From: The Institute of Dentistry, University of Turku, Turku, Finland.

STUDIES ON ORAL ENZYMES

III. FRACTIONATION OF ENZYMES IN HUMAN DENTAL PLAQUE HYDROLYZING DENATURED HEMOGLOBIN

by

KAUKO K. MÄKINEN

INTRODUCTION

The hydrolytic enzymes in human plaque that possess proteolytic activity may be associated with the "solubility" of various hard tissues in the oral cavity. Several investigators have identified protein-splitting enzymes in saliva, but so far no one has ascertained their nature. The investigation on oral enzymes was started in this laboratory because an anticipated relationship between certain hydrolytic enzymes in the dental plaque and some oral diseases. Some aminopeptidase-like enzymes found in saliva and plaque were described in the two previous papers on this subject (*Mäkinen,* 1966 a, b).

Chauncey et al. (1954) have studied such enzymes, and they have demonstrated that one of the salivary proteases is capable of hydrolyzing hemoglobin, fibrin and casein. This proteolytic activity occurred at both acid and alkaline pH. *Sreebny* (1954) has observed protease activity in frozen-dried rat submaxillary glands against casein, azocoll, gelatin and benzoyl-L-arginine amide;

This work was supported by a research grant from the Yrjö Jahnson Foundation, Helsinki.

these enzyme(s) did not hydrolyze native collagen. In this paper endopeptidase-like enzyme activities in the human dental plaque debris will be described.

MATERIAL AND METHODS

The reagents[1]) and general methods used in this investigation were mostly the same as in the first two papers in this series. There were, however, a few changes.

1. Preparation of plaque

The plaque material was collected from nine pre-determined areas on the teeth. — These areas are given in Results. — This meant that the obtained amounts of plaque were smaller (about 20—50 mg) than in the previous study, although the collection time was the same, or about a fortnight, in both cases. After weighing, the bacterial plaque was suspended in 1 ml of cold (+ 1—+ 4° C) 0.154 M NaCl solution and the suspension was centrifuged in cold (23,500 × g, 15 min.). The clear supernatant was used as an enzyme preparation in various experiments. In addition, mixed plaque material was used as enzyme source in an experiment where the hydrolysis of four undenatured proteins were tested. The preparation of this enzyme solution is described in the previous paper (*Mäkinen,* 1966 b).

2. Determination of endopeptidase-like activity

The enzyme activity was determined by the method of *Anson* (*Rick,* 1963), with certain modifications. The enzymes were allowed to hydrolyze substances from denatured hemoglobin or from undenatured proteins, which are soluble in trichloroacetic acid, and whose tyrosine and tryptophane content was determined according to the method of *Folin-Ciocalteu,* as used by *Anson.* The original reagent volumes suggested by *Anson* were reduced to one hundredth, and the incubation time was prolonged to 24 and 48 hours at 37° C. Finally, tyrosine and tryptophane content was determined with a Beckman Microcolorimeter by reading the intensity of the color at 578 mμ. In the case of

[1]) The same abbreviations are used as in the first two papers in this series (*Mäkinen,* 1966 a, b).

hemoglobin, the protein was denatured as suggested by *Anson*. The other proteins used as substrates for plaque enzymes were simply weighed to the same concentration (2 g per 100 ml) in the same solution as hemoglobin, without adding urea and NaOH.

3. Substrate specificity

The substrate specificity of the enzyme peaks hydrolyzing hemoglobin was determined using various amino acid β-naphthylamides and α- or β-naphthylesters of certain carboxylic acids. The method (cf. paper No. I) was slightly changed in that the hydrolysis of naphthylesters were stopped by adding, first, 10 μl of 0.1 % diazotized 4-amino-3:1-dimethylazobenzen (Fast Garnet GBC Salt), and then after a 10 minutes standing, 40 μl of 1 M acetic buffer. The color intensity was then determined as described before (*Mäkinen*, 1966 a).

4. Affector studies

The effects of certain enzyme inhibitors and activators were studied in a reaction mixture composed of 50 μl of universal buffer, pH 7.0 (cf. paper No. I in this series *Mäkinen*, 1966 a), 10 μl of enzyme solution (from the pooled fractions obtained in gel filtration), and 10 μl of affector solution. The reaction was stopped with trichloroacetic acid. The affector concentrations were 0.2×10^{-3} M and 0.1×10^{-3} M. A single exception was formed by PCMB, tested in the concentrations 0.2×10^{-4} M and 0.2×10^{-5} M.

5. Reagents

These were purchased from the sources listed in the previous papers, except for hemoglobin (lyophilized, salt free), casein (Hammersten), egg albumin (2 × cryst.), bovine albumin (cryst.) and lactalbumin (Edible), which were products of Mann Research Laboratories, Inc. (New York, USA).

RESULTS

1. Hydrolysis of denatured and undenatured proteins

Table I shows the hydrolysis rates of various protein molecules and of denatured hemoglobin. The results presented clear-

Table I.

Ability of plaque proteases to hydrolyze various protein molecules. The values are in comparative relation with the rate, marked 100, at which denatured hemoglobin was hydrolyzed. Incubation time 24 hours.

Substrate	Hydrolysis rate
Denatured hemoglobin	100
Casein	4.5
Egg albumin	20
Bovine serum albumin	7.5
Lactalbumin	8.5

ly show that the denatured molecules were much more rapidly broken down by the mixed plaque enzyme preparation used in the experiment, than were the undenatured protein molecules.

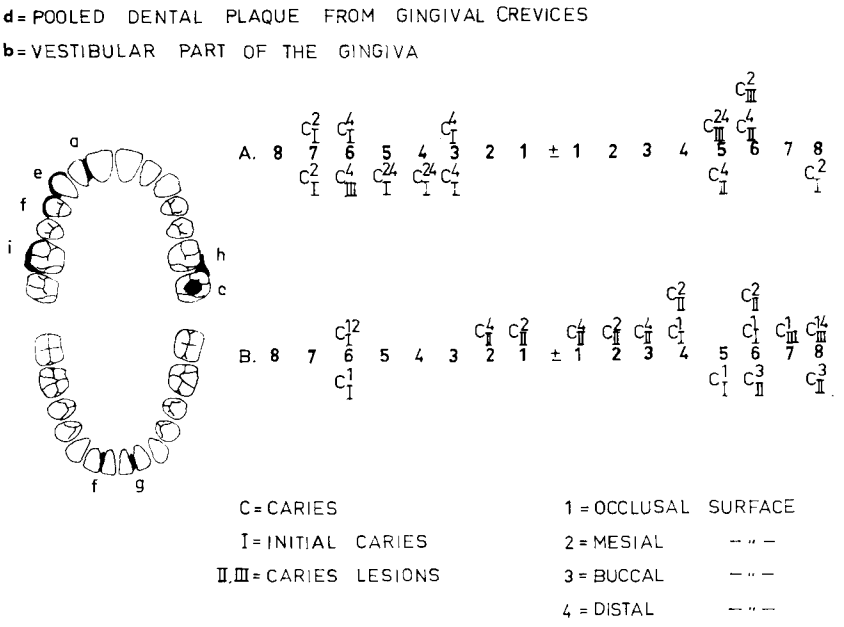

Fig. 1. Areas on the teeth and in the oral cavity from which the plaque material was collected. Results of a roentgenological and clinical investigation of the subject are given beside the schematic representation of the teeth. The places from which the plaque was collected are marked a—i.

2. Occurence of hemoglobin splitting enzymes

Fig. 1 shows the areas on the teeth from which the plaque was collected, together with a roentgenological and clinical explanation of the caries status of the subject. Figs. 2 and 3 present progress curves showing enzyme reactions in which unfractionated plaque material collected from different places on the teeth functioned as enzyme preparates in the hydrolysis of denatured hemoglobin. The curves in Fig. 2 demonstrate that the hydroly-

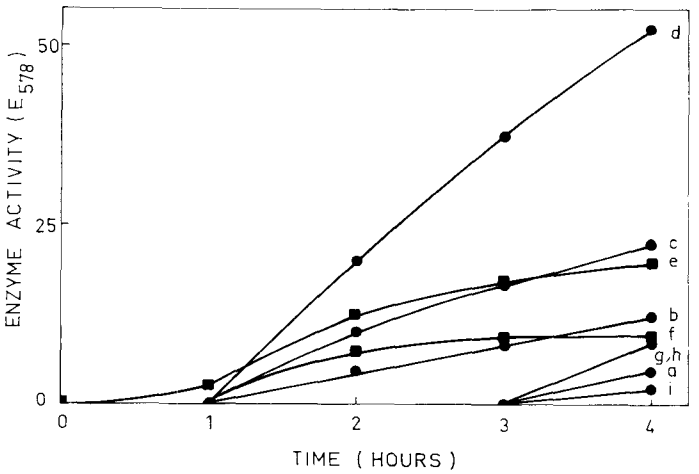

Fig. 2. Progress curves showing eight reactions in which denatured hemoglobin was hydrolyzed by different plaque preparations. The letters a—h refer to areas in the oral cavity shown in fig. 1.

sis of denatured hemoglobin by different plaque preparations proceeded linearly in most cases for at least 4 hours. Fig. 3 presents experiments in which some of the most active plaque preparations were tested for 48 hours. The curves show that the samples collected from the gingiva crevices (curve d in Figs. 2 and 3) always had the highest activity. The bending of this curve in Fig. 3 may be due to a decrease in the degree to which the enzyme(s) was (were) saturated by the substrate.

The results show that the endopeptidase-like enzymes in the bacterial plaque, which degrade denatured hemoglobin, are ac-

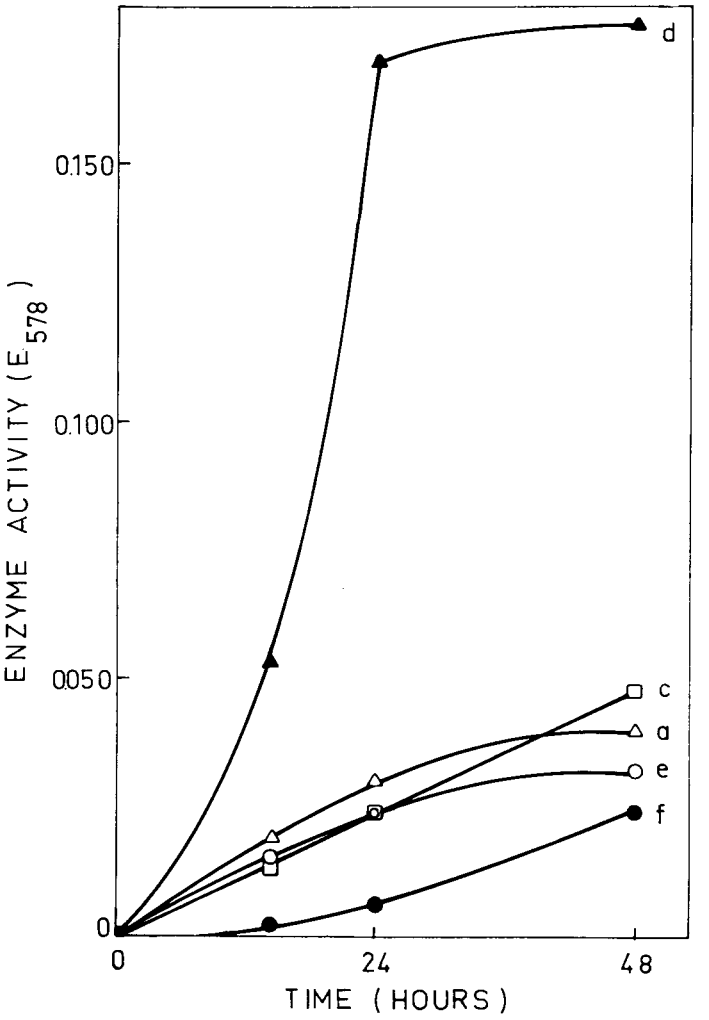

Fig. 3. Progress curves showing five reactions in which denatured hemoglobin was hydrolyzed by different plaque preparations. The letters refer to areas in the oral cavity shown in Fig. 1.

tive for at least 48 hours at 37° C. The results do not, however, indicate the existence of any correspondences between the occurrence and quality of carious lesions and the endopeptidase activity described.

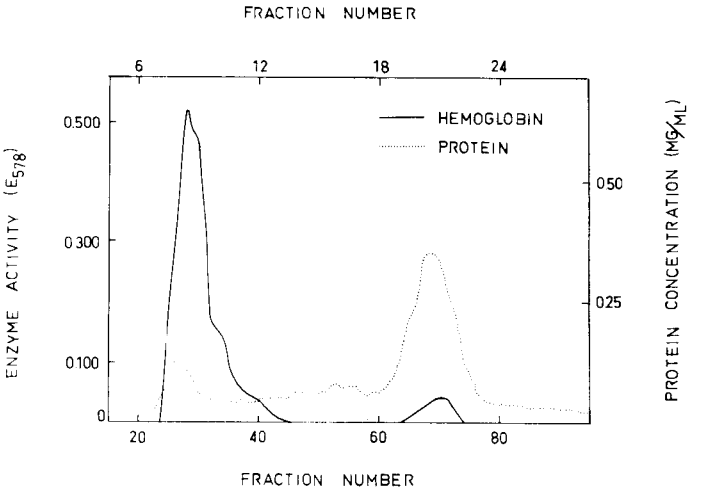

Fig. 4. Sephadex G-200 chromatogram of bacterial plaque. Column: 56×0.7 cm; sample: 0.75 ml of a protein solution the preparation of which is given in Methods; elution: 0.0075 M TRIS-HCl, pH 7.0; flow rate: 0.1 ml/min.; hydrostatic pressure: 10 cm; temperature +4°C; fraction volume: 0.3 ml. Blue Dextran was fractionated into tubes 22—26. Incubation time 24 hours.

3. Fractionation of enzymes hydrolyzing hemoglobin

Fig. 4 shows the results obtained in column chromatography using Sephadex G-200 gel. Two distinct enzyme peaks were observed, the highest of which belonged to an enzyme or enzymes with a molecular weight of about 200000 or more. The activity of the second enzyme peak was constantly low. In Sephadex G-100 filtration these two activities were fractionated together with Blue Dextran from the column.

4. Substrate specificity of enzyme peaks degrading denatured hemoglobin

The ability of the two enzyme peaks obtained in Sephadex G-200 filtration to hydrolyze various amino acid β-naphthylamides is shown in Table II. Table III shows the ability of the first enzyme peak to hydrolyze various naphthylesters. It is seen that most of the substrates tested were cleft at least to a certain degree.

Table II.

Ability of two enzyme peaks obtained in Sephadex G-200 filtration to hydrolyze various amino acid β-naphthylamides. The figures in the columns a and b are relative to the rate of hydrolysis of ala-β-NA, marked 100. The figures in the column c are directly comparable to those in column a. Incubation time 24 hours.

Substrate	Peak I	Peak II	
	a	b	c
Ala-β-NA	100	100	3.7
Arg-β-NA	27	50	1.7
Asp-β-NA	2.9	0	0
α-aspartyl-β-NA	5.7	67	2.3
β-aspartyl-β-NA	1.1	0	0
Cystine di-β-NA	0	33	0
γ-Glu-β-NA	5.7	33	1.1
β-Glu-β-NA	6.9	0	0
Gly-β-NA	14	0	0
His-β-NA	6.9	0	0
OH-pro-β-NA	9.2	47	1.6
Ileu-β-NA	5.7	20	0.7
Leu-β-NA	44	0	0
Leu-4-methoxy-β-NA	24	50	1.7
Lys-β-NA	32	15	1.5
Met-β-NA	84	0	0
N-α-benzoyl-DL-arg-β-NA	5.2	33	1.1
Orn-β-NA	11	67	2.3
Phe-β-NA	26	33	1.1
Pro-β-NA	81	33	1.1
Ser-β-NA	7.5	0	0
Thr-β-NA	11	0	0
N-tosyl-arg-β-NA	0	0	0
Try-β-NA	6.3	0	0
Tyr-β-NA	17	0	0
Val-β-NA	6.3	0	0

Table III.

Ability of the first enzyme peak obtained in Sephadex G-200 filtration to hydrolyze various naphthylesters. The values are in comparative relation to the rate of hydrolysis of α-naphthylvalerate, marked 100.

Substrate	Hydrolysis rate
BPANE	0
β-naphthylacetate	50
β-naphthylpropionate	54
β-naphthyllaurate	0
β-naphthylpalmitate	0
β-naphthylstearate	0
α-naphthylvalerate	100
α-naphthyllaurate	21

Table IV.

Effects of various compounds on the hydrolysis of denatured hemoglobin by two pooled enzyme fractions obtained in Sephadex G-200 filtration. Zero indicates that no effect could be observed; positive and negative figures show percentage of increase and decrease, respectively, in the rate of hydrolysis.

Compound		Peak I	Peak II
EDTA	a	0	0
	b	0	0
Co^{++}	a	+50	+100
	b	+30	+90
Mn^{++}	a	+50	+80
	b	+30	+60
PMSF	a	—60	0
	b	—40	0
TPCK	a	0	0
	b	0	0
PCMB	a	0	0
	b	0	0
NEM	a	0	0
	b	0	0

5. Affector studies

Table IV shows results of experiments testing the effects of certain compounds on the first and most active enzyme peak obtained in Sephadex G-200 filtration. As can be seen from the table, the rate of hydrolysis of denatured hemoglobin increased in the presence of certain divalent metal cations. Common enzyme inhibitors, such as EDTA, NEM and PCMB, did not affect the reaction, whereas PMSF clearly inhibited it. The affector characteristics indicate that at least the first of these two enzyme peaks is heterogeneous.

DISCUSSION

The main purposes of this investigation were to study the hemoglobin splitting activity of bacterial plaque collected from various places in the oral cavity, and to establish some affector characteristics of the proteases occurring in mixed plaque material and hydrolyzing denatured hemoglobin. The results obtained show that bacterial plaque produces enzymes capable of hydrolyzing denatured hemoglobin quite rapidly, and other protein molecules of varied nature, as well. According to Table I protein molecules denatured with urea may be more suitable substrates for these proteases than undenatured ones. The hydrolysis of undenatured protein could be several dozen times faster than that of the undenatured proteins.

Experiments with plaque material collected from different surfaces in the oral cavity proved that the enzyme activity depended more on the stability and age of the plaque than on the occurrence and quality of carious lesions. The abundant material obtained from the mucous membrane of the vestibular part had a fairly low activity, as compared to the material for instance from the gingiva crevice or from the last upper molar on the left side. The lowest activities were, on the whole, obtained with material from areas continuously washed by saliva, whereas the deeper and more inaccessible cavities and crevices showed the highest activity. The progress curves presented in Figures 2 and 3 indicate that the enzymes involved may be fully active even after 48 hours of action also in their place of formation.

As to the fractionation of enzymes hydrolyzing denatured hemoglobin, it is evident that at least the first and most active enzyme peak, obtained in Sephadex G-200 filtration, contains several enzymes possessing proteolytic activity. This appears in the affector studies, too: for instance, EDTA caused no inhibition, although the presence of certain divalent metal cations promoted the breakdown of hemoglobin. This activity was quite effectively inhibited by PMSF, which is known to inhibit trypsin and chymotrypsin-like enzymes (*Fahrney et al.*, 1963). Hence, the investigated enzyme peak may contain a protease, bearing an active serine residue, which is essential for its activity.

Tables II and III demonstrate that the enzymes studied are unspecific, or else the corresponding protein peaks are heterogeneous. In addition, the amino acid β-naphthylamides, hydrolyzed most rapidly by the first enzyme peak, were the same as those hydrolyzed rapidly by the salivary and plaque aminopeptidases, described in the two previous papers (*Mäkinen*, 1966 a, b), that is, the naphthylamides of alanine, arginine, leucine, lysine, methionine, and proline. The substrate specificity of the later and smaller peak seemed to differ from that of the first. This would indicate that the two peaks did, in fact, correspond to different enzyme activities: thus they would not result from a single activity, divided into two owing to conditions in the gel column, as was suggested in a previous paper (*Mäkinen*, 1966 a). Table III demonstrates that naphthylesters bearing long aliphatic side chains were hydrolyzed at a low rate only, or not at all. The effect, for instance, of taurocholate on the hydrolysis of these substrates has not been tested so far: accordingly, nothing can be said about a possible participitation of lipase-like enzymes in these enzyme peaks.

SUMMARY

The main purpose of this study was to demonstrate the existence in human dental plaque of an enzyme activity hydrolyzing denatured hemoglobin. It was found that the breakdown of urea-denatured hemoglobin by plaque enzymes was more rapid than that of undenatured casein, egg albumin, serum albumin, and lactalbumin. Enzyme activities hydrolyzing denatured hemoglo-

bin were more plentiful in the places in the oral cavity containing older and more stable bacterial plaque than in areas being continuously washed by saliva. No clear relationship was observed between the hemoglobin-splitting activity and the occurrence and degree of carious lesions. The corresponding enzymes were found to be active during at least 48 hours at + 37° C. Fractionation of the mixed plaque material revealed two enzyme peaks hydrolyzing denatured hemoglobin. The molecular weight of the enzymes occurring in the first and highest peak is suggested to be about 200.000 or more, as deduced from the fractionation pattern obtained on Sephadex columns. Both enzyme peaks were found to hydrolyze many kinds of amino acid β-naphthylamides and naphthylesters, which indicates that they are either unspecific or heterogeneous. This is also indicated by the fact that the hydrolysis of denatured hemoglobin by the first enzyme peak was inhibited by phenylmethyl sulphonylfluoride and activated by certain divalent metal cations, while EDTA, N-ethylmaleimide and p-chloromercuribenzoate had no effect on the reaction.

RÉSUMÉ

ETUDES SUR LES ENZYMES DE LA BOUCHE

III. FRACTIONNEMENT DES ENZYMES SUR UNE PLAQUE DENTAIRE HUMAINE HYDROLISANT L'HÉMOGLOBINE DÉNATUREE

Le but principal de cette étude a été de mettre en évidence dans la plaque dentaire humaine l'existence d'une activité enzymatique hydrolysant l'hémoglobine dénaturée. L'auteur a constaté que les enzymes de la plaque décomposaient plus rapidement l'hémoglobine dénaturée par l'urée que la caséine, l'albumine de l'oeuf, l'albumine du sérum et la lactalbumine non dénaturées. Les activités enzymatiques hydrolysant l'hémoglobine dénaturée étaient plus intenses aux endroits où la cavité buccale contenait des plaques plus anciennes ou plus stables que dans les régions continuellement lavées par la salive. Il n'existait pas de rapport évident entre l'activité de décomposition de l'hémoglobine et l'incidence et le degré des caries. Les enzymes correspondant se sont révélés être actifs pendant au moins 48 heures à + 37° C. Le fractionnement du mélange de plaques mettait en évidence deux pics

enzymatiques hydrolysant l'hémoglobine dénaturée. Le poids moléculaire des enzymes existant dans le premier pic, le plus élevé, serait d'environ 200000 ou plus, à en juge par le fractionnement obtenu sur colonnes de Sephadex. Il est apparu que les deux pics enzymatiques pouvaient hydrolyser beaucoup de sortes de β-naphtylamides et de naphtylesters d'aminoacides, ce qui indique qu'ils sont soit sans spécificité soit hétérogènes. Cela ressort aussi du fait que l'hydrolyse de l'hémoglobine dénaturée par le premier pic enzymatique était inhibée par le phénylméthylsulphonylfluorure, et activée par certains cations de métaux divalents, tandis que l'EDTA, que l'N-éthylmaléimide et le p-chloromercuribenzoate restaient sans effet sur la réaction.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

III. DIE TRENNUNG VON DEN DAS DENATURIERTE HÄMOGLOBIN IM MENSCHLICHEN PLAQUE HYDROLYSIERENDEN ENZYMEN

Mit dieser Untersuchung wurde die Aktivität von Enzymen, die das denaturierte Hämoglobin aus menschlichem Plaques spalten, demonstriert. Die Spaltung von mit Harnstoff denaturiertem Hämoglobin zu Aminosäuren und Peptiden war schneller als die vom nichtdenaturierten Casein, Eiweissalbumin, Serumalbumin und Laktalbumin. Die Aktivität von Enzymen, welche denaturiertes Hämoglobin im Munde spalten war grösser an solchen Stellen, wo ältere und stabilere Plaques gefunden wurde als an Stellen, die vom Speichel besser bespült werden. Ein klares Verhältnis zwischen der Aktivität und dem Vorkommen von kariösen Herden bzw. der Qualität von Herden konnte nicht festgestellt werden. Die genannten Enzyme können mindestens 48 Stunden bei 37 Grad C aktiv sein. Die Trennung brachte zwei Hämoglobin-spaltende Aktivitäten zum Vorschein, von denen das erstere einem Enzym mit einem Molekulargewicht von etwa 200.000 oder mehr entspricht. Die beiden Enzymfraktionen hydrolysieren mehrere Aminosäure-β-naphthylamide und Naphthylester, was die Unspezifität dieser Enzyme oder die Heterogenität der entsprechenden Fraktionen zeigt. Das erstere und aktivere von beiden Enzymen der Fraktion wurde durch Phenylmethylsulfonylfluorid gehemmt und durch einige bivalente Metallka-

tionen aktiviert. Äthylendiamintetraessigsäure, N-äthylmaleimid und p-Chlormercuribenzoat hatten dagegen in der Reaktion keine Wirkung.

REFERENCES

Chauncey, H. H., V. M. Johnson & V. F. Lisanti, 1954: Proteolytic enzymes of saliva (Abstract) J. dent. Res. *33:* 652.

Fahrney, D. E. & A. M. Gold, 1963: Sulphonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α-chymotrypsin and trypsin. J. Am. Chem. Soc. *85:* 997.

Mäkinen, K. K., 1966 a: Studies on oral enzymes. I. Fractionation and characterization of aminopeptidases of human saliva. Acta odont. scand. *24:* 579.

—»— 1966 b: Studies on oral enzymes. II. Fractionation and characterization of aminopeptidases in human dental plaque. Acta odont. scand. *24:* 605.

Rick, W. in "Methods of Enzymatic Analysis", 1963, (ed. by Bergmeyer, H.U.), p. 808, Verlag Chemie, Weinheim.

Sreebny, L. M., 1954: Characterization of a proteolytic enzyme in the submaxillary gland of the white rat (Abstract) J. dent. Res. *33:* 685.

Address: *Institute of Dentistry,*
University of Turku,
Turku 3,
Finland.