ACTA CHEMICA SCANDINAVICA 20 (1966) 2013–2018

# Demonstration of a Proteolytic Enzyme, Salivain, in Rat Saliva

P. J. RIEKKINEN and T. O. EKFORS

*Department of Anatomy, University of Turku, Turku, Finland*

An alkaline proteolytic enzyme was shown to be excreted into the submandibular saliva of the rat and the same enzyme was identified in the mouth and cardial stomach by using immunoelectrophoresis.

The enzyme is similar to salivain, a proteolytic enzyme purifed from rat submandibular gland, in regard to pH optimum, modifier characteristics, molecular weight, mobility in starch gel electrophoresis and substrate specificity. It also precipitates the anti-salivain serum.

After stimulation by intraperitoneal injections of isoprenaline the enzyme was found to be excreted into saliva at a concentration of about ten times that found in gland tissue homogenate.

In the literature there are several reports about the proteolytic activity of saliva.[1–3] A particularly strong protease activity has been found in the saliva of rodents.[4] However, the origin and chemical characteristics of the proteolytic enzymes excreted into saliva have not been studied.

Earlier we have demonstrated several different trypsin-like proteases in rat submandibular gland.[5] We have purifed and characterized two of them, *i.e. salivain* [6] and *glandulain*.[7,8] The present study concerns the relation of the gland enzymes to the salivary enzyme activity.

## MATERIALS AND METHODS

*Collection of saliva.* Submandibular saliva was collected from adult male rats weighing on average 300 g. The rats had been fasted 24 h before collection. They were anaesthetized with ether, the submandibular ducts were prepared and cannulated with glass cannulas, 0.4 mm in diameter. The excretion of saliva was stimulated by injecting isoprenaline, 0.1 mg/kg, intraperitoneally.

Samples of whole saliva, collected directly from the mouth, and samples of the contents of the cardial part of the stomach, were obtained from rats fed immediately before the experiment and held under ether anaesthesia. The saliva was diluted with distilled water and centrifuged at 3000 rpm for 15 min, and the clear supernatant was studied.

*Substrates, modifiers and activity determinations.* The following substrates were used: $N^{\alpha}$-benzoyl-DL-arginine $\beta$-naphthylamide (BANA) and $N^{\alpha}$-benzoyl-DL-arginine *p*-nitroanilide (BAPA), $N^{\alpha}$-benzoyl-DL-arginine ethyl ester (BAEE), *p*-toluenesulphonyl-L-argi-

nine methyl ester (TAME), $N^{\alpha}$-benzoyl-DL-phenylalanine β-naphthylester (BPANE) and $N^{\alpha}$-carbobenzoxy-DL-phenylalanine *p*-nitroanilide (Nutr. Biochem. Corp. Cleveland, Ohio, U.S.A.). Human haemoglobin was a gift from Kabi AB (Stockholm, Sweden) and casein from E. Merck AG (Darmstadt, Germany).

Substrate stock solutions and the determinations of the hydrolysis rates were made as earlier described.[5,6]

The following modifiers were used: Tetra-N-butyl ammoniumiodide (TNBA), tetra-N-methyl ammoniumiodide (TNMA), cysteine, iodoacetic acid, EDTA, $CACl_2$ (from different commercial sources). Ovomucoid trypsin inhibitor was obtained from Sigma Chem. Corp.

Protein determinations were performed according to the method of Lowry.[9]

*Chromatography.* DEAE-cellulose (Whatman, Floc DE 50) was obtained from W. & R. Balston LTD (England) and Sephadex G-100 (particle size 40—120 μ) from Pharmacia (Uppsala, Sweden). Columns were prepared according to the manufacturer's instructions.

*Starch gel electrophoresis.* Hydrolysed starch was obtained from Connaught Research Laboratories (Toronto, Canada). Runs were performed in a continuous buffer system. Gels (12 g starch in 100 ml of 75 mM Tris-HCl buffer pH 8.6) were prepared in the usual manner. Protein and activity bands were visualized as earlier described.[6] The voltage was 15 V/cm and the running time 4 h.

*Immunoelectrophoresis.* Immunoelectrophoresis was performed in 1 % agar (Agar purified, sterilised and free from inhibitors, obtained from E. Merck AG, Darmstadt, Germany). Gels were prepared in 25 mM veronal buffer pH 8.2 and the run was performed in the same buffer, voltage 6 V/cm, and the running time 4 h. Antibody to salivain was obtained by the following procedure: Purified preparation [6] (0.2 mg in Freund's adjuvans) was injected intradermally into a rabbit six times at 6-day intervals. The serum of the rabbit diluted 1:2 in distilled water was used as antibody and the serum of an uninjected rabbit diluted likewise as control. Immune serum for glandulain was made as described earlier.[7]

## RESULTS

*Hydrolysis rate of BAPA by submandibular saliva.* The hydrolysis rate in 0.1 M glycine-NaOH buffer at pH 9.2, and with a final BAPA concentration of 0.5 mM, was 1100 mμmole/mg/min.

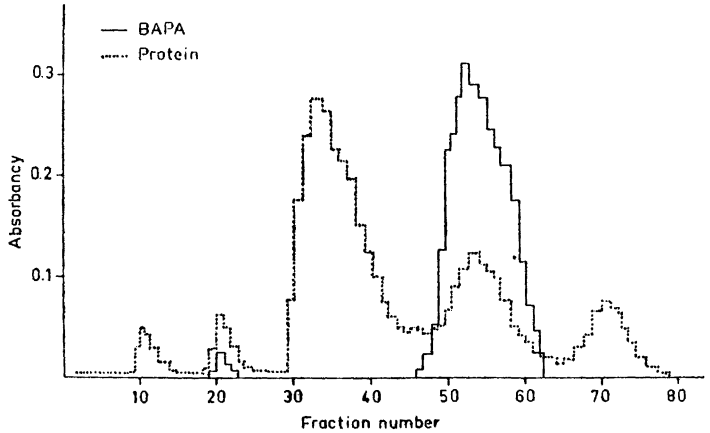


*Fig. 1.* DEAE-cellulose chromatography. The column (2 × 30 cm) was equilibrated with 10 mM Tris-HCl buffer pH 7.0. Submandibular saliva containing 13 mg protein was applied to the column. A linear NaCl concentration (0.02 M to 0.75 M) in the elution buffer was used; the flow rate about 0.5 ml/min.

*DEAE-cellulose chromatography.* Submandibular saliva diluted in distilled water was applied to a DEAE-cellulose column. The distribution of the enzymatic activity was determined using BAPA as substrate. The result is seen in Fig. 1. It is evident that a large part of the protein was eluted from the column before the activity. The fractions showing principal activity (48—58) were pooled and used for the following studies. At the position of the second protein peak there was also low activity towards BAPA. The characteristics of this activity and its relation to the gland enzymes were not studied.

*Sephadex G-100 chromatography.* In order to compare the molecular weight of the salivary enzyme with that of purified salivain the pooled preparation after DEAE-cellulose chromatography and salivain were run in identical conditions through a Sephadex G-100 column. The result is seen in Fig. 2. No difference between the enzymes could be demonstrated in respect to the rate of gel filtration.

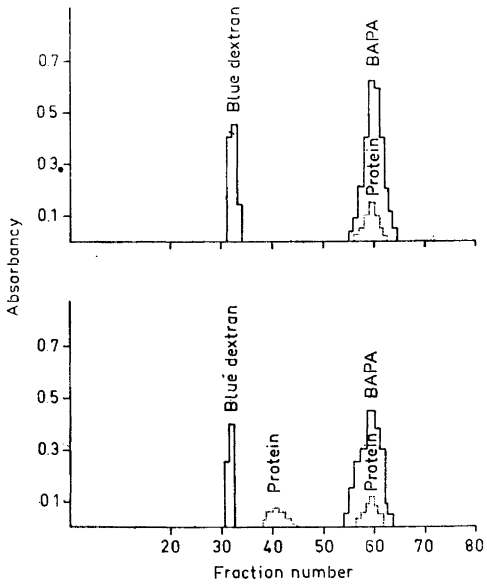


*Fig. 2.* Sephadex G-100 chromatography. A 3 ml sample of the pool obtained from the DEAE-cellulose chromatography containing 0.6 mg protein and a crystal of blue dextran was applied to the column 2 × 160 cm). Elution was performed with 10 mM Tris—HCl buffer pH 7.0, 0.1 M NaCl, and the flow rate 0.25 ml/min. Eighty 6 ml fractions were collected. The colour of blue dextran was measured at 7500 Å. Salivain (0.2 mg in 3 ml) was chromatographed on the same column under identical conditions.

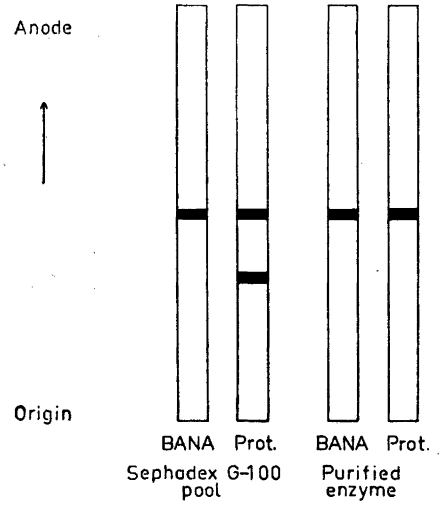


*Fig. 3.* Starch gel electrophoresis at pH 8.6. For details see materials and methods.

*Starch gel electrophoresis.* In parallel starch gel electrophoretic runs, the experimental conditions being the same, both the salivary enzyme and the purified salivain behaved in the same way (Fig. 3).

*Immunoelectrophoresis.* After the DEAE-cellulose chromatography, the pooled preparations of whole saliva, cardial stomach contents, and salivain were subjected to electrophoresis in agar gel for 4 h. The antibody of salivain was then applied in slits in the middle of the gels. The result after 24 h diffusion time is seen in Fig. 4. The precipitation lines were identical with each other.

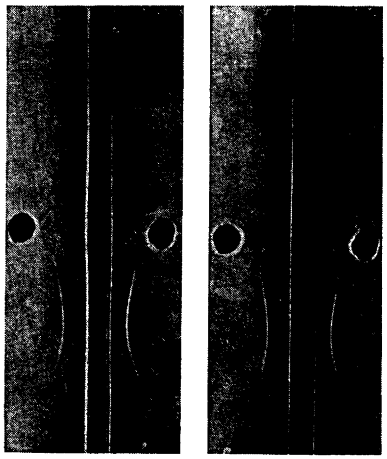

*Fig. 4.* Immunoelectrophoresis. The precipitation lines are from left to right: Salivain, the pooled preparation of saliva after DEAE-cellulose chromatography, whole saliva, and cardial stomach contents. For experimental conditions see text.

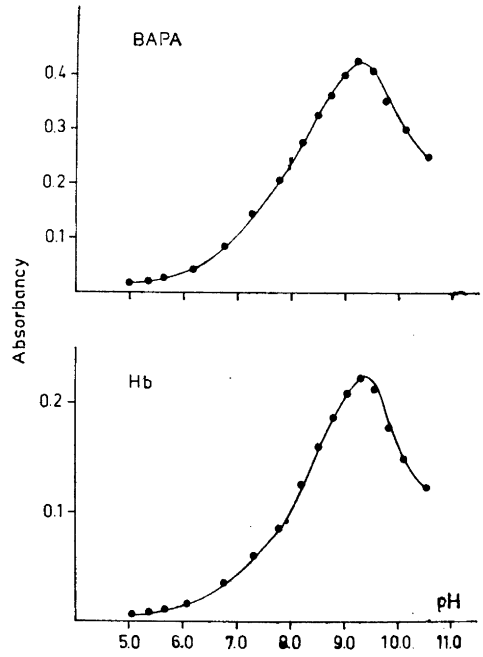


*Fig. 5.* pH-Dependence of the hydrolysis of BAPA and hemoglobin by the pooled preparation of saliva after DEAE-cellulose chromatography.

*pH-Optimum.* The pH-optima of the hydrolysis of BAPA and haemoglobin by the pooled DEAE saliva fractions were about 9.2—9.3 (Fig. 5).

*Modifier characteristics.* Table 1 lists the effect of modifiers on the hydrolysis of BAPA by the pooled preparation after DEAE-cellulose chromatography. In order to facilitate the comparison between the effects and those of the same modifiers on salivain some experiments published earlier [6] were repeated. All the effects on salivary enzyme were almost the same as on salivain. The activating effect of $CaCl_2$ on the BANA hydrolysis of salivain was also found with the salivary enzyme.

*Table 1.* The effect of various modifier substances on the hydrolysis of BAPA by the pooled preparation of saliva after DEAE-cellulose chromatography and by salivain. The figures are the percentage changes observed.

| Modifier | Concentration | Pooled saliva preparation after DEAE-cellulose chromatography | Salivain |
|---|---|---|---|
| $CaCl_2$ | 4 mM | 0 | 0 |
| EDTA | 2 mM | 0 | −10 |
| Cysteine | 0.2 mM | 0 | 0 |
| Iodoacetic acid | 0.2 mM | 0 | 0 |
| Ovomucoid | 0.2 mg/ml | 0 | 0 |
| TNBA | 10 mM | +60 | +50 |
| TNMA | 10 mM | 0 | 0 |

*Table 2.* The relative hydrolysis rates of various substrates by the pooled preparation of saliva after DEAE-cellulose chromatography and by salivain, taking the rate of hydrolysis of BANA as 1. The assays were made under the same experimental conditions.

| Substrate | Concentration | Relative hydrolysis rates | |
|---|---|---|---|
| | | Pooled saliva preparation after DEAE-cellulose chromatography | Salivain |
| BANA | 0.25 mM | 1 | 1 |
| BAPA | 1 mM | 13.7 | 13.7 |
| BAEE | 5 mM | 1770 | 1700 |
| TAME | 5 mM | 265 | 215 |
| BPANE | 0.08 mM | 0.23 | 0.30 |
| Hb (den.) | 1 % | 2.9 | 2.5 |
| Casein | 1 % | 3.7 | 3.8 |

*Substrate specificity.* Table 2 shows the relative hydrolysis values of a number of substrates by the salivary enzyme, taking the hydrolysis rate of BANA as 1. For comparison the equivalent values of salivain similarly calculated are given, too. The pooled preparation after DEAE-cellulose chromatography was found to hydrolyse generally the same substrates at the same relative rates as salivain. The hydrolysis rate of BAPA by the pooled preparation after DEAE-cellulose chromatography was about 3000 mμmole/mg/min. In addition to the data given in the table it was noted that the enzyme did not hydrolyse N-carbobenzoxy-DL-phenylalanine *p*-nitroanilide.

## DISCUSSION

The activity toward BAPA by rat submandibular gland homogenate at pH 9.2 is about 120 mμmole/mg/min, as we have reported,[6] whereas activity toward BAPA in saliva after isoprenaline stimulation was found to be about

1100 mμmole/mg/min. This means that the activity is nearly ten times more concentrated in submandibular saliva and that the activity is really excreted into the saliva.

The studies on the pooled preparation after DEAE-cellulose chromatography show that salivary enzyme hydrolyses BAPA and haemoglobin optimally at pH 9.2—9.3. All the tested modifiers had almost the same effects on the salivary enzyme as on salivain. The relative hydrolysis rates of various substrates were approximately equal. Slight differences may be due to the fact that the pooled preparation still contained contaminating protein. After Sephadex G-100 chromatography the activity peak was in the same fraction in both runs and so it is evident that the molecular weights of the enzymes are the same. In starch gel electrophoresis the activity bands were obtained 6.5 cm from origin in both cases. In immunoelectrophoresis the precipitation lines of salivain, the DEAE pool, the whole saliva, and the cardial stomach contents were in exactly the same place when the same anti-salivain serum was used, whereas antibody of glandulain did not give precipitation lines in identical conditions. On the basis of the evidence listed above we can conclude that the alkaline protease in rat submandibular saliva is salivain.

Studies have shown that the pH 9.2 protease is not present in rat sublingual and parotis glands,[10] hence salivain is excreted only from the submandibular glands.

The high pH optimum does not seem to accord with the possible digestive function of salivain. Junqueira [11] has found, however, that alkaline conditions persist in the rat stomach several hours after feeding. Under these conditions salivain can split protein for a considerable period even in the stomach. It is also known that salivain is remarkably active at neutral pH. Junqueira has reported that according to his calculations the rat digests more than half of the ingested protein by saliva. It is evident that this digestion is due principally to the proteolytic activity of salivain.

## REFERENCES

1. Voss, O. *Z. physiol. Chem.* **197** (1931) 42.
2. Sreebny, L. M. *J. Dental Res.* **32** (1953) 686.
3. Resnik, J., Willent, N. O. and Shaw, J. H. *J. Nutr.* **66** (1958) 411.
4. Junqueira, L. C. U. In Sreebny, L. M. and Meyer, J. Eds., *Salivary Glands and Their Secretions*, Macmillan, New York 1964, p. 105.
5. Riekkinen, P. J. and Hopsu, V. K. *Ann. Med. Exptl. Fenniæ (Helsinki)* **43** (1965) 6.
6. Riekkinen, P. J., Ekfors, T. O. and Hopsu, V. K. *Biochim. Biophys. Acta* **118** (1966) 604.
7. Riekkinen, P. J., Ekfors, T. O., Hollmén, T. and Hopsu-Havu, V. K. *Enzymologia. In press.*
8. Riekkinen, P. J., Ekfors, T. O. and Hopsu-Havu, V. K. *Enzymologia. In press.*
9. Lowry, O. H., Rosebrough, N. J., Farr, A. R. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
10. Riekkinen, P. J. and Ekfors, T. O. *Unpublished results.*
11. Junqueira, L. C. U. In *Secretion and Excretion*, Springer, Berlin-Heidelberg-New York 1965, p. 42.

Received April 27, 1966.