Some properties of proteolytic enzymes in human saliva

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Paraffin stimulated whole mixed saliva from seven subjects was collected. The peptidases found in the supernatant and released from the saliva sediment were characterized by the following properties: pH-optimum on gelatin and haemoglobin as substrates, pH-stability and the influence of inhibitors and activators. Saliva was precipitated with acetone, treated with sodium deoxycholate and fractionated on Bio-gel P-100 and P-150. The data obtained indicate the presence of several peptidases in human saliva hydrolyzing the substrates α-N-benzoyl-L-arginine ethyl ester-HCl, *p*-tosyl-L-arginine methyl ester-HCl, L-lysine-*p*-nitroanilide dihydrobromide and gelatin.

Key-words: Saliva; peptidases

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Proteolytic enzymes have been studied in rat and mouse submandibular glands (*Riekkinen, Ekfors & Hopsu-Havu*, 1966; *Riekkinen, Ekfors, Hollmén & Hopsu-Havu, 1967; Riekkinen & Niemi*, 1968; *Ekfors & Hopsu-Havu*, 1972; *Ekfors, Malmiharju & Hopsu-Havu*, 1972) and also in human saliva (*Chauncey*, 1961; *Chauncey & Shannon*, 1966; *Mäkinen*, 1966; *Söder*, 1972).

In human saliva most of the proteolytic activity was found in the sediment obtained after centrifugation (*Chauncey* 1961, *Söder*, 1972). This »bound» enzyme could be released from the sediment by using a detergent, sodium deoxycholate (*Lindqvist, Söder, Modéer & Lundblad*, 1974), but has not yet been characterized.

The purpose of the present investigation was to characterize the peptidases from human saliva.

MATERIAL AND METHODS

Saliva

Paraffin — stimulated whole mixed saliva (165 ml) was collected from each of 4 men and 3 women. The subjects had to rinse their mouths before collection procedure, 3—4 hours after oral hygiene. The saliva samples were pooled after the collection. From the pooled saliva, 150 ml was withdrawn and centrifuged at 10,000 g for 15 min at 4°C. The sediment was washed twice with 0.05 M tris-HCl buffer, pH 8.1 and centrifuged. The supernatants were pooled (e_1). The sediment was incubated with solid sodium deoxycholate (DOC), 0.4 % (w/v) (Merck A.G., Darmstadt, Germany) in 0.05 M tris-HCl buffer pH 8.1 for 3 hours at 25°C, centrifuged, washed twice with the same buffer and centrifuged. The supernatant fluid and the wash supernatants were pooled

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(e_2). The fractions e_1 and e_2 were precipitated separately with two volumes of acetone (—18°C). The precipitates were collected by centrifugation at 10,000 g and each dissolved in 35 ml distilled water. The concentrated fractions e_1 and e_2 were stored frozen in 5 ml aliquots until used.

For the chromatography study 1000 ml of the pooled saliva was incubated with 0.4 % DOC for 4 hours at 25°C. At the end of the incubation period the solution was centrifuged at 10,000 g for 15 min at 4°C. The sediment was washed twice with 0.05 M tris-HCl buffer pH 8.1 and harvested by recentrifugation. The saliva supernatant and the supernatants from the washes were pooled (1230 ml) and used for purification.

Enzyme assays

The peptidases were determined on following substrates: gelatin (USP gran Fisher S-C Co., N.J., USA) (*Hultin*, 1946, 1948), haemoglobin (Sigma Chemical Company, St. Louis, Miss., USA) (*Anson* 1938, *Ruyssen and Lauwers*, 1963), α-N-benzoyl-L-arginine ethyl ester-HCl (BAEE) (Sigma) (*Kétzdy, Lorand & Miller*, 1965), *p*-tosyl-L-arginine methyl ester-HCl (TAME) (Nutritional Biochemical Corporation, Cleveland, Ohio, USA) (*Hummel*, 1959) and L-lysine-*p*-nitroanilide dihydrobromide (LPA) (Nutritional Biochemical Corporation) (*Erlanger, Kokowsky & Cohen*, 1961). All of these assay systems have been described previously (*Lindqvist et al.*, 1974).

To study the stability of the gelatinolytic activity 0.5 ml aliquots of fractions e_1 and e_2 were incubated in 0.25 ml of 0.1 M buffers of different pH for 30 min. at 25°C. The pH was determined in the mixture. At the end of the incubation period 1.0 ml of 0.4 M tris-HCl buffer, pH 8.8 was added to each tube. From each buffer-enzyme solution 1.0 ml was withdrawn in order to determine the remaining gelatinolytic activity at pH 8.8.

Inhibitors and Activators

Their influence of the following substances was tested on the gelatinolytic activity at final concentrations of 1 mM and 10 mM: ethylenediaminetetraacetic acid disodiumsalt (EDTA) (Merck A.G.) sodium lauryl sulphate, iodoacetic acid, L(+)-cysteine-HCl, ascorbic acid (Hoffman La-Roche & Co., Basle, Switzerland), acetic acid anhydride. Soybean trypsin inhibitor (3 × cryst.) (Worthington Biochemical Corporation, Freehold, N.J., USA) was used at a final concentration of $5 \cdot 10^{-3}$, 10^{-2} and $5 \cdot 10^{-2}$ % (w/v).

The effect of the following metal ions were also tested on the gelatinolytic activity at a final concentration of 1 mM and 10 mM: Cd^{2+}, Zn^{2+}, Mn^{+2}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+} and Fe^{3+} (added as chlorides).

Gel filtration

The supernatant of DOC-treated saliva (1230 ml) was saturated with two volumes of acetone (—18°C). The mixture was centrifuged and the precipitate was dissolved in 56 ml 20 mM tris-HCl buffer, pH 8.1, used in gel filtration chromatography.

The gel filtration procedure was performed on Bio-Gel P-100 (100—200 mesh) and P-150 (50—100 mesh) (Bio-Rad, Richmond, California, USA). The gels were treated according to the manufacturer's instructions and equilibrated with 20 mM tris-HCl buffer, pH 8.1, 0.5 M NaCl and 0.02 % (w/v) sodium azide as a bactericidal agent. The first gel filtration was performed on a Bio-gel P-100 column (4.5 × 92 cm) at 12—14°C

and the effluent collected in fractions of 10.0 ml using a fraction collector (LKB, Stockholm, Sweden). The absorbance of the effluent was continuously monitored at 254 nm with a LKB Uvicord Ultra-Violet Flow-Through Spectrophotometer. The optical densities of the fractions at 280 nm and 260 nm were also determined. The protein content was determined by the method of *Warburg & Christian* (1942). Spectrophotometric measurements were performed with a Zeiss spectrophotometer PMQ II.

Fractions from P-100 chromatography numbered 44—50 (a), No 51—58 (b), No 59—66 (c) and No 67—78 (d) were pooled. These pools were concentrated and chromatographed separately on a Bio-gel P-150 column (1.93 × 51.2 cm) at 12—14°C. In these experiments an expression for the partition coefficient (K_{av}) was used (*Laurent & Kilander*, 1964).

RESULTS

Influence of pH on the proteolytic activity of fractions e_1 and e_2 on gelatin and haemoglobin

The influence of pH on the activity of these two fractions was tested with gelatin

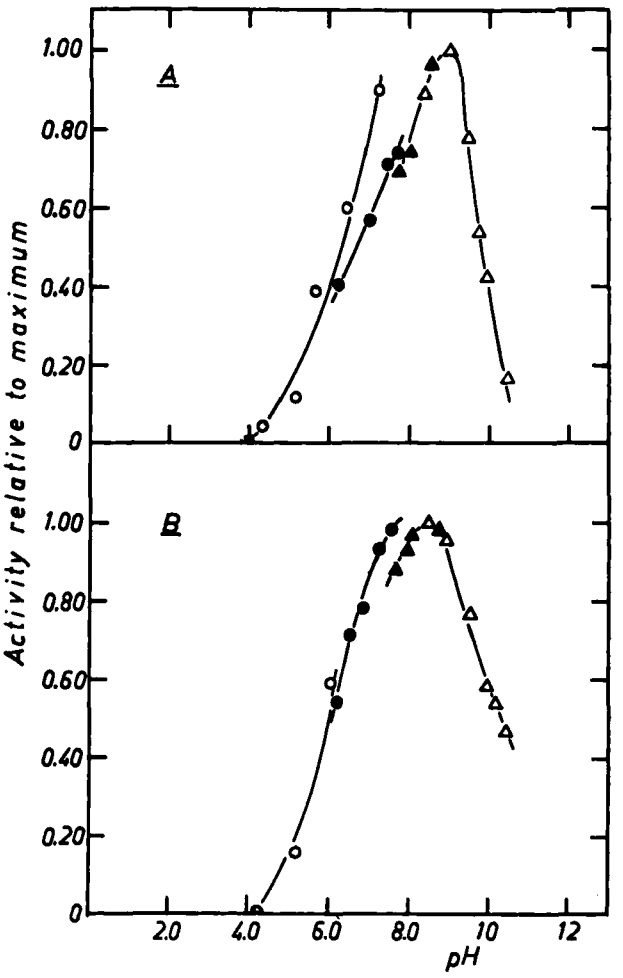

Fig. 1. The influence of pH on the gelatinolytic activity of fraction e_1 and e_2.
A = The effect of pH on the gelatinolytic activity of fraction e_1
B = The effect of pH on the gelatinolytic activity of fraction e_2
O—O = 0.2 M Sodium acetate-acetic acid buffer
●—● = 0.2 M Sodium arsenate--HCl buffer
▲—▲ = 0.2 M Tris-HCl buffer
△—△ = 0.2 M Glycine-NaOH buffer
■—■ = 0.2 M Glycine-HCl buffer

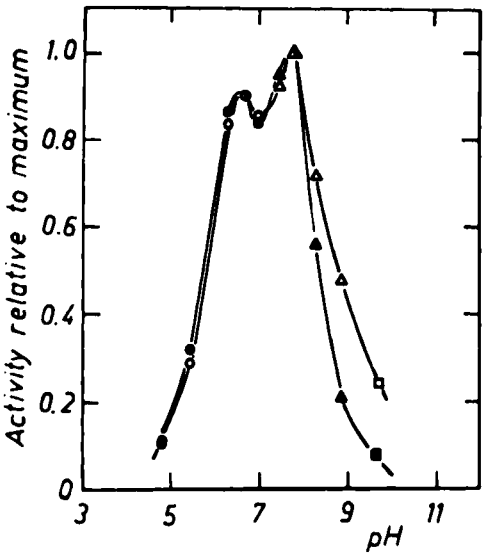

Fig. 2. The influence of pH on the hydrolysis of haemoglobin of fraction e_1 and e_2
Filled bars = The effect of pH on the activity in fraction e_1
Opened bars = The effect of pH on the activity in fraction e_2
○,● = 0.2 M Phosphate buffer
△,▲ = 0.2 M Tris-HCl buffer
□,■ = 0.2 M Glycine-NaOH buffer

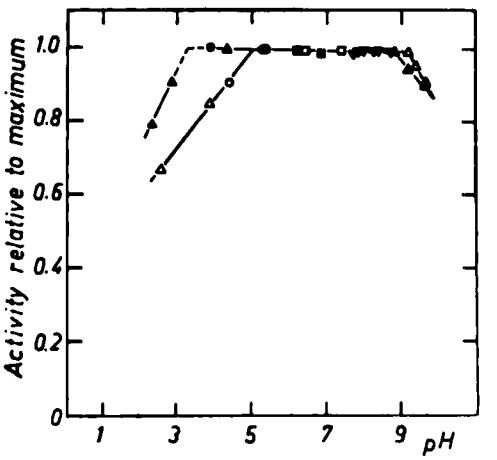

Fig. 3. The effect of pH on the stability of gelatinolytic activity of fractions e_1 and e_2. The enzyme solutions were incubated with buffers of different pH for 30 min at 25°C and the remaining activities were determined at pH 8.8.
Opened symbols = The effect of pH on the stability of the activity of fraction e_1.
Filled symbols = The effect of pH on the stability of the activity of fraction e_2.
△,▲ = Glycine-HCl buffer
○,● = Sodium acetate-acetic acid buffer
□,■ = Sodium arsenate-HCl buffer
▽,▼ = Tris-HCl buffer
△,▲ = Glycine-NaOH buffer
Buffer molarities as for Fig. 1.

(Fig. 1) and haemoglobin as substrates (Fig. 2). Both e_1 and e_2 possessed maximal gelatinolytic activity between pH 8 and 9. At alkaline pH the decrease in activity was slower for fraction e_2 than for e_1 (Fig. 1). Two pH-optima for the hydrolysis of haemoglobin were found to be identical for fractions e_1 and e_2. The first optimum was reached around pH 6.5 and the second at pH 7.8 (Fig. 2).

The stability of the gelatinolytic activity of fractions e_1 and e_2 at different pH values

The pH-stability of the activity in the two fractions is shown in Fig. 3. While the activity of fraction e_1 was stable within the pH range 5.0 to 9.3, that of fraction e_2 was stable between pH 3.5 to 9.0. Only small decreases in activity were noticed when the enzymes were exposed to a pH lower than 3.0 and 5.0 for e_2 and e_1, respectively (Fig. 3).

Influence of moderators on the gelatinolytic activity in fractions e_1 and e_2

The effect of metal ions on the activity is shown in Table I. From the table it can be seen that addition of cadmium, zinc, copper and nickel ions at concentration of 1 mM and 10 mM resulted in inhibition of the activity of both e_1 and e_2, whereas addition of manganese, magnesium and calcium ions activated fraction e_2 at the concentrations tested.

Table II presents data on the effect of various inhibitors on the gelatinolytic activity of e_1 and e_2. The strongest inhibi-

Table I. *The influence of metal ions on the gelatinolytic activity of fraction e_1 and e_2. Activities were determined at pH 8.8*

Metal ions	Final conc.	Gelatinolytic activity as % of control	
		Fraction e_1	Fraction e_2
Ba^{2+}	1 mM	99	102
	10 mM	101	92
Ca^{2+}	1 mM	105	111
	10 mM	103	123
Mg^{2+}	1 mM	99	120
	10 mM	97	122
Zn^{2+}	1 mM	70	67
	10 mM	27	5
Mn^{2+}	1 mM	106	113
	10 mM	95	124
Cu^{2+}	1 mM	79	97
	10 mM	44	55
Ni^{2+}	1 mM	54	70
	10 mM	28	64
Cd^{2+}	1 mM	42	71
	10 mM	19	28
Fe^{3+}	1 mM	99	99
	10 mM	95	94

Table II. *The effect of various inhibitors on the gelatinolytic activity of fraction e_1 and e_2. Activities were determined at pH 8.8*

Substances	Final conc.	Gelatinolytic activity as % of control	
		Fraction e_1	Fraction e_2
Iodoacetic acid	1 mM	80	99
	10 mM	59	20
Acetic acid	1 mM	82	104
anhydride	10 mM	71	93
Sodium lauryl	1 mM	84	104
sulphate	10 mM	15	47
L-cysteine	1 mM	85	98
	10 mM	77	75
Ascorbic acid	1 mM	79	96
	10 mM	73	87
EDTA	1 mM	79	93
	10 mM	68	77
Soy bean	$5 \cdot 10^{-3}$ %	75	57
inhibitor	$5 \cdot 10^{-2}$ %	64	42
	$1 \cdot 10^{-2}$ %	63	42

tion of the enzyme in fraction e_1 was effected by 10 mM sodium lauryl sulphate whereas 10 mM iodoacetic acid was the strongest inhibitor of fraction e_2. Soybean trypsin inhibitor produced a stronger inhibition of the activity of fraction e_2 than e_1.

Gel filtration

The proteins of DOC-treated human saliva were fractionated into three main peaks on Bio-Gel P-100 (Fig. 4:1) with absorbance maxima in fractions 49, 70 and 138. The enzymes hydrolyzing the substrate LPA were mainly eluated close to the void volume. The peptidases hydrolyzing the substrates TAME and gelatin were detected in a wider range in contrast to the enzymes hydrolyzing LPA. Hydrolysis of TAME and gelatin was present in fractions 43—80.

The chromatographic pattern of Bio-Gel P-150 separations of the pools (A, B, C, D) are shown in Fig. 4:2. The activities of the fractions on gelatin and BAEE were expressed in relation to the fraction with maxima. As can be seen from the Fig., peptidases were present in the whole range with K_{av} up to 0.40. Each separation contained several peptidases with molecular weights too close to be separated by this technique. The results from these chromatography experiments indicate differences in substrate specificity between the peptidases.

DISCUSSION

Of the proteolytic activity in human saliva around 50 % is bound to the saliva sediment (*Lindqvist et al.*, 1974). It has been possible to solubilize the peptidase »bound» to saliva sediment (*Lindqvist et al.*, 1974) by use of DOC (*Allan & Crumpton*, 1971). The properties of the

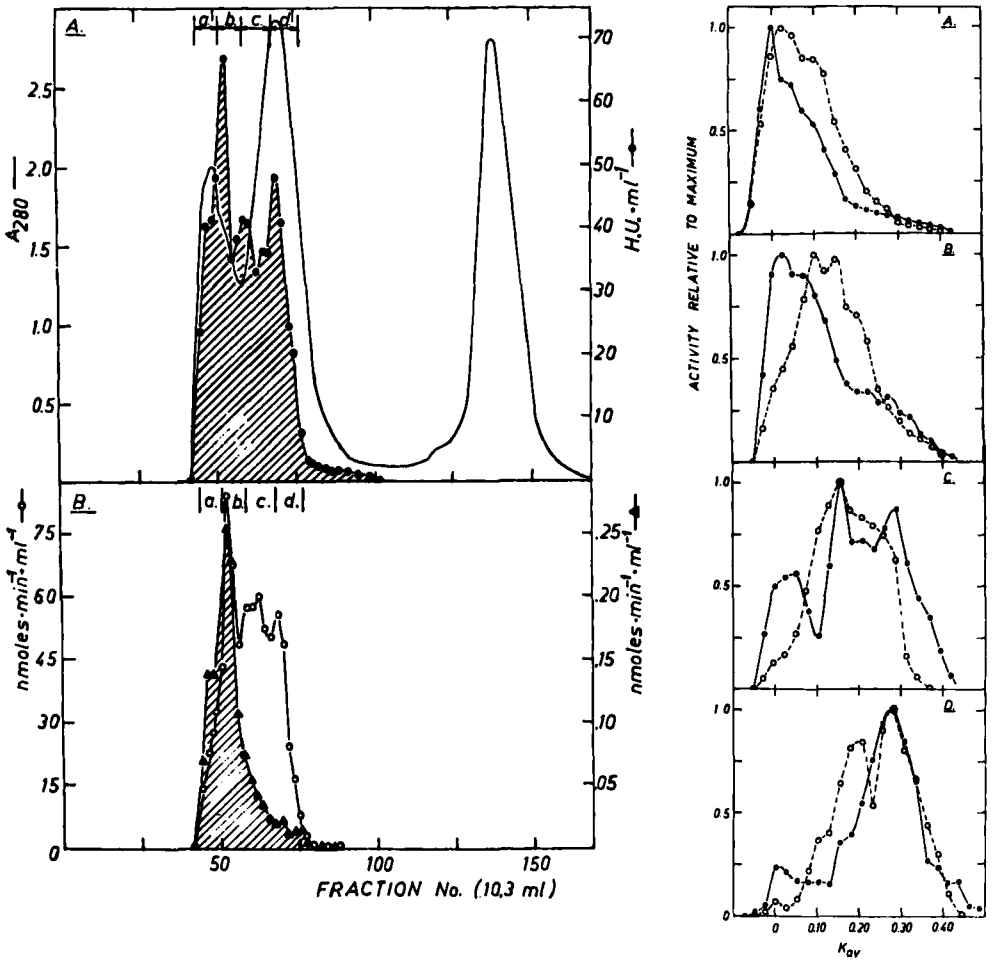

Fig. 4:1. Gel filtration of acetone-precipitated DOC treated saliva (56 ml) on a 4.5 × 92 cm Bio-Gel P-100 column. Eluant was 0.02 M tris-HCl, 0.5 M NaCl, 0.02 % (w/v) sodium azide buffer pH 8.1. Flow rate was 20 ml/h. Fraction volume, 10.3 ml.
A = The proteolytic activity was assayed on gelatin at pH 8.8 (—●—). A_{280} = ————.
B = The proteolytic activity was assayed on TAME at pH 8.1 (—O—) and LPA at pH 8.1 (—▲—).

Fig. 4:2. Gel filtration experiments of pools a, b, c and d from Fig. 4:1 on a Bio-Gel P-150 column (1.93 × 51.2 cm).
(A) 44 ml of pool a (121 mg protein) was concentrated against sucrose to 2.9 ml, (B) 66 ml of pool b (123 mg protein) concentrated to 4.5 ml, (C) 50 ml of pool c (107 mg protein) concentrated to 4.5 ml (3.5 ml was applied) and (D) 84 ml of pool d (314 mg protein) concentrated to 6.0 ml (4.0 ml was applied). Eluant was 0.02 M tris-HCl, 0.5 M NaCl 0.02 % (w/v) sodium azide buffer pH 8.1. The proteolytic activity assayed on BAEE at pH 8.1 (o—o), on gelatin at pH 8.8 (•—•). Flow rate was 30 ml/h. Fraction volume, 10.3 ml.

peptidases released by DOC, named e_2, could be compared with the peptidases soluble in 10,000 g supernatant called e_1.

In preliminary experiments acetone (—18°C) was added to saliva supernatant in the concentration range 35—70 % saturation. The proteolytic activity (95—100 %) was recovered at an acetone con-

centration of 62—70 % saturation. This is in agreement with other studies in which organic solvents have been used for the initial steps in purifying proteolytic enzymes without any great loss of activity (*Moriya, Yamazaki & Fukushima*, 1966); *Angeletti et al.*, 1973).

The pH activity curves on gelatin and haemoglobin were almost the same for the enzymes in fraction e_1 and e_2. One broad maximum of activity between pH 8—9 was found when gelatin was used as substrate (Fig. 1), closely resembling one of the pH-optima of the gelatinolytic activity in dental plaque material (*Söder*, 1967). Two identical pH-optima for both fractions e_1 and e_2 were detected when haemoglobin was used as substrate (Fig. 2). These two maxima reflect the presence of more than one enzyme in each fraction. One of the proteolytic enzymes had a similar pH-optimum to one of the proteases isolated from dental plaque material (*Söder*, 1966).

Differences between the peptidases in the two fractions were found with regard to the influence of metal ions and the effect of inhibitors (Table I and II).

The absence of any effect of calcium ions on the activity in fraction e_1 might depend on the presence of optimal concentrations of this divalent ion in the supernatant e_1. The inhibitor studies showed that reducing agents like ascorbic acid and L-cysteine exerted a strong inhibition of the gelatinolytic activity in both fractions, whereas the thiol reagents such as iodoacetic acid produced only a markedly strong inhibition of the activity in fraction e_2. This suggests that one of the enzymes released from the sediment could possess an active sulfhydryl group. The chelating agent showed a similar inhibition to the reducing agents (Table II). Another difference was found with regard to the influence of pH on the stability of the peptidases. The peptidases in fraction e_2 were more stable at low pH than the peptidases in fraction e_1.

The data obtained from the gel filtration experiments showed the presence of several peptidases in human saliva hydrolysing the substrates BAEE, TAME, LPA and gelatin (Fig. 4:1 and 4:2), which is in agreement with an earlier report (*Lindqvist et al.*, 1974). The result illustrates the problems of separating proteins present in human saliva using methods based on physicochemical properties. Macromolecules in saliva may adsorb small polypeptides which could increase the difficulty of purification (*Schrager & Oates*, 1971). One of the peptidases possessed a high molecular weight as the enzyme was detected in the void volume, but proteins present in solution in high concentration tend to aggregate into higher molecular weight forms (*Levine & Ellison*, 1973). However, it may represent the same enzyme as *Söder* (1972) found after isoelectric focusing of the supernatant of submaxillary and whole saliva and which had the same pI 4.0 as one of the proteolytic enzymes in plaque material with a molecular weight more than 150,000. It is reasonable to assume that some of the proteolytic activity found in plaque material could also be detected in saliva.

The characteristics of the peptidases studied indicated that a trypsin-like enzyme was present in saliva.

The source of these enzymes remains unclear although some of the activity is probably derived from cellular elements (*Lindqvist et al.*, 1974). Trypsin-like enzymes have been found in mast cells and are firmly bound to protein heparin complex (*Lagunoff*, 1968). By electron microscop *Barnett* (1973) showed that mast cells were present in human gingiva,

However, trypsin-like enzymes have also been isolated from mouse submandibular gland (*Ekfors et al.*, 1972).

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