Phosphopeptides derived from human salivary acidic proline-rich proteins

Biological activities and concentration in saliva

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Human saliva contains a large number of phosphopeptides derived by cleavage of acidic proline-rich proteins (APRPs). These peptides were purified by column chromatography and they constituted 0.5 % of APRPs in parotid saliva, but 11 % of APRPs in saliva expectorated from the mouth (whole saliva), indicating that there is considerable cleavage of APRPs after secretion from the gland. Similarly to APRP, the phosphopeptides bind Ca^{2+}, but they accounted for only 4 % of protein-bound Ca^{2+} in whole saliva. APRPs as well as the phosphopeptides inhibited formation of hydroxyapatite, but, whereas 19–20 μg of APRP was needed for 50 % inhibition, only 0.7–3.3 μg of purified peptides was needed for the same degree of activity, and the phosphopeptides accounted for 18 % of total inhibitory activity in whole saliva. All phosphopeptides adsorbed on hydroxyapatite in vitro, and adsorption of phosphopeptides on tooth surfaces in vivo could also be demonstrated, indicating that they would be able to inhibit unwanted mineral formation on the tooth surface in vivo. Degradation of APRPs after secretion therefore does not lead to a loss of their biological activities.

INTRODUCTION

Human saliva contains a family of proline-rich proteins (PRPs) that on the basis of charge and structural properties can be subdivided into acidic proline-rich proteins (APRPs), basic proline-rich proteins (BPRPs) and glycosylated proline-rich proteins (GPRPs) (for review see Bennick, 1989). All three types, each of which comprise several proteins, are present in parotid saliva, where they account for 70 % of total protein (Kauffman & Keller, 1979), and some PRPs are also present in sub-mandibular–sublingual secretion (Robinson et al., 1989). The isoforms of APRP include PRP1 (proline-rich protein 1), PRP2 (proline-rich protein 2), PIFs (parotid isoelectric-focusing variant, slow), Dbs (double band, slow) and Pa (parotid acidic protein), and each of these except Pa gives rise to smaller PRPs named PRP3, PRP4, PIFf and Dbf respectively by a post-ribosomal cleavage whereby a C-terminal 44-residue fragment is removed. This fragment corresponds to the only BPRP in submandibular–sublingual saliva (Robinson et al., 1989). All APRPs share a common N-terminal region, which has many acidic amino acids, including two phosphoserine residues, and this region of the proteins varies only in the residues at positions 4 and 50, which can be either aspartic acid or asparagine (Hay et al., 1988), and in the presence of either isoleucine or leucine at position 26 (Azen et al., 1987).

Various functions have been associated with APRPs, including binding of Ca^{2+} with a strength suggesting that they may regulate the concentration of ionic Ca^{2+} in saliva (Bennick et al., 1981). They also inhibit formation of hydroxyapatite (Moreno et al., 1979) and bind strongly to dental enamel (Hay, 1967), forming part of the so-called dental pellicle (Kousvelari et al., 1980). These activities, which indicate that the proteins may be important for mineral homoeostasis in the mouth, are all located in an N-terminal 30-residue tryptic fragment called peptide TX. Indeed, TX is more active with regard to Ca^{2+} binding and inhibition of hydroxyapatite formation than the protein from which it is derived (Hay et al., 1979; Bennick et al., 1981). It is therefore tempting to speculate that an N-terminal fragment similar to the TX peptide represents the final active product of APRP synthesis.

Interestingly, APRP adsorbed on hydroxyapatite can mediate attachment of some oral micro-organisms, such as Actinomyces viscosus, which is prominent in human plaque. Plaque is the accumulation of micro-organisms on the tooth surface, and is the immediate cause of dental caries, gingivitis and periodontal disease. A. viscosus, which has been associated with gingivitis and root caries, binds to the C-terminal region of APRP (Gibbons et al., 1988), and a cleavage of APRP that would remove the hydroxyapatite-binding N-terminal phosphorylated region would be expected to destroy the ability of APRP to mediate binding of A. viscosus to the dental enamel surface, thereby affecting colonization of teeth by this micro-organism.

In a previous publication (Minaguchi et al., 1988) we have demonstrated that whole saliva, i.e. saliva that is collected by expectoration from the mouth, contains a large number of different phosphopeptides derived from the N-terminal region of APRPs, and many of these peptides are formed by cleavage of the proteins after secretion into the mouth. Moreover work by Hay & Gron (1977) suggests that post-secretory cleavages of APRPs do not necessarily lead to loss of ability to inhibit hydroxyapatite formation. To evaluate the importance of post-synthetic cleavages of APRPs, we have therefore determined the amount and biological activities of APRP-derived phospho-peptides in parotid saliva and whole saliva.

EXPERIMENTAL

Materials

Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories (Missisauga, Ont., Canada), and Stains-All and pancreatric-trypsin inhibitor were from Sigma Chemical Co. (St.

Abbreviations used: PRP, proline-rich protein; APRP, acidic proline-rich protein; BPRP, basic proline-rich protein; GPRP, glycosylated proline-rich protein.
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Louis, MO, U.S.A.). Mono Q HR 10/10 column, Q-Sepharose and AH-Sepharose 4B were obtained from Pharmacia (Dorval, P.Q., Canada, and dialysis tubing (M_r cut-off 1000) was from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). $^{45}CaCl_2$ was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), and ethylene oxide was from Eastman–Kodak Co. (Rochester, NY, U.S.A.). Hydroxyapatite was a gift from Dr. E. Moreno (Forsyth Dental Centre, Boston, MA, U.S.A.).

Collection of whole saliva and parotid saliva, dialysis of saliva and determination of protein concentration

Saliva was collected by expectoration as described by Mina-guchi et al. (1988) into tubes kept on ice. Within 2 min of collection, the tubes, containing approx. 4 ml, were placed in a 100 °C water bath for 10 min to inactivate proteinases. In preliminary experiments it was found that this treatment did not generate any additional phosphopeptides. The tubes were then cooled in ice and centrifuged at 9000 g for 10 min, and the supernatant, which is referred to below as whole saliva, was removed.

Parotid saliva was collected at least 1 h after a meal as described by Bennick & Connell (1971), except that the collection vessel contained 1.0 mg of pancreatic trypsin inhibitor/100 ml of saliva. A solution of 0.2 M-EDTA, pH 7.2, was added to parotid saliva or heat-inactivated whole saliva to a final concentration of 10 mM (Bennick & Connell, 1971), and the samples were dialysed exhaustively against water and freeze-dried. To test whether small peptides were retained inside the bag, the diffusate was concentrated and subjected to gel electrophoresis on a 35% acrylamide gel (Minaguchi et al., 1988). No peptides were detected in the diffusate.

The amount of APRP in whole saliva was determined by single radial immunodiffusion (Bennick & Cannon, 1978) and crossed immunoelectrophoresis (Bennick et al., 1983). All protein concentrations were assessed from A_{220} assuming $A^{0.1\%}$ 1.0 as previously determined for APRPs (Bennick et al., 1981).

Column chromatography

Whole saliva. A 30 ml sample of freeze-dried whole saliva was redissolved in 2 ml of 0.25 M-NH_4HCO_3 and loaded on a Q-Sepharose HR 10/10 column (8 ml bed volume). The column was equilibrated in 0.25 M-NH_4HCO_3 and developed at a flow rate of 0.5 ml/min with a two-step linear gradient of NH_4HCO_3: step 1, 0.25–0.59 M (total volume 12 ml); step 2, 0.59–1.0 M (total volume 78 ml). Further purification of 11.5 mg of one of the Q-Sepharose fractions was carried out on a Mono Q anion-exchange column (8 ml bed volume). The column was equilibrated in 0.25 M-NH_4HCO_3 and developed with a three-step linear gradient of NH_4HCO_3: step 1, 0.25–0.45 M (volume 12 ml); step 2, 0.45–0.67 M (volume 68 ml); step 3, 0.67–1.0 M (volume 10 ml). The columns were monitored by measuring A_{220}, A_{224} or A_{230}, depending on the concentration of the eluate.

Parotid saliva. Freeze-dried parotid saliva (60 ml) was re-dissolved in 8 ml of 10 mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl and applied to an AH-Sepharose 4B column (25 ml bed volume) to which had been coupled antibodies to APRP (Minaguchi et al., 1988). Bound protein was eluted with 0.1 M-formic acid and freeze-dried. A 50 mg sample of bound protein was dissolved in 4 ml of 0.25 M-NH_4HCO_3 and loaded on a Mono Q column (8 ml bed volume). The column was equilibrated in 0.25 M-NH_4HCO_3 and developed at a flow rate of 0.5 ml/min with a linear gradient (80 ml) of the same salt ranging from 0.25 M to 1.0 M. Further fractionation was obtained on the same column with a stepwise linear gradient of NH_4HCO_3 from 0.25 M to 0.51 M (7.5 ml), from 0.51 M to 0.74 M (70 ml) and from 0.74 M to 1.0 M (7.5 ml) at a flow rate of 0.5 ml/min.

PAGE

Electrophoresis on 35% gels was done as described by Minaguchi et al. (1988). After staining with Stains-All (Green et al., 1973), which under these conditions only reveals phospho-peptides (Minaguchi et al., 1988), some of the gels were also stained with Coomassie Brilliant Blue R250 as described by Smith et al. (1975) to evaluate further the presence of proteins and peptides.

To analyse individual peptides, sections of the gels containing a single peptide were cut out, crushed in 3 ml of 50 mM-NH_4HCO_3 and incubated at 22 °C for 24 h before removal of the super-natant. This procedure was repeated twice. To obtain sufficient material the peptide was applied in nine or ten lanes. The supernatants were combined, dialysed, passed through a Milli-pore filter and freeze-dried. For each peptide the same procedure was performed on a blank piece of the same weight and from the same region of the stained gel. Preliminary experiments demon-strated that higher recovery was obtained by this method than by electro-elution.

Ca^{2+} binding

Ca^{2+} binding to protein was studied by equilibrium dialysis as described by Bennick (1976), except for the use of dialysis bags with a nominal retention of molecules with M_r 1000 or larger. The free Ca^{2+} concentration was 1 mM, since preliminary experi-ments had shown that at this concentration proteins and peptides from whole saliva were saturated with Ca^{2+}. This is also similar to the concentration of total calcium in whole saliva as estimated by Gron (1973). Two buffers were used, 5 mM-Tris/HCl buffer, pH 7.5, and 5 mM-Tris/HCl buffer, pH 7.5, containing 41 mM-NaCl. The ionic strength of the latter buffer is similar to that of whole saliva (Gron, 1973).

Adsorption of peptides from whole saliva on hydroxyapatite

Freeze-dried saliva was redissolved in 10 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl. To each tube containing 3 mg of hydroxyapatite was added 3 ml of 4-fold-diluted, 2-fold-diluted, undiluted or 5-fold-concentrated saliva. The tubes were incubated at 37 °C for 30 min and centrifuged at 3000 g for 6 min, and the supernatants were removed. The pellets were each washed twice in 2 ml of buffer and the supernatants were combined. Each pellet was dissolved by incubation in 0.2 M-EDTA, pH 7.1, at 37 °C for 1 h. The supernatants and dissolved pellets were dialysed extensively against 50 mM-NH_4HCO_3 and freeze-dried.

Adsorption of peptides from whole saliva on enamel fragments

Enamel fragments showing no signs of dental decay were cut from extracted human teeth and sterilized in an atmosphere saturated with 1% (v/v) ethylene oxide for at least 4 h. Cut surfaces were covered with nail varnish, and extraneous proteins that had adsorbed on the enamel surface before extraction (old pellicle) were removed as described previously (Bennick et al., 1983) by a single extraction with 0.32 M-HCl. The enamel fragments were then divided into four sets: two sets were mounted on an appliance that covered the palate of the volunteer and it was worn in the mouth for periods of 15 or 60 min. The proteins adsorbed on the fragments (new pellicle) were extracted as described previously (Bennick et al., 1983). The other two sets were extracted without exposure in the mouth. The extracts were exhaustively dialysed and freeze-dried.

Inhibition of hydroxyapatite formation

The ability of whole saliva and parotid saliva as well as fractions isolated from these secretions to inhibit the seeded

precipitation of hydroxyapatite on the surface of calcium mono-hydrogen phosphate dihydrate $(CaHPO_4,2H_2O)$ crystals was studied as described by Hay *et al.* (1979). The phosphate concentration in the supernatant of the suspension of calcium monohydrogen phosphate dihydrate crystals, determined by the method of Lowry & Lopez (1946), was used as a measure of inhibition. The initial phosphate concentration was 2.5 mM as a result of dissolution of calcium monohydrogen phosphate to the point of saturation. It increased to 6.5 mM after 10–20 h because of hydrolysis of calcium monohydrogen phosphate and formation of the more basic hydroxyapatite. If precipitation is inhibited the concentration of phosphate in solution remains at 2.5 mM. Control samples included buffer without protein and samples with added 0.001 μM-polyaspartate, which causes 100 % inhibition (Hay *et al.*, 1979). To compare the activities in different samples, the amount of protein or peptide necessary for 50 % inhibition was calculated.

RESULTS

Separation of whole saliva on Q-Sepharose resulted in four fractions. WFr1, WQ2, WQ3 and WQ4 (Fig. 1). WFr1 was further purified on a Mono Q column (Fig. 2) and, on the basis of the gel-electrophoretic profile, tubes containing only phospho-peptides were pooled (WQ1). Gel electrophoresis (Fig. 3) demon-strated that the starting material, whole saliva (lane 3), contained a large number of peptides, of which the most prominent had the same or lower mobility than that of a mixture of PRP3 and PIFf $(M_r\ 11000)$ shown in lane 1. Fractions WQ1, WQ2, WQ3 and WQ4 (lanes 4–7 respectively) all showed many bands, but, except for a few in WQ1, they all had higher mobilities than APRP and probably a wide range of M_r values smaller than 11000, since their mobilities ranged from that of the illustrated APRPs to less than that of the 3500-M_r TX fragments shown in lane 8. The peptides labelled with lower-case letters in Fig. 3 were further purified by gel elution. All proteins and peptides stained blue with Stains-All, indicating that they contained phosphate (Minaguchi *et al.*, 1988), and further staining of the gel with Coomassie Brilliant Blue did not reveal any additional bands in WQ1, WQ2, WQ3 and WQ4, demonstrating the absence of non-phosphorylated proteins and peptides.

The parotid-saliva protein that bound to the immunosorbent column was further fractionated on a Mono Q column (Fig. 4). Although most of the protein was eluted with 0.4–0.6 M salt, several small peaks, which only contained phosphopeptides, as
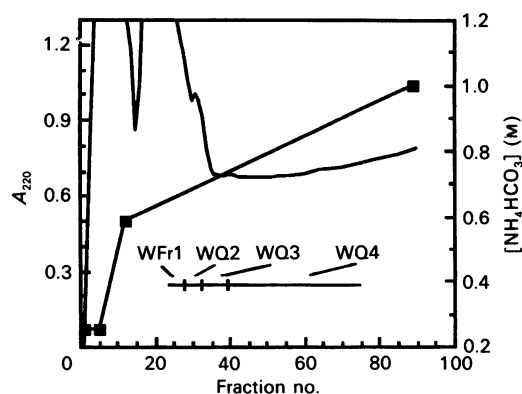

Fig. 2. Fractionation of WFr1 on Mono Q

The column was monitored by measuring A_{230} (———). The gradient is also shown (■—■). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min. Tubes were pooled to give fraction WQ1 as indicated by the horizontal bar.

shown by gel electrophoresis, were eluted at higher salt concen-trations. These peaks were pooled (PQ1) and subjected to further chromatography on a Mono Q column (Fig. 5), whereby the fractions labelled PQ2-1, PQ2-2, PQ2-3, PQ2-4, PQ2-5 and PQ2-6 were obtained. PAGE (Fig. 6) demonstrated that the starting material, parotid saliva, only contained a few fast-migrating bands (lane 1), but it is difficult to illustrate the presence of additional minor components, since the gel in that case would be overloaded with proteins that have mobilities similar to the APRPs shown in lane 11. A large number of bands
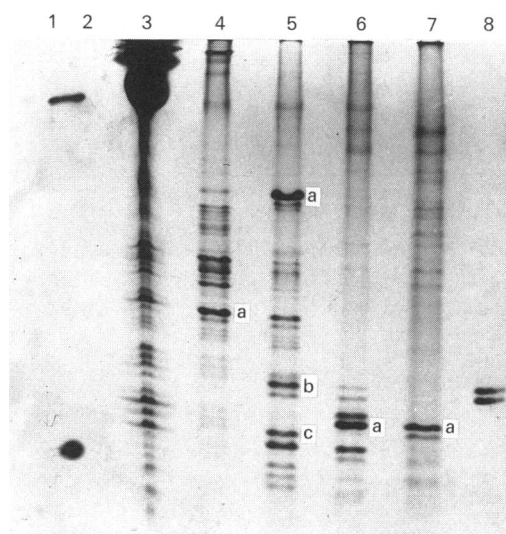
Fig. 3. Electrophoresis of fractions from Q-Sepharose and Mono Q columns

The 35 % gel was stained with Stains-All. Lane 1, mixture of PRP3 and PIFf, M_r 11000 (4 μg); lane 2, Bromophenol Blue dye; lane 3, whole saliva (1 ml); lane 4, WQ1 (48 μg); lane 5, WQ2 (62 μg); lane 6, WQ3 (100 μg); lane 7, WQ4 (74 μg); lane 8, mixture of two *N*-terminal TX peptides, M_r 3500, obtained from PRP3 and PIFf (2 μg). All the bands stained blue. The bands labelled with a lower-case letter were further purified by gel elution.

Fig. 1. Fractionation of whole saliva on a Q-Sepharose column

The column was monitored by measuring A_{220} (———). The gradient is also shown (■—■). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min and pooled to give WFr1, WQ2, WQ3 and WQ4 as shown.
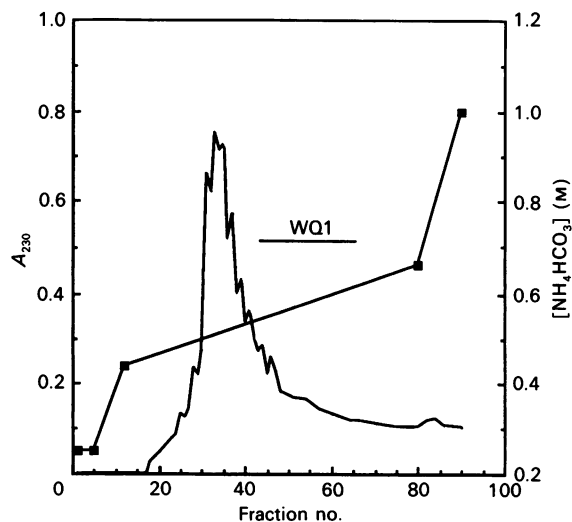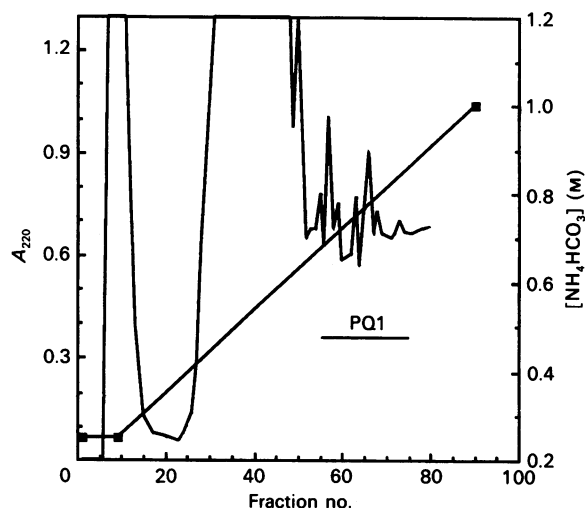
Fig. 4. Purification of immunoreactive fraction from parotid saliva on a Mono Q column

The bound fraction obtained by chromatography on an AH-Sepharose 4B column to which antibodies of APRPs had been coupled was further separated on a Mono Q column. The column was monitored by measuring A_{220} (———). The gradient is also shown (■—■). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min. Fractions 55–75 were pooled (PQ1) as shown by the horizontal bar.
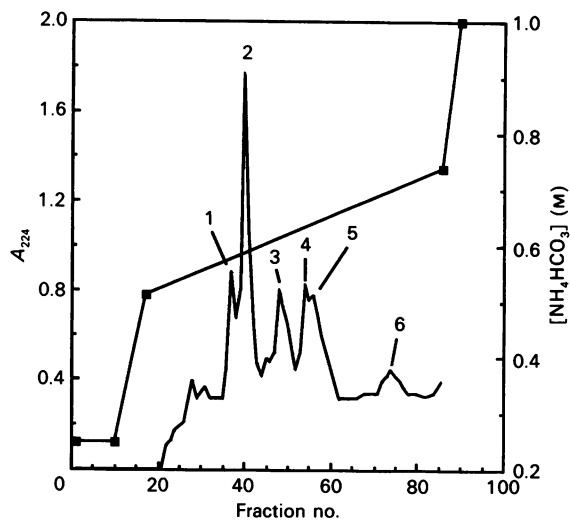

Fig. 5. Chromatography of fraction PQ1 on a Mono Q column

The column was monitored by measuring A_{224} (———). The gradient is also shown (■—■). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min. Tubes corresponding to peaks 1, 2, 3, 4, 5 and 6 were pooled and labelled PQ2-1, PQ2-2, PQ2-3, PQ2-4, PQ2-5 and PQ2-6 respectively.

were visible in the fraction bound to the immunosorbent column (lane 2), but this was not likely to be due to proteolytic digestion during fractionation, since saliva was collected in the presence of pancreatic trypsin inhibitor, which has been shown to prevent most degradation of APRPs by salivary enzymes. Addition of inhibitors of other proteolytic enzymes did not have an effect (Minaguchi et al., 1988). Fractionation of the bound fraction from the immunosorbent column on Mono Q separated APRPs and phosphopeptides, as illustrated by the appearance of fraction PQ1 (Fig. 6, lane 3). Partial separation of the phosphopeptides was achieved by further chromatography, as shown by the staining patterns of fractions PQ2-1, PQ2-2, PQ2-3, PQ2-4, PQ2-5 and PQ2-6 (Fig. 6, lanes 4–9 respectively).
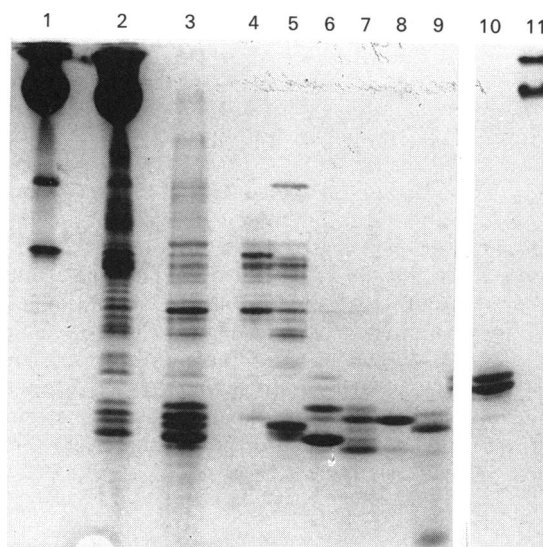

Fig. 6. Electrophoresis of proteins and peptides obtained by fractionation of parotid saliva

The 35% acrylamide gel was stained with Stains-All. Lane 1, parotid saliva (1 ml); lane 2, AH-Sepharose 4B immunosorbent-bound fraction (576 μg); lane 3, PQ1 (24 μg); lane 4, PQ2-1 (4 μg); lane 5, PQ2-2 (7 μg); lane 6, PQ2-3 (3 μg); lane 7, PQ2-4 (4 μg); lane 8, PQ2-5 (3 μg); lane 9, PQ2-6 (4 μg); lane 10, two N-terminal TX peptides, M_r 3500, from acidic PRPs (2 μg); lane 11, mixture of two APRPs, PRP3, M_r 11000 (4 μg), and PRP1, M_r 16000 (7 μg).

Table 1 shows that the total amount of phosphopeptides recovered from whole saliva as represented by the sum of fractions WQ1, WQ2, WQ3 and WQ4 was 14070 μg or 2.6% of protein in whole saliva. Parts of these fractions were subjected to electrophoresis, whereby the purified phosphopeptides WQ1-a, WQ2-a, WQ2-b, WQ2-c, WQ3-a and WQ4-a were obtained in amounts that varied from 4 to 21 μg. From Table 2 it can be estimated that the phosphopeptides obtained from parotid saliva (fraction PQ1) accounted for less than 0.1% of total parotid-saliva protein, and the concentration of phosphopeptides in whole saliva is therefore 26 times higher than in parotid saliva. The total amount of APRP in whole saliva as estimated by crossed immunoelectrophoresis was 0.33 mg/ml, and by single radial immunodiffusion it was determined to be 0.29 mg/ml. The amount of phosphopeptides is therefore 11% of that of APRP in whole saliva. Although the phosphopeptides are derived from APRP, in the absence of estimates of M_r amounts of each peptide and location of the peptides in the APRPs, it is difficult to evaluate how large a percentage of APRP has been degraded. Previously we have estimated the concentration of APRP in stimulated parotid saliva to be 0.7 mg/ml (Bennick & Cannon, 1978), and phosphopeptides would therefore correspond to 0.5% of the APRP concentration, but, since whole saliva is a mixture of various proportions of different secretions, some of which contain APRP, it is difficult to relate the parotid-saliva and whole-saliva concentrations of APRP and thereby estimate the degree of degradation.

All the phosphopeptide-containing fractions WQ1–WQ4 from whole saliva bound Ca^{2+} (Table 3). In agreement with previous observations (Bennick, 1977), the Ca^{2+}-binding activity decreased with increased ionic strength, and together the phosphopeptide fractions accounted for 4.2% (in 5 mM-Tris buffer) or 3.8% (in 5 mM-Tris buffer containing 41 mM-NaCl) of total bound Ca^{2+}. The variation in ionic strength therefore has little if any effect on the percentage of Ca^{2+} bound to the phosphopeptides.

The adsorption of phosphopeptides in saliva on hydroxyapatite

Table 1. Summary of purification of active peptides from whole saliva

For experimental details see the text.

Purification method	Fraction	Total protein recovered (μg)	Amount needed for 50% inhibition (μg)	Relative specific activity	Total units*
Unfractionated	Whole saliva (307 ml)	550000	25.5	1	21570
Q-Sepharose	WQ1†	2080	2.0	13	1040
	WQ2	6020	3.5	7	1720
	WQ3	2740	6.4	4	428
	WQ4	3230	5.3	5	609
Electrophoresis and elution	WQ1-a	21‡	1.4	18	15
	WQ2-a	23§	3.3	8	7
	WQ2-b	13§	1.3	20	10
	WQ2-c	13§	0.7	36	19
	WQ3-a	4 ‖	0.7	36	6
	WQ4-a	6¶	0.7	36	9

* One unit is the amount (μg) needed for 50% inhibition.
† This fraction was obtained by further purification on Mono Q.
‡ From 955 μg of fraction WQ1.
§ From 2740 μg of fraction WQ2.
‖ From 1000 μg of fraction WQ3.
¶ From 1700 μg of fraction WQ4.

Table 2. Summary of purification of active peptides from parotid saliva

For experimental details see the text.

Purification method	Fraction	Total protein recovered (μg)	Amount needed for 50% inhibition (μg)	Relative specific activity	Total units*
Unfractionated	Parotid saliva (1080 ml)	4360000	21.00	1.0	207762
Immunosorbent column	Bound fraction	605000	8.35	2.5	72455
Mono Q 1	PQ1	3500	0.40	53.0	8750
Mono Q 2	PQ2-1	230	2.20	9.5	105
	PQ2-2	760	2.20	9.5	345
	PQ2-3	460	0.35	60.0	1314
	PQ2-4	280	0.15	140.0	1867
	PQ2-5	380	0.15	140.0	2533
	PQ2-6	140	0.26	81.0	538

* One unit is the amount (μg) needed for 50% inhibition.

Table 3. Ca^{2+} binding to whole-saliva protein and phosphopeptides as measured by equilibrium dialysis

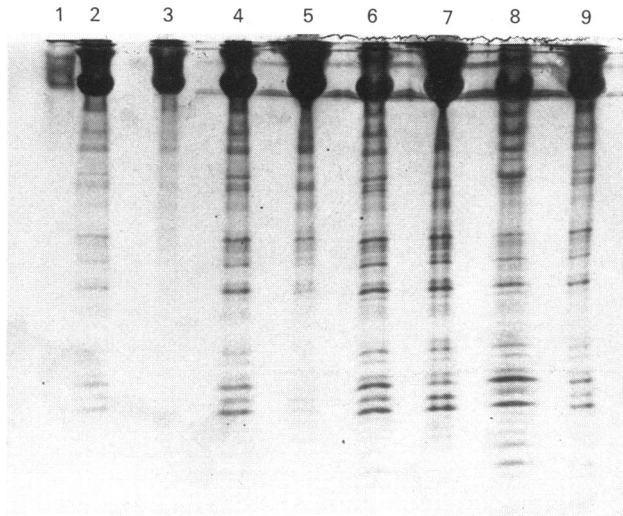| Buffer	Sample	Amount of protein (mg)	Concn. of Ca^{2+} (nmol/ml)	Ca^{2+} bound (nmol/mg of protein)	Ca^{2+} bound (nmol/amount of fraction present in 1 ml of whole saliva)
5 mM-Tris, pH 7.5	Whole saliva	3.6	1000	134	240
	WQ1	0.2	1000	261	8
	WQ2	0.2	1000	245	9
	WQ3	0.2	1000	162	9
	WQ4	0.2	1000	224	10
5 mM-Tris, pH 7.5, containing 41 mM-NaCl	Whole saliva	3.6	1000	43	77
	WQ1	0.2	1000	71	2
	WQ2	0.2	1000	79	3
	WQ3	0.2	1000	50	3
	WQ4	0.2	1000	48	2

Fig. 7. Adsorption of phosphopeptides from whole saliva on hydroxyapatite

Electrophoresis of the saliva supernatant following adsorption and of protein recovered from the hydroxyapatite pellets was performed on a 35 % (w/v) polyacrylamide gel, which was stained with Stains-All. Lanes 1 and 2, saliva diluted 4-fold; lanes 3 and 4, saliva diluted 2-fold; lanes 5 and 6, undiluted saliva; lanes 7 and 8, saliva concentrated 5-fold. Odd-numbered lanes contain supernatant from the hydroxyapatite suspension and even-numbered lanes protein eluted from hydroxyapatite. Lane 9, whole saliva concentrated from 1 ml. All protein recovered was applied in lanes 1–6. In lane 7, one-quarter of the supernatant was applied and in lane 8 one-half of the protein extracted from the mineral was used.
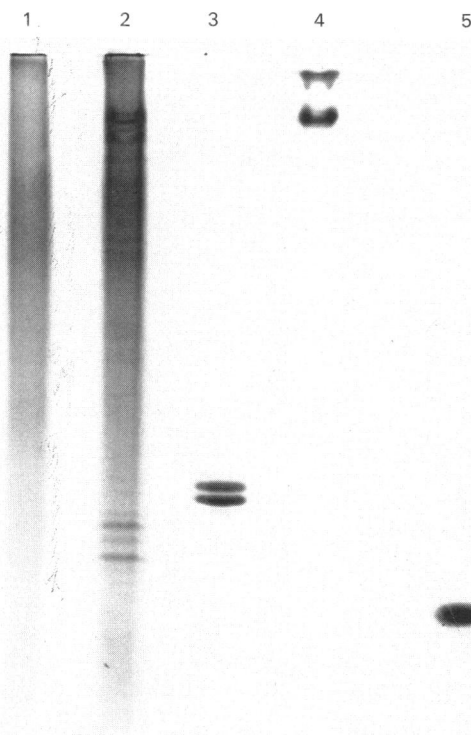

Fig. 8. Adsorption in vivo of phosphopeptides from whole saliva on enamel fragments

Electrophoresis of proteins extracted from enamel fragments on 35 % (w/v) polyacrylamide gel stained with Stains-All. Lane 1, extract of fragments that had not been exposed in the mouth; lane 2, extract of proteins adsorbed on the fragments for 15 min; lane 3, mixture of two N-terminal TX peptides (M_r 3500) from PRP3 and PIFf; lane 4, mixture of APRPs PRP1 (M_r 16000) and PRP3 (M_r 11000); lane 5, Bromophenol Blue dye.

is illustrated in Fig. 7. There were no apparent differences between the band patterns of whole saliva (Fig. 7, lane 9) and protein that had adsorbed on hydroxyapatite (Fig. 7, lanes 2, 4, 6 and 8) demonstrating that all of the phosphopeptides bound to the mineral. When 4-fold-diluted saliva was exposed to hydroxyapatite no bands could be seen in the saliva after adsorption, indicating that all or most of the peptides had bound to the mineral (Fig. 7, lane 1). After exposure of undiluted saliva and 2-fold-diluted saliva to hydroxyapatite, slowly migrating bands were visible, but there was little or no staining of fast-moving peptides, demonstrating that they had been more extensively adsorbed (Fig. 7, lanes 4 and 2). This could be due to a higher affinity of these peptides for hydroxyapatite or to a relatively lower abundance than the slowly migrating peptides.

Since it is of interest to determine if this adsorption occurs in vivo, the experiment illustrated in Fig. 8 was performed. Exposure of the enamel fragments in the oral cavity for 15 min caused adsorption of slowly migrating peptides as well as rapidly migrating peptides (Fig. 8, lane 2). The mobility ranged between those of the tracking dye and the APRP, with some peptides migrating faster than the N-terminal TX peptide (Fig. 8, lanes 3, 5 and 4). These peptides originated from saliva, since they were not seen in extracts of enamel fragments that had not been exposed in the mouth (Fig. 8, lane 1). Similar results were obtained when the enamel fragments were kept in the mouth for 1 h, indicating that the peptides persist on the enamel surface for at least 1 h.

The ability of whole saliva to inhibit hydroxyapatite formation is illustrated in Fig. 9(a), together with the activity of the phosphopeptide-containing fractions WQ1, WQ2, WQ3 and WQ4. To evaluate further the ability of the phosphopeptides to inhibit hydroxyapatite formation, selected peptides were purified and assayed (Fig. 9b). All the peptides, which included rapidly migrating components as well as slowly migrating components, were able to inhibit hydroxyapatite formation in considerably smaller amounts than proteins from whole saliva. Whereas 25.5 μg of whole-saliva protein was needed for 50 % inhibition, only 2.0–6.4 μg of WQ fractions and 0.7–3.3 μg of purified peptides were necessary to obtain the same degree of inhibition (Table 1). This represents up to 36-fold purification compared with whole saliva. It is noteworthy that the peptides can be ranked identically according to either gel-electrophoretic mobility or inhibitory activity. Fig. 3 shows that the order of increasing mobility is WQ2-a, WQ1-a, WQ2-b, WQ3-a, WQ4-a and WQ2-c. From Table 1 the ranking according to increasing activity is also WQ2-a, WQ1-a, WQ2-b, WQ3-a, WQ4-a and WQ2-c. These results indicate that the fastest-migrating peptides are also the most active. The sum of activities of the phosphopeptide-containing fractions WQ1, WQ2, WQ3 and WQ4 represents 18 % of the total activity in whole saliva.

Other proteins in saliva that inhibit hydroxyapatite formation include native APRP (Moreno et al., 1979), statherin (Schlesinger & Hay, 1977), histatin (Oppenheim et al., 1986) and cystatin (Shomers et al., 1982). These proteins would be expected to account for most of the remaining activity. Inhibitory activity was present in parotid saliva as well (Fig. 9c), where 35 % was found in the immunoreactive fraction, and the phosphopeptide-containing fraction PQ1 accounts for 4 % of activity (Table 2). Similar amounts of parotid-saliva and whole-saliva protein were needed for 50 % inhibition, but the activity of the parotid-saliva phosphopeptide-containing fraction PQ1 was 53-fold higher than that of parotid saliva (Table 2). It is apparent that several inhibitory phosphopeptides were present in parotid saliva, since fractions PQ2-1, PQ2-2, PQ2-3, PQ2-4, PQ2-5 and PQ2-6 were all reactive, showing a degree of purification ranging from 9.5-fold to 140-fold. Whereas 19–20 μg of the purified APRPs PRP3 and
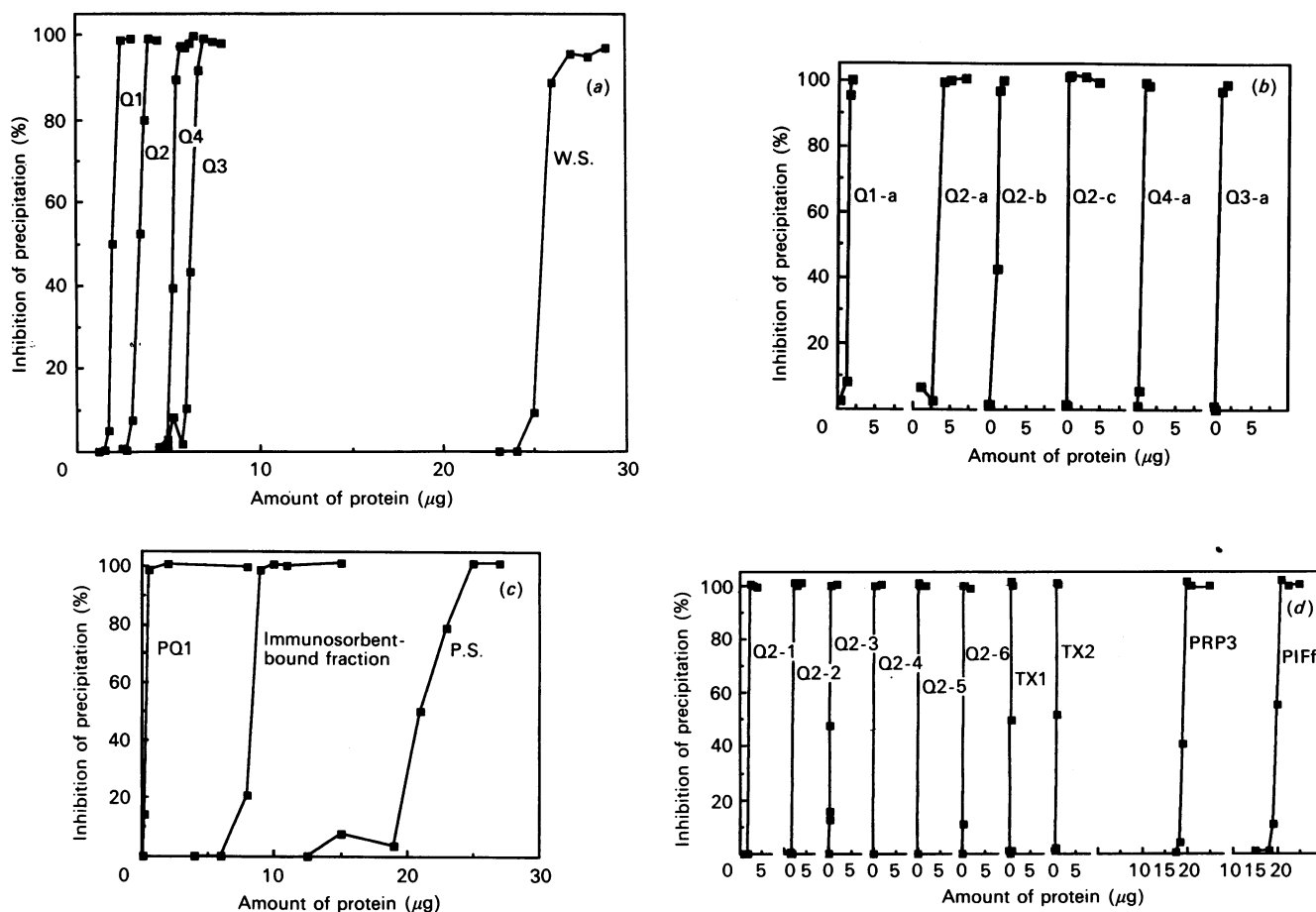
Fig. 9. Inhibition of hydroxyapatite formation by whole saliva and parotid saliva and by peptides from these secretions

Inhibition was assayed by measuring the phosphate concentration in the assay supernatant after 24 h incubation of the suspension of calcium monohydrogen phosphate dihydrate crystals. If there is no inhibition the phosphate concentration is 6.5 mM (0% inhibition of precipitation). In complete inhibition the phosphate concentration remains at the initial 2.5 mM (100% inhibition). (a) Inhibition by whole saliva and its phosphopeptide-containing fractions. Q2, Q3 and Q4 refer to WQ2 to WQ4, which were obtained from a Q-Sepharose column. Q1 refers to WQ1, which was eluted from a Mono Q column. W.S., whole saliva. Experimental values (■) were connected by straight lines and the amount needed for 50% inhibition of hydroxyapatite formation was obtained by interpolation (see Table 1). (b) Inhibition of hydroxyapatite formation by purified peptides from whole saliva. Q1-a, Q2-a, Q2-b, Q2-c, Q4-a and Q3-a refer to peptides WQ1-a to WQ3-a, which were obtained by elution of the correspondingly labelled peptides in Fig. 3. For further explanation see the legend to (a) above. The values obtained for 50% inhibition of hydroxyapatite formation are listed in Table 1. (c) Inhibition of hydroxyapatite formation by parotid saliva and chromatographic fractions from the secretion. P.S., parotid saliva. Immunosorbent-bound fraction and PQ1 (Fig. 4) were obtained as described in the text. For further explanation see the legend to (a) above. The values for 50% inhibition of hydroxyapatite formation were measured by interpolation and are listed in Table 2. (d) Inhibition of hydroxyapatite formation by phosphopeptide-containing fractions from parotid saliva. Q2-1, Q2-2, Q2-3, Q2-4, Q2-5 and Q2-6 refer to fractions PQ2-1 to PQ2-6 obtained by chromatography on a Mono Q column (Fig. 5). PRP3 and PIFf are APRPs previously purified. TX1 and TX2 are the N-terminal peptides of PIFf and PRP3 respectively. For further explanation see the legend to (a) above. The values obtained for 50% inhibition of hydroxyapatite function are listed in Table 2.

PIFf was needed for 50% inhibition, only 0.15–2.2 μg of fractions PQ2-1 to PQ2-6 was necessary for the same degree of inhibition, which is similar to the amount of 0.8 μg that was required for 50% inhibition by the APRP N-terminal TX peptides.

The immunosorbent fraction accounts for 35% of the activity in parotid saliva (Table 1). At least part of the remaining activity is due to the presence of statherin (Schlesinger & Hay, 1977), histatin (Oppenheim et al., 1986) and cystatin (Shomers et al., 1982).

DISCUSSION

The purpose of this paper was to evaluate the importance of the APRP-derived phosphopeptides, and it was therefore necessary to obtain fractions that did not contain any native APRP. Since there was overlap of APRP and phosphopeptides in the

elution from the columns, the fractions containing APRP as well as phosphopeptides were discarded, and the estimations of the phosphopeptides in parotid saliva and whole saliva are therefore minimum concentrations. The phosphopeptides in parotid saliva probably arise by cleavage of APRPs in the gland and are not separate gene products, since no cDNA clones for N-terminal fractions of APRP have been isolated (Maeda et al., 1985) and translation of parotid RNA in vitro resulted only in the formation of a 16000-M_r APRP (Robinson et al., 1989). When whole saliva is collected the transient time of glandular saliva before it is expectorated and heat-inactivated is approx. 2 min. Under these conditions there is a 26-fold increase in the amount of phosphopeptides in whole saliva compared with parotid saliva. Further formation of peptides occurs with increased exposure of glandular saliva to the contents of the mouth (Minaguchi et al., 1988), and it is therefore important to consider the transient time of

saliva in the mouth under physiological conditions. It has been estimated that at a normal flow rate of 0.3 ml/min the saliva present in the mouth is diluted 2-fold within 2 min. At flow rates of 0.05 ml/min and 1.0 ml/min the half-lives are 13 min and 0.7 min respectively (C. Dawes, personal communication), and it would be expected that there is a corresponding variation in the cleavage of APRPs and consequently in the concentration of phosphopeptides. The average thickness of the salivary film covering the oral tissues is 0.07–1.0 mm (Collins & Dawes, 1987), and the velocity of the film at the level of the teeth varies between 0.8 mm/min and 8.0 mm/min at different locations in the mouth (Dawes et al., 1989). It is therefore likely that saliva remains in contact with the teeth for several minutes, which would allow adsorption of the phosphopeptides. Since APRPs bind to hydroxyapatite via the N-terminal phosphorylated region, it is likely that APRPs and phosphopeptides bind to the same sites on hydroxyapatite, but it is clear that the phosphopeptides will bind in the presence of APRPs and that this adsorption occurs in vivo. The amount of Ca^{2+} bound to the phosphopeptide fractions dissolved in 5 mM-Tris/HCl buffer, pH 7.5, varied from 162 nmol/mg to 261 nmol/mg (Table 3). These values are much lower than those previously obtained for the N-terminal TX peptide (2200 nmol/mg) (Bennick et al., 1981), but it is possible that some of the peptides in these fractions have values that are closer to that of peptide TX. At physiological ionic strength (5 mM-Tris/HCl buffer, pH 7.5, containing 41 mM-NaCl) it can be estimated from the values in Table 3 that the phosphopeptide fractions accounts for only approx. 4 % of total protein-bound Ca^{2+} which may not be of physiological importance.

Several phosphopeptides both in parotid saliva and in whole saliva are able to inhibit hydroxyapatite formation, as evidenced by the presence of the activity in all of the fractions obtained from the final column purifications of both fluids as well as in all of the purified peptides. The amount of inhibitory activity located in the phosphopeptides increases from 4 % of total activity in parotid saliva to 18 % in whole saliva, demonstrating the formation of active peptides upon secretion. Although the parotid-saliva and whole-saliva phosphopeptides have the same comparable high inhibitory activity, the small amount of parotid-saliva peptides shows that there are no physiological mechanisms in the gland that convert a significant amount of APRP into their more active phosphopeptides. Such processes are in fact not necessary, because of the rapid cleavage of APRPs into more active peptides after secretion from the gland that ensures that the hydroxyapatite-inhibitory activity in the mouth is maintained, thus preventing unwanted mineral formation. This cleavage also prevents APRPs from acting as a mediator in some bacterial adherence to tooth surfaces. PRPs bind strongly to tannin and protect the digestive system from the deleterious effect of this food component (Mehansho et al., 1987); since this activity is related to the high proline content, it is likely that the C-terminal proline-rich fragment resulting from the cleavage would also bind to tannin. It therefore seems possible that it is of benefit to the host to cleave the APRPs, since useful functions could be maintained, but colonization of the teeth that may lead to oral disease could be impeded. The cleavage occurs rapidly after secretion, and the pattern is similar in different individuals (Minaguchi et al., 1988). There may therefore never have been a need for the human host to evolve proteinases that would effectively cleave the proteins.

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