (2) isolated these components from parotid saliva by cation-exchange chromatography and showed that, as a group, they were rich in basic amino acids. In addition, the group of HRPs was reported to have an unusually high content of histidine. Since these pioneering studies, several laboratories have turned their attention to the HRPs (4, 25, 26), but as yet a

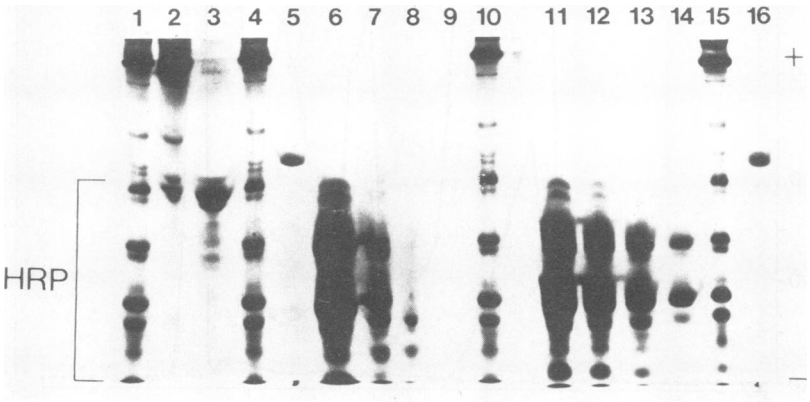

FIG. 5. Cationic PAGE of concentrated parotid saliva gel filtration fractions. Samples were analyzed by the modified cationic PAGE system (see text). (Lanes 1, 4, 10, and 15) Reconstituted parotid saliva (50 μl; 50 mg/ml); (lane 2) Sephadex G-25 (see Fig. 4), pool A (50 μl; 25 mg of lyophilized solids per ml); (lane 3) pool B (50 μl; 10 mg/ml); (lanes 5 and 16) 10 μg of purified human parotid lysozyme; (lanes 6 and 11) pool C (50 μl; 10 mg/ml); (lane 7) pool D (50 μl; 10 mg/ml); (lane 8) pool E (50 μl; 10 mg/ml); (lane 9) pool F (50 μl; 10 mg/ml); (lane 12) pool C (30 μl; 10 mg/ml); (lane 13) pool C (20 μl; 10 mg/ml); (lane 14) pool C (10 μl; 10 mg/ml). Cathode is at the bottom.

biological role(s) for these salivary components has not been established (1).

To determine the biological function(s) of these salivary constituents, it was apparent that isolation and purification of milligram quantities of the HRPs was required. Initially, parotid saliva was therefore examined by cationic PAGE to determine the number of components present in the HRP region. Using the gel electrophoretic technique described by Baum and co-workers (3), we observed that seven major HRPs were continuously secreted in parotid saliva. These results are similar to those reported for a group of parotid proline-rich basic proteins which were also shown to be present in continuously stimulated parotid saliva (16). By modification of the PAGE system, we have been able to visualize as many as 14 polypeptide bands, although the amounts and concentrations of the bands seem to vary with the parotid salivas of different individuals.

Similar to the results of previous investigators, we have found that the HRPs can be absorbed and subsequently eluted from the cation-exchange resin Bio-Rex 70. The most slowly migrating HRP component in gel electrophoresis was recovered in an essentially purified form in agreement with the work of Baum and co-workers (4), whereas the faster migrating components of this region eluted off the column as mixtures of several HRPs. Further processing of these fractions was difficult due to the basic nature and low molecular weight of these components. The high phosphate content of eluting buffers required utilization of a dialysis or ultrafiltration step which resulted in poor recoveries of the HRP.

As an alternative to ion-exchange chromatography, gel filtration was attempted to prepare an enriched preparation of the HRPs which would be free of other salivary components. Several investigators had already shown that chromatography on Bio-Gel yields fractions containing the HRPs (13, 25). When either freshly collected (data not shown) or concentrated parotid saliva was fractionated on Sephadex G-25, a small 280-nm absorption peak which contained a significant amount of 227-nm-absorbing material eluted from the column immediately after the voiding peak and before the salt peak. Analysis of the included peak indicated a highly enriched fraction of the HRP which contained at least 48% basic residues, of which approximately half were histidine. This enriched fraction contained not only the major HRP but also the minor components which migrated electrophoretically in this region. Sodium dodecyl sulfate-PAGE analysis of this fraction revealed contamination with minor amounts of what appeared to be other salivary components. A second fractionation on Sephadex G-25 eliminated these contaminants (data not shown). The HRP mixture could be lyophilized without further treatment or concentrated with an Amicon UM05 membrane.

Since it would be important to establish that each component of the mixture of polypeptides was histidine rich for purposes of drawing conclusions from the results of biological studies, each Coomassie blue-staining band was cut out and eluted from the gel and then hydrolyzed for amino acid analysis. The results obtained suggested that individual components of the mixture were histidine rich, which has been confirmed after partial purification of these components by high-performance liquid chromatography (MacKay, Ph.D. thesis, 1982; MacKay et al., unpublished data). The antibacterial and antifungal properties of these HRPs are discussed in the accompanying two papers (22, 27).

TABLE 2. Amino acid composition of the HRPs isolated by Sephadex G-25 chromatography

Amino acid	Residues per 100 residues	
	Reported analysis[a]	Present analysis, pool C[b]
Aspartic acid	12.9	8.55
Threonine	tr	0
Serine	8.3	8.63
Glutamic acid	8.7	7.84
Proline	2.4	2.46
Glycine	8.9	8.32
Alanine	0.5	3.41
Half-cystine	0	0
Valine	tr	0
Methionine	tr	0
Isoleucine	tr	0
Leucine	2.6	1.09
Tyrosine	12.0	8.83
Phenylalanine	7.6	3.16
Tryptophan	ND[c]	ND
Lysine	8.0	13.25
Histidine	17.2	24.07
Arginine	10.1	10.39

[a] According to Baum and co-workers (4). From 24-h 6 N HCl hydrolysate of HRP 1 purified from human parotid saliva.

[b] See Fig. 4 for details of the Sephadex G-25 chromatography. Each amino acid is the mean of two determinations.

[c] ND, Not determined.

TABLE 3. Histidine content of the human parotid HRPs isolated by cationic PAGE

Polypeptide	% of Histidine[a]
HRP 1	17.8[b]
HRP 2	13.8
HRP 3	24.2
HRP 4	25.2
HRP 5	33.7
HRP 6	35.0
HRP 6a, 6b, 6c	26.5
HRP 7	26.8

[a] Based on the number of residues per 100 residues from 24-h 6 N HCl hydrolysates of isolated HRPs. Results express the mean of two determinations.

[b] All percentage values are calculated on the total number of amino acid residues except for glycine. Control polyacrylamide when electrophoresed and extracted by the method of Gibson and Gracy (11) yields amino acid analyses values of glycine which significantly exceed the expected value listed in Table 2.

ACKNOWLEDGMENT

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