From: The Department of Anatomy, University of Turku, Finland.

STUDIES ON THE ALKALINE TRYPSIN-LIKE ENZYMES IN RAT SUBMANDIBULAR GLAND AND SALIVA

by

V. K. HOPSU-HAVU*

P. J. RIEKKINEN

T. O. EKFORS

INTRODUCTION

The salivary glands are known to contain a proteolytic activity since 1873 when *Hüfner* published his studies. The protelytic activity has been found to vary considerably from one species to another (*Rothschild & Junqueira,* 1951; *Sreebny et al.,* 1955; *Bayerle et al.,* 1960; *Junqueira & de Moraes,* 1965). The presence of a proteolytic activity in the saliva has also been demonstrated (*Voss,* 1931; *Fantl & Weinmann,* 1935). Some species, e. g. human, have a very low activity and the proteolysis has been suggested to be due to contamination from bacteria (*Willstätter et al.,* 1929) and leucocytes. Some other species, e. g. rodents, have a remarkably strong proteolytic activity in the saliva as well as in the salivary glands (*Junqueira & de Moraes,* 1965).

In the studies on salivary glands merely crude homogenates have been used and no attempt to purify the proteolytic activity has been taken. The activity has been considered to be due to one

*) V. K. Hopsu-Havu, in earlier publications V. K. Hopsu, has been aided by grants from Sigrid Juselius Foundation, The Finnish State Medical Research Council, and The National Institutes of Health (Grant no. TW-00103).

enzyme, the properties of which have been studied. The aim of these studies has been to analyse more thoroughly the protease pattern of the rat submandibular gland and saliva with special attention to the trypsin-like enzymes. A number of enzymes have been separated and the presence of still others is likely. The enzymes purified and characterized resemble pancreatic trypsin mainly in their substrate specificity, i. e., all of them split Nα-benzoyl-*DL*-arginine 2-naphtylamide (BANA), p-nitroanilide (BAPA) and methyl ester (BAME) as well as proteins. The technical and methodological details have been published earlier in the reports of these series of works (*Riekkinen & Hopsu,* 1965; *Riekkinen et al.,* 1966; *Riekkinen & Ekfors,* 1966; *Riekkinen,* 1966 a; *Riekkinen et al.,* 1967 a; *Riekkinen et al.,* 1967 b; *Ekfors et al.,* 1967).

RESULTS

Chromatographic fractionation of the submandibular homogenate

Rat submandibular homogenate was chromatographed through a DEAE-cellulose column in order to have a rough idea about its protease composition (*Riekkinen & Hopsu,* 1965). The enzyme assay was carried out with Nα-benzoyl-DL-arginine 2-naphthylamide as substrate. Five peaks were repeatedly obtained and some enzymic characteristics were determined. The results can be summarized as follows:

Peak I: The pH optimum was at 8.2 and the enzymic activity was inhibited strongly by Cu^{2+} ions.

Peak II: The pH optimum was at about pH 7, the enzymic activity was typically sulphhydryl-dependent being activated by cysteine and inhibited by iodoacetic acid.

Peak III: The most alkaline protease, pH optimum at 9.2—9.3 and it was not much affected by a number of modifier substances.

Peaks IV and V: These were most active at acid pH range, at 6.2 and 5.3 respectively. The latter of them was identified as cathepsin B. These enzymes we have so far not further purified and characterized.

On the basis of this DEAE-cellulose chromatography it was concluded that the rat submandibular gland contains at least five separate activities capable of hydrolyzing typical chromogenic trypsin substrates.

Purification and characterization of the enzymes

The purification procedures used are summarized in Fig. 1. Altogether four separate enzymic activities could be readily purified and characterized, i. e., salivain, glandulain, kallikrein-like peptidase and a neutral protease.

Salivain. The most alkaline enzyme, peak III, was first purified and characterized (*Riekkinen et al.,* 1966). The purity of the

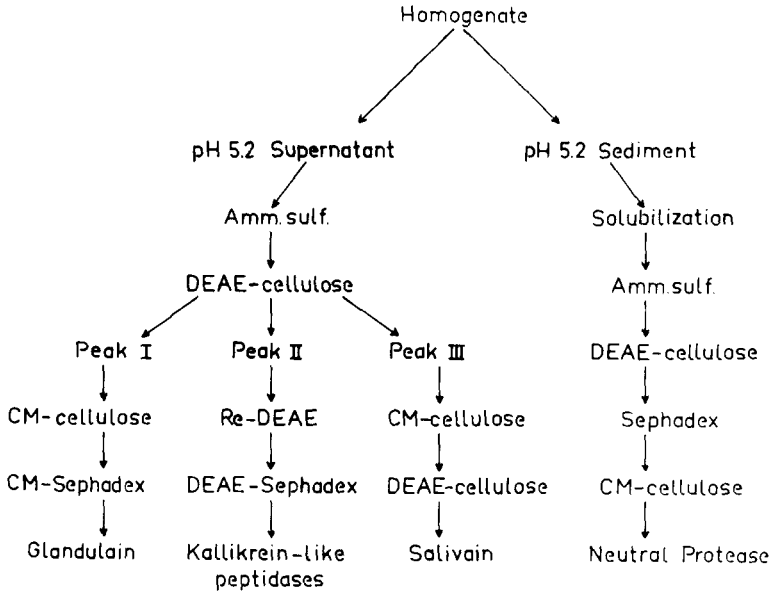

Fig. 1. Purification procedures of submandibular enzymes.

final preparation was assessed by ultracentrifugation and chromatography on Sephadex G-200 (superfine) and CM-Sephadex, as well as by starch gel electrophoresis in which it behaved as a homogenous protein.

Sedimentation constant was 2.5 S. The approximate molecular weight was determined on Sephadex G-100 column and it was found to be about 30000.

The enzyme was most stable at alkaline pH. It hydrolyzed, in general, the same substrates as trypsin. The enzyme hydrolyzed BAPA about four times faster than trypsin, whereas the rate of hydrolysis of BANA was about the same. BAME and BAEE (N-benzoyl-arginine ethylester) were hydrolyzed about ten times

faster than by pancreatic trypsin. Protein substrates, casein, hæmoglobin and bovine serum albumin were hydrolyzed by the submandibular protease at rates comparable with those found with trypsin.

Heavy metals and EDTA had little effect at low concentrations. Organophosphorus compounds inhibited the enzyme at equimolar concentrations, and it can be decided that serine is involved in the active centre of this enzyme as is true with trypsin, chymotrypsin and thrombin, too. Tetra-N-butyl ammonium iodide activated the enzyme over a wide concentration range. SH compounds and reagents had no influence. Lima bean and ovomucoid trypsin inhibitors were without effect but Trasylol, a potent kallikrein and trypsin inhibitor purifed from salivary glands, inhibited this enzyme strongly.

This enzyme would be placed in the EC Class 3.4 (peptide hydrolase) according to the International Union of Biochemistry Commission on Enzyme Nomenclature (Report of the Commission of Enzymes, 1961). Since the enzyme was extracted from the salivary glands and it is known to be present in saliva, the name Salivain was suggested in accordance with the rule suggested by *Greenberg* and *Winnick* (1949).

Glandulain. The next step in the work was to purify the peak I (*Riekkinen et al.*, 1967 a; *Riekkinen et al.*, 1967 b). The procedure was essentially the same as that of salivain, as can be seen in Fig. 1. The only difference was the last chromatography; CM-Sephadex replaced DEAE-Sephadex. The purity of the final preparation was studied by ultracentrifugation and in Sephadex G-200 superfine, DEAE-Sephadex and CM-cellulose chromatographies. In DEAE-Sephadex column the protease preparation was shown to contain a minor inactive protein component. The other chromatographies, starch gel electrophoresis, immunoelectrophoresis and ultracentrifugation demonstrated only one protein component. The molecular weight of the enzyme protein was found to be 21500 and 23000 with Sephadex G-100 and ultracentrifugation analysis, respectively.

The enzyme hydrolyzed the substrates tested optimally at pH 8.0—8.2. It was most stable at around pH 3 and was stabilized by a number of cations and anions. The enzyme readily hydroly-

zed proteins and the same synthetic substrates as trypsin and salivain. The relative hydrolysis rates of the various substrates were, however, characteristically different by all of these enzymes. The complete inhibition of this protease by lima bean and ovomucoid trypsin inhibitors was a clear difference from salivain. Trasylol^R and $CuCl_2$ were other very potent inhibitors. The enzyme was not affected by cysteine, sulphydryl reagents or diethyl p-nitrophenyl phosphate and was slightly activated by tetra-N-butyl ammonium iodide.

The name *Glandulain* was suggested for this enzyme.

The neutral protease. This enzyme was found to be particle-bound and the pH 5.2 sediment was used for further purification (*Riekkinen,* 1966 a). The great deal of other enzymes remain in this step in the supernatant. The pH optimum of this protease was 6.8—7.1. BAPA was hydrolyzed only one third as fast as BANA. Ester substrates were hydrolyzed only 2—3 times more rapidly than the corresponding amides.

Cysteine and mercaptoethylamine activated strongly and SH reagents inhibited drastically the enzymic activity. Bivalent metals were inhibitory and E-600 and DFP had no influence. The approximate molecular weight was determined in Sephadex G-100 gel filtration and was found to be about 48000.

Four isozymic forms of a kallikrein-like peptidase. Three peaks hydrolyzing BAPA were obtained in the purification procedure of salivain and glandulain when the pooled preparation of the fractions of Sephadex G-100 was chromatographed through DEAE-cellulose column (*Ekfors et al.,* 1967). The second peak was found to be no one of the enzymes demonstrated in the DEAE-cellulose chromatography of the homogenate. The most striking feature of this peak was the low activity towards haemoglobin. The preparation of combined fractions of this peak was re-chromatographed through a column of DEAE-cellulose and two partially separated activity peaks were obtained. Both of them were put into a DEAE-Sephadex column in identical conditions and in both cases two activities hydrolyzing BAPA were found. So we had four preparations which we named A, B, C and D according to the order in which they were obtained from chromatographies. In order to ensure the non-identity of the four preparations, a DEAE-cellulose chromatography was run, in which a combined

sample of each of the four preparations was applicated. A complete separation was accomplished. Starch gel electrophoresis gave in principle an identical result so that preparation D moved fastest to the anode at pH 8.6, then came C, B and A. The molecular weight of A, B and D was found to be about 25000 and that of C about 28000 when determined by Sephadex G filtration.

Immunologically the four preparations were identical with each other and salivain since they all gave an identical precipitation line with the rabbit anti-salivain serum.

All the preparations hydrolyzed BAPA optimally at pH 8.2—8.3. Substituded basic aminoacid derivaties BAPA and BANA were hydrolyzed readily by all of the preparations as well as substituted ester substrate BAEE and TAME. The hydrolysis rates of protein substrates were very low. Only relatively small quantitative differences in substrate specificity and modifier properties could be demonstrated between the preparations. The clearest difference from salivain and glandulain was the partial inhibition by lima bean trypsin inhibitor. The low protein hydrolysis rate speaks for strict substrate specificity. It is possible that our preparations represent four isozymic forms of the rat submandibular kallikrein. In order to ensure the identity of the enzyme with kallikrein, however, the biological activity of the preparations must be tested.

Demonstration of salivain in rat saliva

Rat saliva was collected from submandibular ducts in aether anaestehesia and stimulating the excretion by intraperitoneal injections of isoprenaline (*Riekkinen & Ekfors,* 1966). The enzymic activity of collected saliva towards BAPA was ten times higher than that found in the submandibular gland homogenate. Submandibular saliva was fractionated in DEAE-cellulose chromatography and fractions showing peak activity towards BAPA were used for the following studies. In order to get an idea about the relation of the molecular weight of the salivary enzyme and of purified salivain, both were run in identical conditions trough a column of Sephadex G-100. No difference between the enzymes could be demonstrated in respect to the rate of gel filtration. Both preparations behaved equally in starch gel electrophoresis, too. The pH optimum for the hydrolysis of BAPA by the salivary

enzyme was the same as that by salivain, namely 9.2—9.3. Modifier characteristics and substrate specificity studies revealed only minimal quantitative differences between salivain and the salivary preparation.

In immunoelectrophoresis identical precipitation lines could be demonstrated with purified salivain, the salivary preparation obtained in DEAE-chromatography, whole saliva and cardial stomach content supernatant by using the same antisalivain rabbit serum. Anti-glandulain serum, on the other hand, did not give any precipitation lines. On the ground of evidence listed, it is concluded that the alkaline protease in rat submandibular saliva is salivain.

Effect of isoprenaline on the submandibular salivain and glandulain activity

Isoprenaline treatment of rats (injected daily for eight days, 20 mg/kg) caused hyperplasia in submandibular gland tissue and the increase in weight was about fourfold (*Riekkinen*, 1966 b). Isoprenaline decreased drastically the salivain activity in gland tissue, but did not effect remarkably the activity of glandulain. This result agrees well with the finding that salivain is a secretory enzyme, but glandulain not. The latter enzyme was found, on the other hand, to be under the control of male sex hormones.

DISCUSSION

In the light of the evidence presented, it is obvious that the proteolytic enzyme pattern of the rat submandibular gland is much more complicated than has been thought. In order to lighten the comparison between the purified enzymes some properties have been gathered in Table I. Furthemore, this study is concerned only with trypsin-like enzymes, i. e. those capable of hydrolyzing N-substituted basic aminoacid derivatives. Already also a chymotrypsin-like enzyme (*Riekkinen & Hopsu*, 1966) has been purified from the rat submandibular homogenate and there may be other proteases hydrolyzing other kinds of synthetic substrates.

Very little can be said about the function of the enzymes described. The only evident thing is that salivain is a secretory, digestive enzyme. The high pH optimum of salivain does not

Table I. *Some characteristics of the alkaline trypsin-like enzymes purified from the submandibular gland*

Property	Salivain	Glandulain	The neutral protease	The kallikreinlike peptidase
Mol. weight	30000	22000	48000	25000—28000
pH-optimum	9.2—9.3	8.0—8.2	6.8—7.1	8.2—8.3
Inhibitors				
Organophosphorus compounds (E-600)	Inhibited	Not inhibited	Not inhibited	Not inhibited
Natural trypsin inhibitors	Trasylol inhibits	Trasylol, lima bean and ovomucoid inhibit	Lima bean inhibits partially	Lima bean and Trasylol inhibit partially
Others		$CuCl_2$ inhibits (2×10^{-5}M)	Iodoacetamide inhibits (1mM)	
Activators	TNBA	TNBA	Cysteine and mercaptoethylamine	
Substrate specificity*	Proteins	Proteins	Proteins	Proteins (slowly)
	BAEE	TAME	BAEE	BAEE
	TAME	BAPA	TAME	TAME
	BAPA	BAEE	BANA	BAPA
	BANA	BANA	BAPA	BANA
	LysME	LysME and	LysME	LysME
	ArgNA	ArgNA not hydrolysed	ArgNA not hydrolysed	ArgNA not hydrolysed

Abbreviations

ArgNa, *L*-argine 2-naphtylamide
BAEE, Nα-benzoyl-*DL*-arginine ethyl ester
BAPA, Nα-benzoyl-*DL*-arginine p-nitroanilide
BANA, Nα-benzoyl-*DL*-arginine 2-naphthylamide
LysME, *L*-lysine methyl ester
TAME, Nα-toluene p-sulphonyl-*DL*-arginine methyl ester
TNBA, Tetra-N-butyl ammonium iodide

* Substrates in order of hydrolysis rate

seem to be ideal for this function. *Junqueira and de Moraes* (1965) have found, however, that alkaline conditions are retained in the rat stomach several hours after meal. It is also known that salivain is remarkably active at neutral pH. Junqueira has reported that according to his calculations rats digest more than a half of the ingested proteins by saliva. It is evident that this digestion is principally effected by the proteolytic activity of salivain. The other enzymes obtained from the submandibular gland are, on the other hand, structural or metabolic intracellular enzymes and, therefore, to be included under the title cathepsins.

SUMMARY

The pattern of enzymes present in the rat submandibular gland and capable of hydrolysing Nα-benzoyl-DL-arginine β-naphtylamide and other substrates for trypsin-like enzymes, has been analyzed and characterized. A single chromatography of the homogenate on DEAE-cellulose revealed the presence of five separate enzymic species of this kind. Highly purified enzyme preparations of several enzymes were obtained for physical and chemical characterization by employing more elaborate purification procedures. The enzymes were named as salivain, glandulain, a kallikrein-like peptidase (four isozymic forms) and a neutral protease. All of these were identified as separate enzyme species on the basis of their molecular weights, pH optima, substrate specificities, modifier characteristics, etc. Salivain was shown to be a secretory enzyme and glandulain to be under the control of male sex hormones.

RÉSUMÉ

ETUDES SUR LES ENZYMES ALCALINS DU TYPE DE LA TRYPSINE PRÉSENTS DANS LA SALIVE ET DANS LA GLANDE SOUS-MAXILLAIRE DU RAT

Il a été procédé à l'analyse et la détermination des caractères des enzymes présents dans la glande sous-maxillaire du rat et capables d'hydrolyser la β-naphtylamide de Nα-benzoyl-DL-arginine et d'autres substrats utilisés pour les enzymes du type de la trypsine. Par simple chromatographie du broyat homogène sur DAEA-cellulose, la présence de cinq espèces distinctes d'enzymes de ce genre a été mise en évidence. En employant des procédés

plus complexes de purification, on a obtenu plusieurs enzymes sous forme hautement purifiée permettant la détermination des caractères physiques et chimiques. Les enzymes ont été appelés salivaïne, glandulaïne, une peptidase du type kallikréine (quatre formes d'isozymes) et une protéase neutre. Tous ces enzymes ont été caractérisés comme espèces distinctes sur la base de leur poids moléculaire, leur pH optimum, la spécificité en ce qui concerne le substrat, les caractères des effecteurs, etc. Il a été montré que la salivaïne était un enzyme secrétoire et que la glandulaïne était sous la dépendance des hormones sexuelles mâles.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER DIE ALKALISCHE TRYPSIN-ÄHNLICHE ENZYME IN DER SUBMANDIBULARIS UND IN DER SPEICHEL DER RATTE

Die in der Submandibularis der Ratte vorkommenden Enzyme, die N_{α}-benzoyl-DL-Arginine β-Naphthylamide und andere Substrate der Trypsin-ähnlichen Enzyme hydrolysieren können, wurden analysiert und characterisiert. Eine Chromatographie der Homogenate mit DEAE-Cellulose ergab die Anwesenheit von fünf getrennten Enzymen dieser Art. Hochgereinigte Enzympräparate wurden erhalten von vielen Enzymen für die physichalische und chemische Characterisierung durch kompliziertere Reinigungsverfahren. Die Enzyme wurden bezeihnet: Salivain, Glandulain, eine kallikrein-ähnliche Peptidase (mit vier Isoenzymen) und eine neutrale Protease. Diese Enzyme wurden als getrennte Enzymarten identifiziert auf der Basis ihre Molekulargewichte, der pH-Optima, der Substratspezifität und ihres Verhaltens gegenüber Inhibitoren und Aktivatoren. Salivain erwies sich als ein sekretorisches Enzym und Glandulain war einbezogen unter die Kontrolle der männlichen Sexualhormone.

REFERENCES

Bayerle, H. & A. Chryssikopoulos, 1960: Zur Kenntnis des proteolytischen System der Speicheldrüsen. *Z. Naturforsch. 15:* 721.

Ekfors, T. O., P. J. Riekkinen, T. Malmiharju & V. K. Hopsu-Havu, 1967; Four isozymic forms of a peptidase resembling kallikrein purified from the rat submandibular gland. Hoppe Seyler's *Z. physiol. Chem., 348:* 111.

Fantl, P. & J. Weinmann, 1935: Die proteolytische Fähigkeit des Speichels. I Mitteilung: Die Eigenproteolyse des Speichels. Biochem. Zeit., *281:* 37.

Greenberg, D. M. & Winnick, 1949: Plant proteases I. Activationinhibition reactions. *135:* 761.

Hüfner, R., 1873: Cited in R. Willstätter, Hoppe Seyler's Z. physiol. Chem. *185:* 85 1924.

Jungueira, L. C. U. & F. Fava de Moraes, 1965: Comparative espects of the vertebrate major salivary gland biology. Secretion and Excretion, Springer-Verlag, Berlin, Heidelberg, New York, pp. 36—48.

Report of the Commission of Enzymes, 1961: I. U. B. Symposium series *20:* 63. Pergamon Press, Oxford.

Riekkinen, P. J., 1966 a: A neutral trypsin-like protease from the rat submandibular gland. Acta chem. scand. *20:* 1440.

Riekkinen, P. J., 1966 b: Rat submandibular proteases, as influenced by diurnal rhytm, feeding, age, sex, and isoprenaline treatment. Ann. Med. Fenn. *44:* 549.

Riekkinen, P. J. & T. O. Ekfors, 1966: Demonstration of a proteolytic enzyme, salivain, in rat saliva. Acta chem, scand., *20:* 2013.

Riekkinen, P. J., T. O. Ekfors, T. Hollmen & V. K. Hopsu, 1967 a: Purification and physical characteristics of a trypsin-like protease from the rat submandibular gland. Enzymologia *32:* 97.

Riekkinen, P. J., T. O. Ekfors & V. K. Hopsu, 1967 b: Substrate specificity and modifier characteristics of an alkaline protease from rat submandibular gland: Glandulain. Enzymologia *32:* 110.

Riekkinen, P. J., T. O. Ekfors & V. K. Hopsu, 1966 e: Purification and characteristics of an alkaline protease from rat-submandibular gland. Biochim. Bioyhys. Acta *118:* 604.

Riekkinen, P. J. & V. K. Hopsu, 1965: Studies in the salivary glands. I Trypsin-like enzymes. Ann. Med. exp. Fenn. 43: 6.

Riekkinen, P. J. & V. K. Hopsu, 1966: A chymotrypsin-like protease from the rat submandibular gland. Acta chem. scand. *20:* 2169.

Rothschild, H. & L. C. U. Junqueira, 1951: The possible correlation between cathepsin activity and protein synthesis. Arc. Biochem. Biophys. *34:* 453.

Sreebny, L. M., J. Mayer & E. Bachem, 1955: Characteristics of a proteolytic enzyme in the submaxillary and sublingual glands of the albino rat. J. Dent. Res. *34:* 915.

Willstätter, R., E. Bamann & M. Rohdewald, 1929: Über die Enzyme der Speicheldrüse. Hoppe Seyler's Z. physiol. Chem. *180:* 182.

Voss, O., 1931: Über eine trypsinartige Protease im Sekret der menschlichen Parotisdrüse. Hoppe Seyler's Z. physiol. Chem. *197:* 42.

Address: *Department of Anatomy,*
University of of Turku,
Turku 3, Finland