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The role of glandular kallikrein in the formation of a salivary proline-rich protein A by cleavage of a single bond in salivary protein C

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An enzyme was purified from human parotid saliva that can cleave a single arginine–glycine peptide bond between residues 106 and 107 in human salivary proline-rich protein C, hereby giving rise to another proline-rich protein A, which is also found in saliva. The enzyme was purified 2400-fold. It cleaved salivary protein C at the rate of 59 μg of protein/h per μg of enzyme and had amino acid composition, molecular weight and inhibition characteristics similar to those reported for human salivary kallikrein. Confirmation that the enzyme was kallikrein was demonstrated by its kinin-generating ability. Histochemical evidence indicates that a post-synthetic cleavage of protein C by kallikrein would have to take place during passage of saliva through the secretory ducts. In secreted saliva, cleavage of salivary protein C can only be observed after 72 h incubation. In addition, there is no effect of salivary flow rate on the relative amounts of proteins A and C in saliva. On the basis of the experimental observations, it is proposed that *in vivo* it is unlikely that kallikrein secreted from ductal cells plays a significant role in converting protein C into protein A.

Human parotid saliva and submaxillary saliva contain a group of related acidic proline-rich proteins that constitute approx. 28% of total protein in parotid saliva (Bennick & Cannon, 1978). The amino acid sequences of two of the major components, salivary proteins A and C, have been determined (Wong *et al.*, 1979; Wong & Bennick, 1980). The sequence of salivary protein A was identical with the *N*-terminal 106 residues of salivary protein C, which consists of a single polypeptide chain of 150 amino acids. This suggests that salivary protein A may be formed by a post-synthetic cleavage of salivary protein C at the arginine–glycine bond in position 106–107. This is supported by the observation that human parotid saliva contains a peptide with amino acid sequence identical with the sequence of residues 107–150 in salivary protein C (Isemura *et al.*, 1980). Such a peptide was also isolated in the process of sequencing salivary protein C and was named peptide CTZ (Wong & Bennick, 1980). In a previous study (Wong & Bennick, 1980) a proteinase isolated from mouse submaxillary gland (Schenkein *et al.*, 1977) cleaved salivary protein C only between residues 106 and 107. The enzyme did not break any peptide bonds in salivary protein A. A similar enzyme may be present in the human, because Karn *et al.* (1979) found that human parotid saliva contains a factor that converts salivary protein C into a component with the same electrophoretic mobility as salivary protein A. Karn *et al.* (1979) therefore suggested that protein A arose by a post-translational modification of protein C. An attempt in our laboratory to identify a proteinase in a homogenate of human parotid gland capable of converting salivary protein C into protein A was unsuccessful, although degradation of salivary protein C was seen (Wong & Bennick, 1980). Such an enzyme might be detected in saliva, being secreted either from the acinar cells together with proline-rich proteins or from another glandular source. Initial experiments in our laboratory suggested the presence of a proteinase that cleaves salivary protein C between residues 106 and 107. It was therefore decided to purify and characterize the enzyme and to evaluate if it could be responsible for the presence in saliva of protein A by cleavage of protein C subsequent to its biosynthesis.

Abbreviations used: Tos-Arg-OMe, N^{α}-tosyl-L-arginine methyl ester; Bz-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester.

Experimental

Materials

Tos-Arg-OMe, Bz-Arg-OEt, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, EDTA,

EGTA, soya-bean trypsin inhibitor, pancreatic trypsin inhibitor, bradykinin, diethylstilboestol, Sepharose 4B and molecular-weight markers for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were supplied by Sigma Chemical Co. Dialysis tubing of 2000-M_r cut-off was obtained from Spectrum Medical Industries, and UM05 ultrafiltration membranes were from Amicon. Sephadex G-50 was purchased from Pharmacia, and Bio-Gel P-2 from Bio-Rad Laboratories. CNBr was obtained from Eastman. All other reagents were of analytical grade.

Collection of parotid saliva

Parotid saliva was collected from a single donor in 100ml volumes as described by Bennick & Connell (1971).

Purification of salivary proteins

Salivary proteins A and C were purified as described previously (Bennick, 1975, 1977).

Preparation of antiserum

Antiserum to salivary protein C was prepared as described by Bennick (1977).

Assays for proteinase activity

For routine assays hydrolysis of the arginine esters Tos-Arg-OMe and Bz-Arg-OEt was determined as described by Hummel (1959) and Schwert & Takenaka (1955) respectively.

Demonstration of proteolytic activity in human parotid saliva that cleaves proline-rich protein C

By chromatography of human parotid saliva on CM-cellulose (CM-32), a number of fractions were previously obtained (Bennick, 1975). They were assayed for proteinase activity, and the active fractions were pooled and concentrated. A 200μl sample of this salivary fraction was added to 50μl of 1M-Tris/HCl buffer, pH8.5, that also contained 39μg of proline-rich protein C labelled with ^{14}C by reductive methylation (Wong & Bennick, 1980). The sample was incubated for 16.5h at 23°C. The incubation mixture was fractionated on a Sephadex G-50 column (0.9cm × 118cm) that had been calibrated with salivary proteins A and C and peptide CTZ. The radioactivities of 400μl samples of each fraction were counted on a liquid-scintillation counter.

Purification of parotid salivary proteinase

Parotid saliva (500ml) was dialysed against distilled water overnight at 3°C and concentrated to 85ml by ultrafiltration. The concentrated saliva was applied to a Sephadex G-50 column (5cm × 60cm) that was equilibrated and developed with 0.1M-Tris/HCl buffer, pH8.6. Fractions eluted from the column were assayed for proteolytic activity and pooled. An affinity column was prepared by coupling 20mg of pancreatic trypsin inhibitor to a 10ml volume of packed Sepharose 4B activated with CNBr as described by Parikh *et al.* (1974). The column (1.5cm × 6cm) was equilibrated with 0.1M-Tris/HCl buffer, pH8.6, and the proteinase purified by gel filtration was applied. The column was developed with equilibration buffer until unbound proteins had emerged and the enzyme was then eluted with 0.1M-sodium acetate buffer, pH3.3. The enzyme was freeze-dried and desalted on a Bio-Gel P-2 column equilibrated with 0.125M-NH_4HCO_3 and further purified by preparative polyacrylamide-gel electrophoresis (Lee *et al.*, 1982) with a discontinuous buffer system (Davis, 1964) for separation of the components at pH8.3.

The gel was removed from the tube after electrophoresis and the part containing the enzyme was cut out and re-loaded in the tube. The location of the enzyme in the gel had been determined in preliminary experiments. The gel tube that was placed on top of a Bio-Gel P-2 column (1.5cm × 8.0cm) equilibrated in 0.1M-Tris/HCl buffer, pH8.6, was filled with 0.01M-Tris/HCl buffer, pH8.6. Electrodes were placed in the buffer and in the Bio-Gel P-2 column outlet. After electrophoretic migration of the enzyme into the Bio-Gel P-2 resin, the polyacrylamide-gel tube was removed and the Bio-Gel P-2 column was developed with 0.1M-Tris/HCl buffer, pH8.6. The enzyme was freeze-dried, and salt and impurities were removed on another Bio-Gel P-2 column equilibrated with 0.125M-NH_4HCO_2.

Cleavage of proline-rich proteins by parotid salivary proteinase

A 1μg portion of purified proteinase was incubated with 615μg of protein C or 425μg of protein A in 0.125M-NH_4HCO_3 containing 0.02% NaN_3 at 23°C for 69h. The digest was fractionated on the Sephadex G-50 column (0.9cm × 118cm) that had been calibrated with the salivary proteins. Fractions with elution positions identical with those of protein C, protein A or peptide CTZ were pooled and freeze-dried. Samples of these fractions were used for amino acid analysis and for determination of the *N*-terminus (Percy & Buchwald, 1972). From the amino acid composition and the absorption at 220nm of the sample of peptide CTZ an absorption coefficient $A^{0.1\%}_{1mm,220} = 1.0$ was found.

To investigate the rate of cleavage, 665μg samples of protein C were incubated with 0.2μg of parotid proteinase in 0.1ml of 0.125M-NH_4HCO_3 containing 0.02% NaN_3 at 37°C for 8, 16, 24 and 40h. The digests were fractionated on the calibrated Sephadex G-50 column (0.9cm × 118cm) and the degree of degradation of protein C was measured from the amount of peptide CTZ generated.

Characterization of the parotid salivary proteinase

Estimations of the purity and molecular weight of the enzyme were done by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The following M_r markers were used: bovine albumin, egg albumin, pepsin, trypsinogen, β-lactoglobulin and lysozyme. The gel was stained by the procedure of Merril *et al.* (1981).

A sample of the purified enzyme was hydrolyzed for 24h in 6M-HCl in evacuated and sealed tubes. The hydrolysate was analysed for amino acids on a Beckman model 120C analyser.

The nature of the proteinase activity was investigated by incubating the purified enzyme with Bz-Arg-OEt and inhibitors including soya-bean trypsin inhibitor, pancreatic trypsin inhibitor, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, EGTA and EDTA.

Kallikrein bioassay

Rabbit plasma was prepared as described by Ardlie *et al.* (1970), adjusted to pH2.0 with 0.3M-HCl and left at 23°C for 15min. The pH was then adjusted to 7.4 with 0.3M-NaOH. This inactivates the proenzymes of the kinin-generating system.

Kinin-generating activity was measured by contraction of rat uterine horn as described by Movat *et al.* (1971). To standardize contraction solutions containing between 0.2 and 0.6ng of bradykinin/ml were used. To estimate kinin formation by parotid proteinase, 0.5ml of plasma was mixed with 3.5ml of 0.2M-Tris/HCl buffer, pH7.4, containing 0.01 to 0.04μg of purified enzyme. The mixture was incubated at 37°C for 1min and the reaction stopped by heating at 100°C for 15min. Assays of the digests were preceded and followed by application of standard bradykinin solutions.

Cleavage of salivary proline-rich protein C in saliva

Parotid salivary proteinase may cleave salivary protein C in the glandular ducts during secretion, causing the appearance of protein A in secreted saliva. Parotid saliva was therefore collected from a donor as described previously (Bennick & Connell, 1971), and samples were incubated at 23°C for 0, 24, 48, 72, 96 and 168h.

In another experiment, two samples of saliva were incubated for 168h, one of them in the presence of 16μg of pancreatic trypsin inhibitor/ml. Portions were removed after incubation and tested for microbial contamination. The samples were stored at −70°C.

The concentration of acidic proline-rich proteins was measured by single radial immunodiffusion with antiserum to protein C (Bennick & Cannon, 1978). Samples of incubated saliva containing 1.0–1.3μg of acidic proline-rich proteins were subjected to crossed immunoelectrophoresis (Bennick *et al.*, 1982), which results in a pattern of two peaks of immunoprecipitate. The major acidic proline-rich proteins in the donor (A. B.) are proteins A and C, with only very small amounts of related proteins present (Bennick, 1975, 1977). The ratio of the areas under the two peaks can therefore be used to estimate the relative amounts of proteins A and C. To find this ratio, the electrophoretic patterns were traced and the peaks corresponding to proteins A and C were cut out and weighed.

Effect of flow rate of saliva on the relative amounts of proteins A and C

If protein C is cleaved during secretion in the secretory-duct system, the degree of cleavage should depend on the flow rate of saliva. Saliva was therefore collected from the left parotid gland of a donor (A. B.) in the early afternoon to minimize the effect of diurnal variations (Ferguson *et al.*, 1973). Samples were collected without stimulating the flow every 5min for 45min. Flow of saliva was then stimulated by sucking sour lemon drops, and samples were collected every 3min for 30min. Pancreatic trypsin inhibitor was added to the collection tubes to inhibit the proteinase (unstimulated saliva, 6μg/tube; stimulated saliva, 48μg/tube). The volumes of the fractions were measured and the flow rates were calculated. The concentration of acidic proline-rich proteins was determined as described in the preceding experiment.

To measure the amount of proteinase in saliva, the experiment was repeated, but without addition of proteinase inhibitor. The samples were assayed for Bz-Arg-OEt-hydrolytic activity.

Results

Demonstration of proteinase in parotid saliva

Bz-Arg-OEt-hydrolytic activity was located between the first and the second protein peak eluted from the CM-cellulose column. Separation on a Sephadex G-50 column of a ^{14}C-labelled sample of protein C digested with this chromatographic fraction resulted in a ^{14}C-labelled peak in the elution position of peptide CTZ and a shoulder on the protein C peak with the elution position of protein A, demonstrating hydrolysis of protein C. This proteinase assay is cumbersome in routine analyses, for which the Bz-Arg-OEt-hydrolytic assay was preferred.

Purification of parotid salivary proteinase

Fractionation of 1.3g of parotid salivary protein on Sephadex G-50 resulted in the elution profile presented in Fig. 1. The proteolytic activity was located in the void volume. Subsequent purification of the proteinase by affinity chromatography resulted in the separation of most of the other proteins

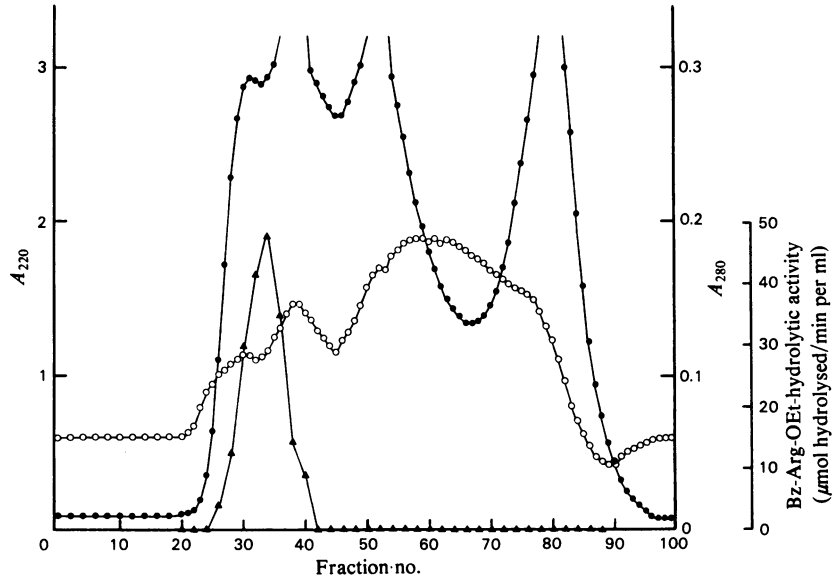

Fig. 1. *Gel filtration of concentrated parotid saliva on Sephadex G-50*
The column (5 cm x 60 cm) was equilibrated and then eluted with 0.1 M-Tris/HCl buffer, pH 8.6, at a flow rate of 0.9 ml/min. Fractions of volume 13 ml were collected. Enzyme activity was determined with Bz-Arg-OEt as a substrate. The absorbances were measured in a cell with a 1 mm optical pathway. ●, A_{280}; ○, A_{280}; ▲, Bz-Arg-OEt-hydrolytic activity.

Table 1. *Summary of yields and purification of proteinase from human parotid saliva*
For full experimental details see the text. Protein was determined from amino acid analysis of the samples. The enzyme activity unit was defined as 1 μmol of Bz-Arg-OEt hydrolysed/min.

Purification step	Volume (ml)	Concn. of protein (mg/ml)	Total amount of protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification (fold)
Ultrafiltered concentrated parotid saliva (original volume 500 ml)	85	15.61	1327	2.73	3623	100	1
Sephadex G-50 chromatography	182	2.61	474	6.38	3031	83.7	2.3
Pancreatic trypsin inhibitor affinity chromatography	3	0.11	0.33	2235	737.6	20.4	819
Preparative gel electrophoresis followed by Bio-Gel P-2 chromatography	6	0.00996	0.0598	6548	391.6	10.8	2399

from the enzyme (Fig. 2). Further purification was obtained by preparative polyacrylamide-gel electrophoresis and Bio-Gel P-2 chromatography. This resulted in co-elution of the enzymic activity and all material absorbing at 280 nm. To remove impurities from the enzyme preparation, it was applied to another Bio-Gel P-2 column. The elution pattern of this column (Fig. 3) shows that u.v.-absorbing components had been removed from the enzyme.

A summary of the purification is shown in Table 1. The final yield from 500 ml of saliva was 60 μg of proteinase. This preparation had an activity 2400-fold higher than that of unfractionated parotid protein.

Cleavage of salivary proline-rich proteins by parotid salivary proteinase

Chromatography of a 69 h parotid-salivary-proteinase digest of salivary protein A on Sephadex G-50 resulted in the elution profile illustrated in Fig. 4(*a*). No breakdown of the proline-rich protein was observed. A similar digest of salivary protein C

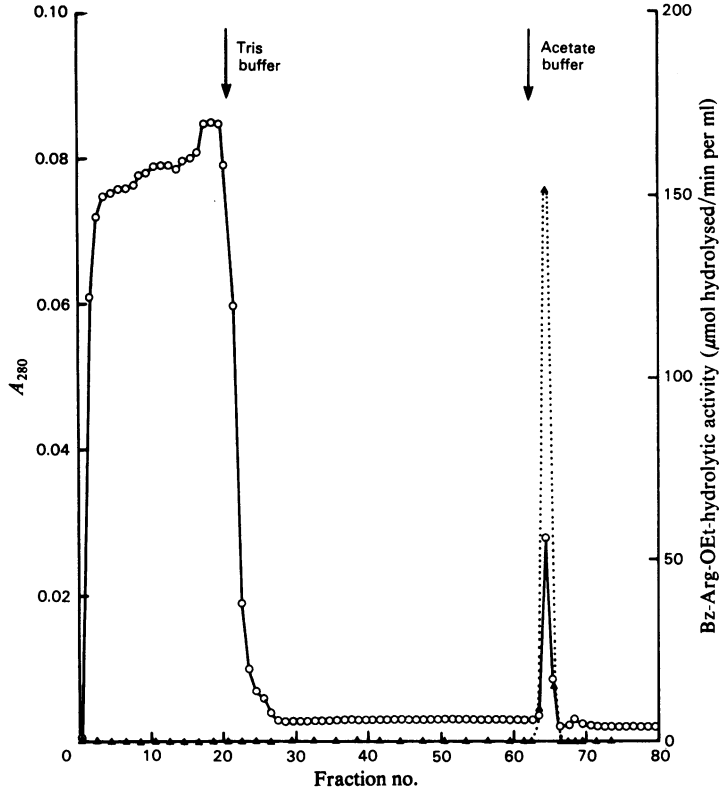

Fig. 2. *Affinity chromatography of the preparation of parotid salivary proteinase obtained from Sephadex G-50 filtration*
Pancreatic trypsin inhibitor was coupled to Sepharose 4B as described in the text. The pancreatic-trypsin-inhibitor–Sepharose 4B column (1.5 cm × 6 cm) was equilibrated with 0.1 M-Tris/HCl buffer, pH 8.6, and developed with the same buffer starting from the position indicated by the arrow at a flow rate of 2 ml/min. A total of 62 fractions of volume 10 ml were collected. The column was then eluted with 0.1 M-sodium acetate buffer, pH 3.3, at a flow rate of 2 ml/min. Fractions of volume 5 ml were collected. Enzyme activity (▲) was determined with Bz-Arg-OEt as a substrate; A_{280} (○) of the fractions was measured as indicated in the legend to Fig. 1.

Table 2. *Amino acid composition and N-terminus of salivary protein C, salivary protein A, peptide CTZ and the products of parotid-salivary-proteinase digestion of salivary protein C*

			Parotid-salivary-proteinase digest		
Residue	Protein C	Protein A	G-50 fraction no. 32	G-50 fraction no. 43	Peptide CTZ
Lys	2	1	1.1	1.1	1
His	3	2	2.0	1.0	1
Arg	6	4	4.8	2.5	2
Asp	11	11	11.5	0	0
Ser	6	5	4.3	0.9	1
Glu	40	29	30.0	11.5	11
Pro	41	24	20.5	14.6	17
Gly	31	20	20.4	11.1	11
Ala	1	1	1.1	0	0
Val	3	3	2.9	0	0
Ile	2	2	2.1	0	0
Leu	3	2	3.5	0	0
Phe	1	1	1.0	0	0
N-Terminus	Blocked	Blocked	Blocked	Gly	Gly

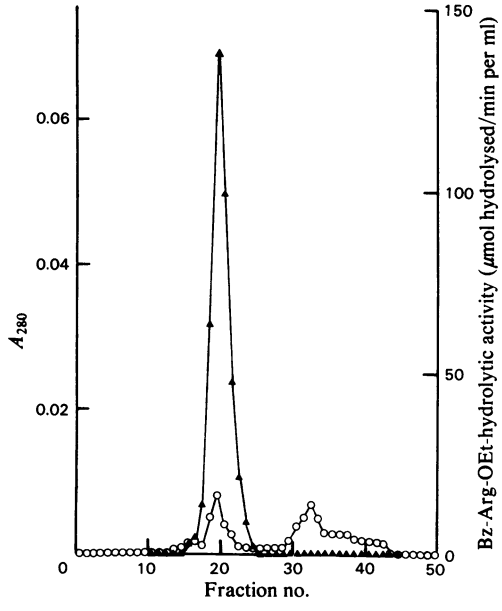

Fig. 3. *Bio-Gel P-2 filtration of parotid salivary proteinase obtained by preparative gel electrophoresis*
The column (1.6cm × 30cm) was equilibrated and subsequently eluted with 0.125M-NH_4HCO_3 at a flow rate of 1ml/min. Fractions of volume 1ml were collected. Enzyme activity (▲) was determined with Bz-Arg-OEt as a substrate; A_{280} (O) was determined as indicated in the legend to Fig. 1.

resulted in the disappearance of most of the protein eluted in the position of protein C and the appearance of protein peaks with elution positions identical with those of salivary protein A (fraction 32) and peptide CTZ (fraction 43) (Fig. 4*b*). Fraction 32 had an amino acid composition almost identical with that of protein A and both had a blocked *N*-terminus. Fraction 43 had amino acid composition and *N*-terminus identical with those of peptide CTZ (Table 2). The enzyme therefore cleaves only the arginine–glycine bond in position 106–107 in salivary protein C and it is unable to cleave any bonds in salivary protein A. Under the experimental conditions, 16.7μg of peptide CTZ was generated/h per μg of parotid salivary proteinase (Fig. 5), corresponding to cleavage of 59μg of salivary protein C/h per μg of parotid proteinase.

Characterization of parotid salivary proteinase

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified enzyme gave rise to a single band. A molecular weight of 38900 was estimated by comparing the electrophoretic mobility of the parotid salivary proteinase with the protein markers. For comparison, salivary kallikrein purified by Modéer (1977) was found to have a molecular weight of 38000.

The amino acid composition of parotid salivary proteinase is presented in Table 3, together with those of salivary kallikrein (Fujimoto *et al.*, 1973) and mouse submaxillary-gland proteinase (Levy *et al.*, 1970). The composition of salivary kallikrein (Fujimoto *et al.*, 1973) is very similar to that of the enzyme purified in the present study except for somewhat different amounts of lysine and glycine residues. The composition of both of these enzymes also show a close resemblance to that of the submaxillary-gland proteinase.

The effects of various inhibitors on parotid-saliva proteinase are presented in Table 4. The virtual absence of inhibition by *N*-ethylmaleimide, EGTA and EDTA, but decreased activity in the presence of phenylmethane sulphonyl fluoride, indicates that the enzyme is a serine proteinase. The lack of effect of soya-bean trypsin inhibitor on the enzymic activity but inhibition by pancreatic trypsin inhibitor is typical of glandular kallikrein (Webster & Pierce, 1961).

Contraction of the rat uterus horn in response to synthetic bradykinin gave a linear response in the range 0.2–0.6ng/ml. Rabbit plasma digested with parotid salivary proteinase also caused muscular

Table 3. *Amino acid composition of parotid salivary proteinase, human salivary kallikrein and mouse submaxillary-gland proteinase*
N.D., Not determined.

Amino acid	Composition (residues/100 residues) Parotid salivary proteinase (present work)	Human salivary kallikrein (Fujimoto *et al.*, 1973)	Mouse submaxillary-gland proteinase (Levy *et al.*, 1970)
Lys	3.50	7.48	7.12
His	2.72	2.72	2.14
Arg	3.22	3.40	1.42
Asp	11.27	9.52	11.03
Thr	5.38	5.44	5.69
Ser	9.85	8.44	6.41
Glu	12.50	11.56	7.83
Pro	3.70	4.76	7.47
Gly	13.32	8.84	10.68
Ala	6.39	8.84	5.34
CyS	0.51	1.36	3.20
Val	6.54	6.80	4.63
Met	1.68	1.36	2.14
Ile	2.30	3.40	3.92
Leu	9.64	7.48	10.68
Tyr	2.16	2.72	2.85
Phe	5.31	3.40	3.20
Trp	N.D.	2.04	4.27

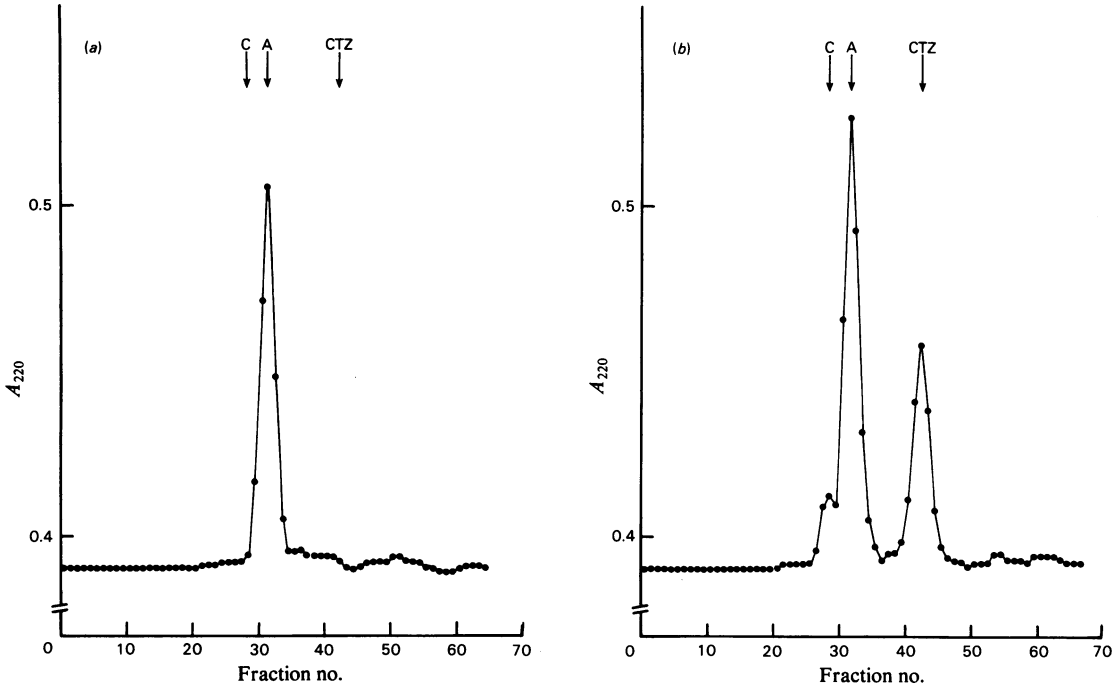

Fig. 4. *Elution profiles of 69 h parotid-salivary proteinase digests of salivary proteins A (a) and C (b) on Sephadex G-50*
The column (0.9 cm × 118 cm) was eluted with 0.1 M-formic acid at a flow rate of 0.2 ml/min. Fractions of volume 1 ml were collected. The arrows indicate the elution volumes of salivary proteins A and C and peptide CTZ obtained by previous calibration of the column.

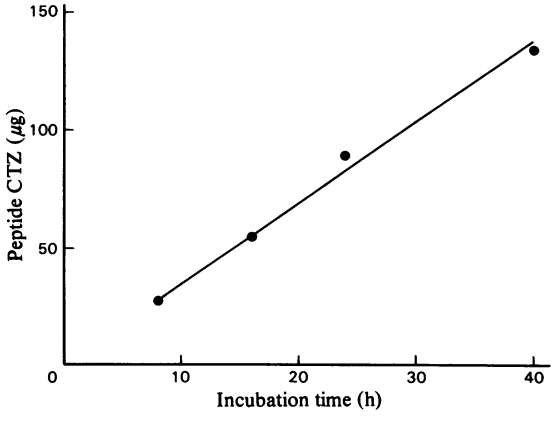

Fig. 5. *Rate of degradation of salivary protein C by the parotid salivary proteinase*
The amount of peptide CTZ generated from 665 μg of protein C by 0.2 μg of enzyme was evaluated from chromatograms of the digests as illustrated in Fig. 4(*b*). The quantity of peptide CTZ was calculated from the volume and A_{220} of the chromatographic fractions and the absorption coefficient of peptide CTZ.

Table 4. *Effects of various inhibitors on the activity of parotid salivary proteinase*
Activity was measured by preincubation of 1 μg of parotid salivary proteinase for 30 min at 23 °C with the indicated quantities of inhibitors in a 200 μl volume before assay with the substrate Bz-Arg-OEt as described in the Experimental section.

Inhibitor	Quantity (μg)	Esterase activity (% of control)
Soya-bean trypsin inhibitor	20	100
	40	100
	80	100
Pancreatic trypsin inhibitor	4	11
	8	0
	20	0
Phenylmethanesulphonyl fluoride	2	78
	9	54
	35	18
N-Ethylmaleimide	25	100
	125	100
	250	88
EGTA	380	99
	761	95
EDTA	373	100
	745	92

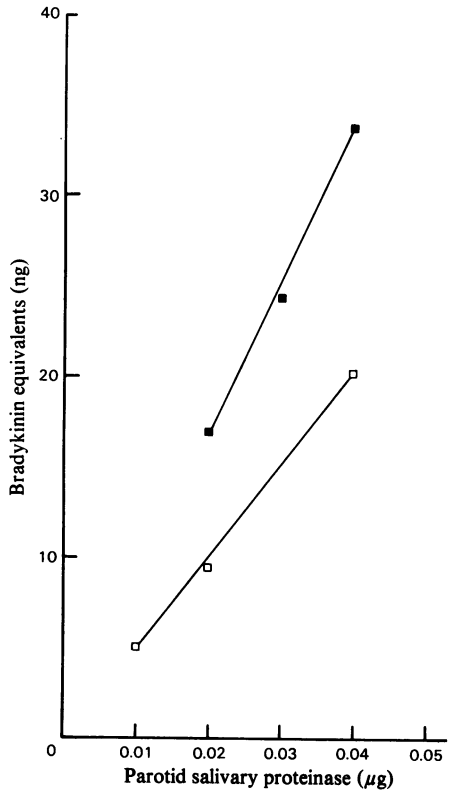

Fig. 6. *Ability of parotid salivary proteinase to generate bradykinin*

Purified parotid salivary proteinase was incubated with acid-treated rabbit plasma, and the incubation mixture was assayed for its ability to induce muscular contraction as described in the text. Muscular contraction was calibrated with known amounts of bradykinin, which allowed calculation of the bradykinin equivalents generated by the parotid salivary proteinase. The results obtained with three different amounts of proteinase in two separate experiments are illustrated.

contraction. In two experiments, a linear relationship was found between the amount of parotid salivary proteinase added to the substrate and the degree of contraction. By comparing contractions obtained with bradykinin and the proteinase digest of plasma, the equivalents of bradykinin generated by parotid salivary proteinase could be calculated (Fig. 6). The different rates were due to variation in the response of the uteri. Under conditions of the assay, 512 and 841 μg of bradykinin equivalents were generated/min per mg of parotid salivary proteinase in the two experiments.

Cleavage of protein C in saliva

Crossed immunoelectrophoresis of parotid saliva demonstrated two immunoreactive components. The fastest-migrating peak corresponded to salivary protein A and the slowest was identical in position with protein C. The results of incubation of saliva are listed in Table 5. Apart from a slightly lower ratio at 24h, no change can be seen during the first 72h of incubation. This is followed by a slow increase in the ratio from 1.2 at 72h to 2.0 at 168h. This conversion is caused by the proteinase, since the saliva sample incubated with pancreatic trypsin inhibitor showed no increase in the ratio (Table 5). No bacteria were detected in any of the saliva samples.

Table 5. *Conversion of salivary protein C into salivary protein A by incubation of saliva for various periods of time*

Stimulated parotid saliva was incubated at 23°C for the length of time indicated and then subjected to crossed immunoelectrophoresis. The relative amounts of proteins A and C were estimated from the areas under the immunoprecipitate peaks. For experimental details see the text.

Expt. no.	Conditions	Length of incubation (h)	Area of protein A peak / Area of protein C peak
1	No additions	0	1.18
	No additions	24	1.11
	No additions	48	1.18
	No additions	72	1.19
	No additions	96	1.38
	No additions	168	2.02
2	16 μg of pancreatic trypsin inhibitor added/ml of saliva	168	1.07
	No additions	168	1.47

To evaluate the effect of flow rate on the relative amounts of proteins A and C in saliva, the last three samples collected of unstimulated and of stimulated parotid saliva were subjected to crossed immunoelectrophoresis. The flow rates and the ratios of the areas corresponding to proteins A and C are given in Fig. 7, which also illustrates flow rates and Bz-Arg-OEt-hydrolytic activities in a parallel experiment. The mean flow rate of stimulated saliva was 20 times that of unstimulated saliva, which had an esterase activity approx. 2.1-fold higher than that of stimulated secretion. The A/C ratio was slightly higher in stimulated than unstimulated saliva.

Discussion

The amino acid composition, molecular weight, inhibition and kinin-generating ability of the parotid salivary proteinase all indicate that the enzyme is in fact kallikrein. There is a close similarity in the composition of the human salivary kallikrein and the mouse submaxillary-gland proteinase. Moreover, both enzymes will cleave only the arginine–glycine

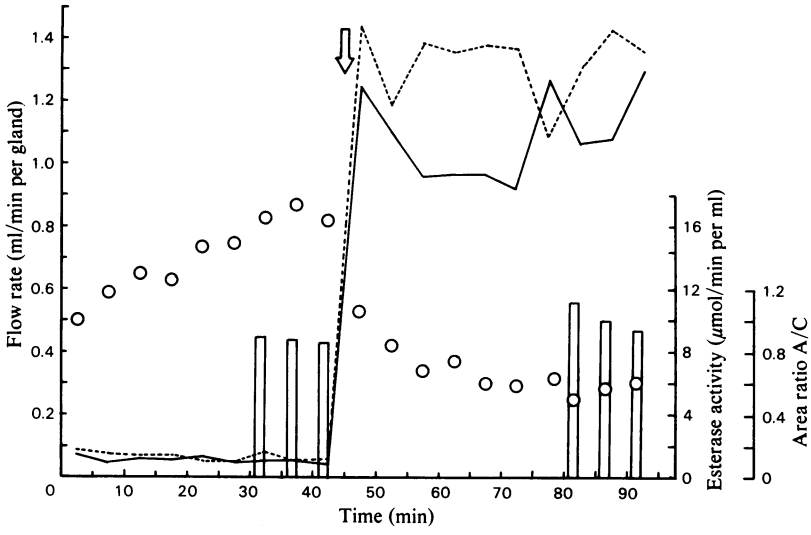

Fig. 7. *Effect of salivary flow rate on the relative amounts of salivary proteins A and C on the Bz-Arg-OEt-hydrolytic activity in saliva*

Unstimulated parotid saliva was collected as described in the text. This was followed by collection of stimulated parotid saliva from the time indicated by the vertical arrow. The histogram bars illustrate the ratios of the areas of of proteins A/C and the corresponding flow rate is indicated by the continuous line. In the second experiment the Bz-Arg-OEt-hydrolytic activities (O) were measured. The flow rate in this experiment is illustrated by a broken line.

bond in salivary protein C. The presence of a number of related enzymes in mouse submaxillary glands capable of cleaving arginyl substrates has been reported (Ekfors *et al.*, 1972; Schenkein *et al.*, 1977), including nerve growth factor γ-subunit, epidermal-growth-factor-binding protein and kallikrein. The enzymes may be involved in post-synthetic proteolytic modification of precursor proteins (Bothwell *et al.*, 1979). Human urinary kallikrein has been shown to activate inactive renin (Sealey *et al.*, 1978), and kallikrein may play a role in the conversion of proinsulin into insulin (ole-Moi Yoi *et al.*, 1979). The function of kallikrein in the human salivary glands is not clear. The enzyme may play a role in vasodilation (Hilton, 1970) and in electrolyte secretion (Ørstavik, 1978), but this does not explain why it is secreted in saliva. Because salivary kallikrein cleaves a single bond in salivary protein C, it is tempting to suggest that it acts as a processing enzyme. This is not in agreement with the glandular location of the enzyme. Acidic proline-rich proteins have been demonstrated in acinar cells of the human parotid gland and in serous demilunes of the submandibular gland (Bennick *et al.*, 1977; Kousvelari *et al.*, 1980), but, at least in the submandibular gland of human and other species, kallikrein is present only in the cells lining the ducts (Schachter *et al.*, 1980), thereby excluding a role of kallikrein as a processing enzyme for proteins synthesized in acinar cells. Protein A could be generated during secretion by the action of kallikrein on protein C as it passes through the glandular duct system. The total concentration of acidic proline-rich proteins in human saliva is approx. 500 mg/l (Bennick & Cannon, 1978), and we have estimated from large-scale fractionations that the concentrations of proteins A and C in saliva are approximately equal. Proteins A and C are by far the major components of the donor's acidic proline-rich proteins, and the concentration of protein A is therefore close to 250 mg/l. The total volume of the human parotid ducts has been estimated to vary from 0.6 ml to 1.0 ml (Ericson & Hedin, 1970). This means that, in the present study, the proteins secreted from the acinar cells may have spent as little as 0.5 min or as much as 18 min in the duct system. If all protein A present in saliva is due to conversion of protein C in the ducts, the rate of generation of protein A should be between 14 and 500 μg/min per ml. This is in sharp contrast with the observation that in saliva cleavage of protein C was only detectable after 72 h. If ductal kallikrein were instrumental in converting protein C into protein A, it would be expected that the higher esterase activity

of unstimulated saliva and the longer time this secretion spends in the ducts would cause cleavage of a larger amount of protein C than in stimulated saliva, giving rise to a higher A/C ratio, particularly since the total concentration of acidic proline-rich protein is lowest in unstimulated secretion (Bennick & Cannon, 1978). The A/C ratio was, however, found to be lower in unstimulated than in stimulated saliva.

In spite of the ability of kallikrein to cleave a single bond in protein C, the rate of cleavage of this protein in saliva is so low that it is unlikely that enzyme secreted from the glandular ducts play a significant role in converting salivary protein C into protein A. The results do not exclude the possibility that protein A is formed from protein C by a post-ribosomal cleavage in the acinar cells, either by kallikrein, which until now has escaped detection, or by another enzyme.

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