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STUDIES ON ORAL ENZYMES

I. FRACTIONATION AND CHARACTERIZATION OF AMINOPEPTIDASES OF HUMAN SALIVA

by

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INTRODUCTION

Knowledge of the oral enzymes, exluding amylase, is still rather scanty. Numerous papers have been published on this subject, but only few of these enzymes have been described in a more detailed manner. Among the pioneers in this field were *Chauncey et al.* (1954), who were able to determine the distribution and origin of salivary enzymes. According to them the salivary enzymes may originate from several sources: from the salivary glands, bacteria, oral tissues, and from ingested substances.

Important work on the oral enzymes has also been done by *Sreebny et al.* (1950) who have studied various glycolytic enzymes in saliva, and *Eggers-Lura* (1944, 1949) who has described a great number of salivary enzymes. A comprehensive survey of the literature on this subject is to be found in "Saliva and its Relation to Oral Health", by *Afonsky* (1961).

From a biochemical point of view, it is interesting to study the oral enzymes in relation to dental caries and periodontal disease. At first glance, there seems to exist two possibilities as to the role

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of these in the development of such diseases. First, the enzymes may produce their effect indirectly by catalyzing the formation of end-products, for instance, organic acids, which may disintegrate the structure of the teeth and the connective tissue. Another possibility is offered if the organic and inorganic material of these tissues is considered a direct substrate in an enzymic catalysis. In both cases the enzymes might have the same origin, that is the oral microorganisms. However, no further conclusion can be made without conducting thorough biochemical studies of the caries process and periodontal disease, irrespective of whether the diseases are produced directly, indirectly, or simultaneously, by the oral enzymes in one or the other manner.

The first part of the present study is devoted to a group of proteolytic enzymes in human saliva, the aminopeptidases, its aim being a description of some important properties of these enzymes, followed by a closer study of the most interesting enzymes. The following papers will describe other enzyme groups that may be of odontological interest.

MATERIAL AND METHODS

All the methods used in this and the following investigations were carried out in ultramicro scale, mainly because the amount of plaque enzymes available in many of the studies to be presented in future papers was restricted. For comparison all the studies, even those concerning saliva, have been carried out in the same scale. Thus the amounts of salivary enzymes obtained as pooled fractions in column chromatography were also small.

1. Reagents

The reagents were purchased from E. Merck AG (Darmstadt, Germany) with the following exceptions. N-ethylmaleimide and the amino acid β-naphthylamides were purchased from Mann Research Laboratories, Inc. (New York 6, USA), except L-cystine-di-β-naphthylamide, L-leucyl-β-naphthylamide, L-leucyl-4-methoxy-β-naphthylamide and N-α-benzoyl-DL-arginine-β-naphthylamide, which were obtained from Koch-Light Laboratories Ltd

(Colnbrook, England). Diazotized 4-amino-3:1′-dimethylazobenzen (Fast Garnet CBC Salt) was a product of G. T. Gurr (London, England), and sodium borohydride of Fluka AG (Buchs SG, Switzerland). Bovine serum albumine was purchased from Sigma Chemical Company (St. Louis, Mo., USA). The water used in these studies was double distilled and passed through a mixed bed ion-exchange column (Zerolit 225 and FF, Zerolit Limited, London, England). All the naphthylamide substrates were L-compounds, except N-α-benzoyl-DL-arginine-β-naphthylamide.

Abbreviations:

ala = alanine
arg = arginine
asp = aspartic acid
glu = glutamic acid
gly = glycine
his = histidine
OHpro = hydroxyproline
ileu = isoleucine
leu = leucine
lys = lysine
tosyl = toluensulphonyl
TANA = N-toluensulphonyl-L-arginine-β-naphthylamide
BANA = N-α-benzoyl-DL-arginine-β-naphthylamide
EDTA = ethylendiamine tetra acetic acid
PMSF = phenylmethyl sulphonylfluoride
met = methionine
orn = ornithine
phe = phynylalanine
pro = proline
ser = serine
thr = threonine
try = tryptophane
tyr = tyrosine
val = valine
β-NA = β-naphthylamide
PCMB = p-chloromercuribenzoate
NEM = N-ethylmaleimide
TPCK = L-1-toluensulphonylamido-2-phenylethyl-chloromethyl ketone
TRIS = tris(hydroxymethyl) aminomethane
DEAE = diethylaminoethyl

2. Preparation of gel filtration and ion exchange columns

The gel filtration was conducted on Sephadex columns. In the preparation of gels and the packing of the columns, the instructions of the manufacturer were followed (Pharmacia, Uppsala, Sweden).

DEAE-cellulose chromatography was carried out on columns packed with a product of Schleicher and Schüll (Dassel/Kr. Einbeck, Germany). The commercial cellulosic adsorbent was sieved into more homogeneous fractions and the fraction between 140 and 200 mesh was separated for further treatment. In preparing the material, in packing the columns, and in conducting the fractionations, the instructions presented by *Peterson & Sober* (1962) have been followed, except for the pressure applied in the packing. The DEAE-columns used in these studies were packed under a pressure accomplished by a 200 × 1 cm glass tube with a 50 ml funnel on top of it. The tube was joined with a rubber stopper to the column. The cellulose suspension was poured into the funnel and the level of the mixture was kept as high as possible during the packing. Accordingly, the pressure used was fairly constant the whole time.

3. Collection of saliva

Whole saliva from one person (male) with open caries lesions, but without apparent inflammatory changes was collected by stimulating the secretion with paraffin (melting point 52° C) or with the ground substance of chewing gum. The gum was chewed for 3—4 hours before the collection of saliva began. Portions of 50 ml were collected daily between 9 and 11 a clock a.m., after a thorough brushing of the teeth. When paraffin was used, the first 5 ml were not included. The material was allowed to run in a 50 ml plastic bottle placed in an ice bath (0—4° C), freezed immediately afterwards between — 20 and — 30° C and stored until used (after one to eight weeks). The caries status of the subject used in these studies will be given separately (*Mäkinen*, 1966 c).

4. Preparation of saliva for column chromatography

After thawing, a 50 ml sample of saliva was centrifuged (10 minutes, 48000 × g) and the sediment was discarded. In order

to minimize the volume, a 100 % ammonium sulphate precipitation was performed by dissolving the dry salt carefully in the protein solution. After 60 minutes, the mixture was centrifuged (10 minutes, 48000 × g) and the supernatant was discarded. The precipitate was suspended in 10 ml of water, and the undissolved portion was spinned down (10 minutes, 48000 × g). The clear supernatant was used in column chromatography and in studies concerning the specific activity. The protein concentration of such a solution was constantly about 0,6 mg/ml. All these procedures, from the thawing of the saple to the chromatography, were carried out in cold (0—5° C).

5. Determination of enzyme activity

The estimation of the aminopeptidase activity was performed in a micro mixture containing the following substances: (a) 50 μl universal buffer, pH 7.0 (*Long*, 1961), (b) 25 μl 10^{-3} M substrate solution in which the solid material was first dissolved or suspended in 10 ml ethanol and afterwards made up to 100 ml with water, (c) 25 μl water and (d) 10 μl enzyme preparation from each fraction obtained in column chromatography or some other enzyme solution. The mixtures were incubated for different periods at + 37° C and the reaction was stopped by adding 50 μl 0.1 % Garnet Salt GBC in 1 M acetate buffer, pH 4.2, which contained 10 % Tween 20. The color intensity was read with a Beckman Spinco Spectrocolorimeter (equipped with a 100 μl flow cuvette) at 525 mμ. A standard curve was prepared with free β-NA by plotting the colorimeter readings against the β-NA concentration. All the solutions were stored in cold and pipetted into cold test tubes in an ice bath (0—4° C) after which the tubes were carried out in cold (0—5° C).

6. Determination of specific activity of aminopeptidases in saliva and plaque

In order to elucidate the origin of salivary aminopeptidases the specific activities of the enzymes involved were estimated and the obtained values compared to those obtained with plaque aminopeptidases. The collection and preparation of the plaque material

will be described in detail in the following paper. Both plaque and saliva were obtained from the same person at approximately the same time, and the enzyme preparations were stored under identical conditions as described above. The specific activity was calculated as μmoles of β-NA liberated per mg protein per hour.

7. Fractionation on Sephadex and DEAE-columns

All fractionations were carried out between 0 and 5° C using glass columns equipped with G-1 sinters (approximate pore diameter 100 μ). As to the individual fractionations, more detailed technical data will be given in connection with each separate experiment. After estimating the aminopeptidase activity and protein concentration, the fractions were pooled to form different preparations containing separated enzyme activities. These pooled fractions were numbered with Roman figures and then used as enzyme solutions in various tests. The separate experiments include information showing how the fractions were pooled.

8. pH Optimum

The pH optimum of the hydrolysis of six different substrates by the pooled enzyme fractions was studied by substituting the normal universal buffer mentioned earlier with the same buffer of varying pH.

9. Effects of various affector compounds

In order to obtain information about the affector characteristics of the enzymes involved, the effects of the following compounds were tested: $CuCl_2$, $ZnCl_2$, EDTA, NEM and sodium borohydride. In addition, the effects of PCMB, TPCK and PMSF on some enzyme reactions were studied. The affector concentrations in the reaction mixture were as follows: (a) 0.2×10^{-3} M, (b) 0.1×10^{-3} M, (c) 0.2×10^{-4} M, except in the case of $CuCl_2$, where the affector concentrations were (a) 0.2×10^{-4} M, (b) 0.1×10^{-4} M, and (c) 0.2×10^{-5} M, and in the case of PCMB, where the concentrations were (a) 0.2×10^{-4} M, (b) 0.2×10^{-5} and (c) 0.2×10^{-6} M. The water (25 μl), normally present in the reaction mixture, was substituted by affector solutions. For

comparison, a pair of tubes without affectors were prepared for each experiment. All the tested compounds were used as water solutions or suspensions.

10. Determination of protein concentration

In this study the protein concentration was determined using the Folin-Ciocalteu method (*Layne,* 1963). Bovine serum albumine (crystallized and lyophilized) was used as a standard. The original method was modified by reducing the reagent volumes to 20 μl in the sample, to 100 μl in the alkaline $CuSO_4$-carbonate-tartrate solution, and to 10 μl in the Folin-Ciocalteu reagent.

RESULTS

1. Fractionation of the enzymes

Figures 1—6 show the results obtained in column chromatography. The Sephadex G-100 filtration (Figs. 1—2) showed that the majority of the aminopeptidase-like enzymes in the test material used probably consist of proteins possessing fairly high molecular weight (near 100,000 and more); in column chromatography they appeared to a large extent in the same fractions as the Blue Dextran used in testing the void volume of the gel columns.

Figs. 3—4 show typical fractionation patterns obtained on Sephadex G-200 columns. Here the fractionation was more complete than with G-100 gel, and only a small fraction of the total activity of the preparate came with Blue Dextran. Pro-, arg-, and lys-β-naphthylamides were hydrolyzed only by a few, the other three substrates by many enzymes.

DEAE-chromatography (Figs. 5—6), however, brought out the largest number of enzyme activities. Here one of the most interesting results was the appearance of an iminopeptidase-like enzyme in quite a pure form hydrolyzing pro-β-NA. Its detachment from the DEAE-column required a raising of the NaCl concentration to almost 1.0 M. The lower peaks on both sides of the maximum were apparently caused by changes in the ion exchange column, and may thus represent the same enzyme.

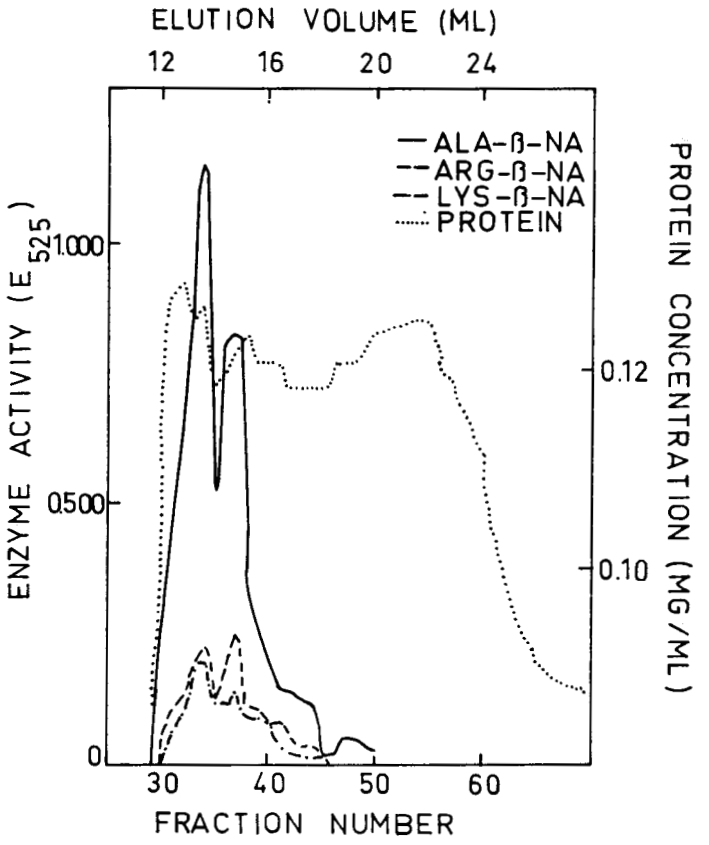

Fig. 1. Sephadex G-100 chromatogram of saliva. Column: 56×0.7 cm; sample: 1 ml of protein solution (preparation described in Methods); elution: 0.05 M TRIS-HCl, pH 7.0; flow rate: 0.12 ml/min; hydrostatic pressure: 10 cm; temperature + 4°C; fraction volume: 0.3 ml. Blue Dextran was fractioned into tubes 29—33. Incubation time 24 hours.

2. Determination of pH-curves

pH-curves for the enzymes separated in DEAE-chromatography are shown in Figs. 7—11, from which can be seen that pHs between 6 and 8 gave optimal hydrolyzation with a majority of the substrates tested. Optimum pH was about 7 in most cases. The only marked exception seems to be pro-β-NA which was rapidly broken down by enzymes also at the alkaline site in the DEAE-

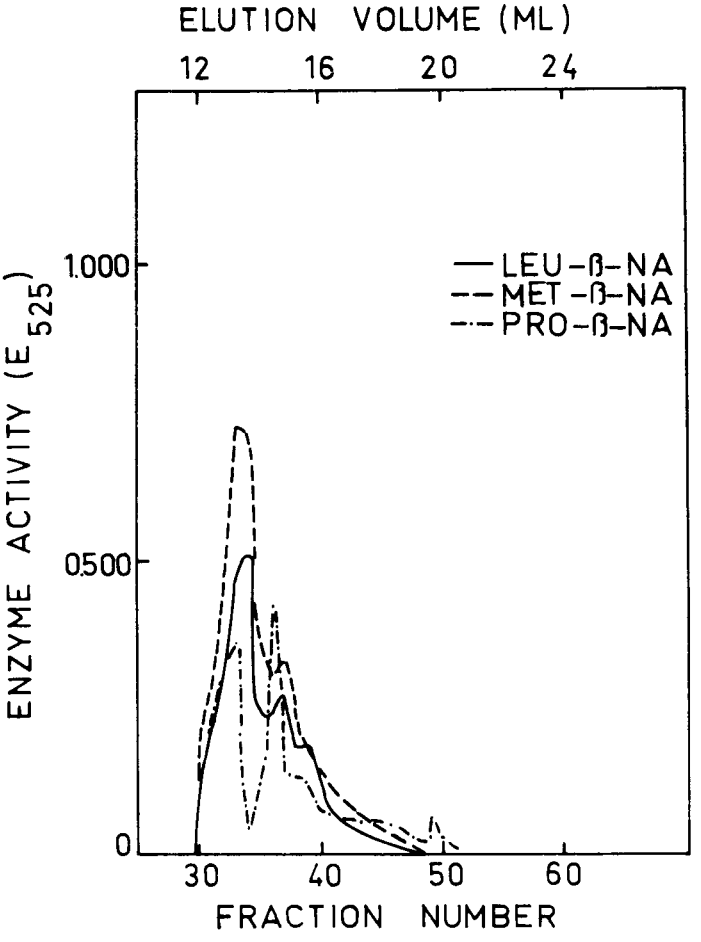

Fig. 2. Explanations as for Fig. 1.

peaks I and VI. The pH-curves (Figs. 7 and 10) of the hydrolysis of pro-β-NA differed markedly in shape from those obtained by other enzyme peaks. However, also the substrates ala-β-NA and lys-β-NA were hydrolyzed fairly rapidly at the alkaline site (Figs. 7 and 8).

Figs. 7—11 also show that the substrate concentrations in these

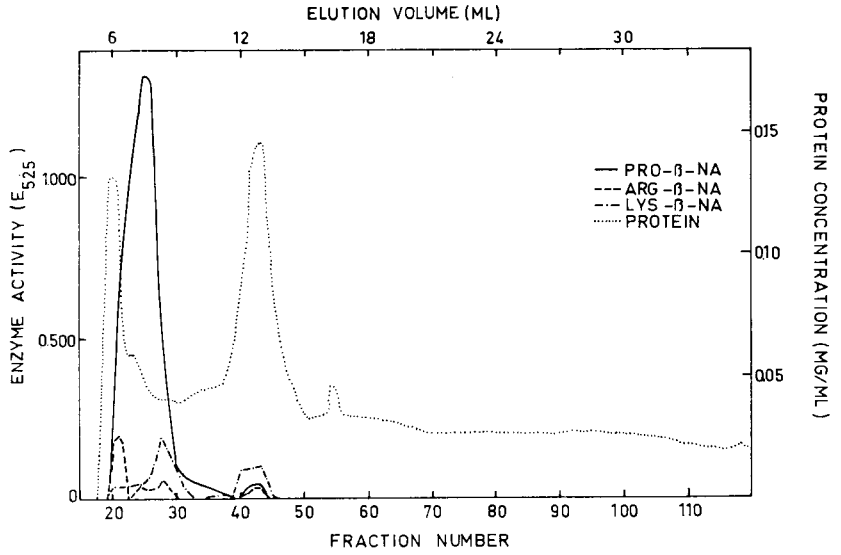

Fig. 3. Sephadex G-200 chromatogram of saliva. Column: 56×0.7 cm; sample: 1 ml protein solution (preparation described in Methods); elution: 0.05 M TRIS-HCl, pH 7.0; flow rate: 0.12 ml/min; hydrostatic pressure: 10 cm, temperature: +3°C; fraction volume: 0.3 ml. Blue Dextran was fractioned into tubes 19—21. Incubation time 24 hours.

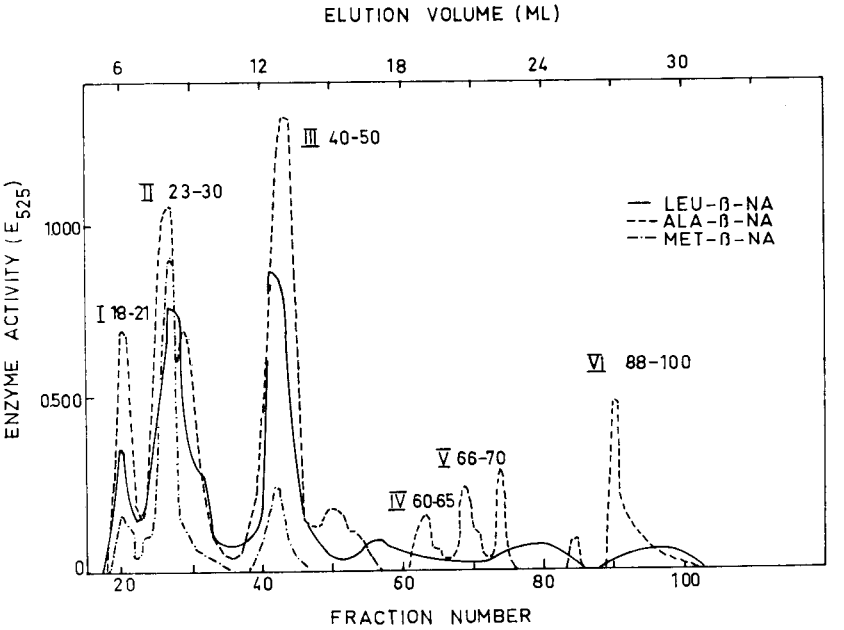

Fig. 4. Explanations as for Fig. 3.

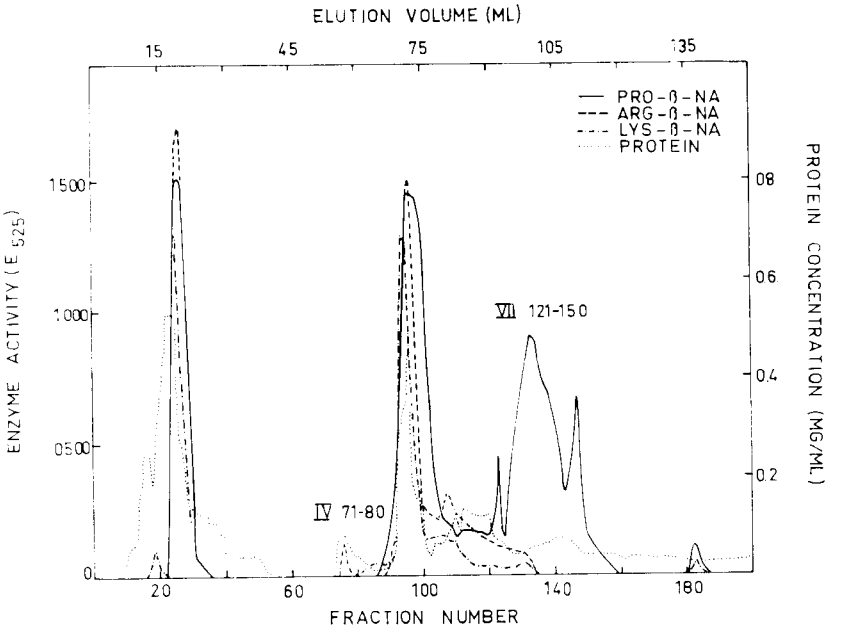

Fig. 5. DEAE-chromatogram of saliva. Column: 36×1.5 cm; sample: 3 ml of protein solution (the initial 1 ml preparation was first transferred into 0.005 M TRIS-HCl buffer, pH 7.0, by passing it through a Sephadex G-25 column (16×1.5 cm) with the aid of the buffer mentioned, and was marked with Blue Dextran, when the collected volume was 3 ml; elution: 0.005 M TRIS-HCl buffer, pH 7.0, containing a sodium chloride gradient from 0.005 to 0.5 M, thereafter increasing the concentration directly to 1.0 M; flow rate: 0.1 ml/min; hydrostatic pressure: 120 cm; temperature: +2°C; fraction volume: 0.75 ml. Incubation time 24 hours.

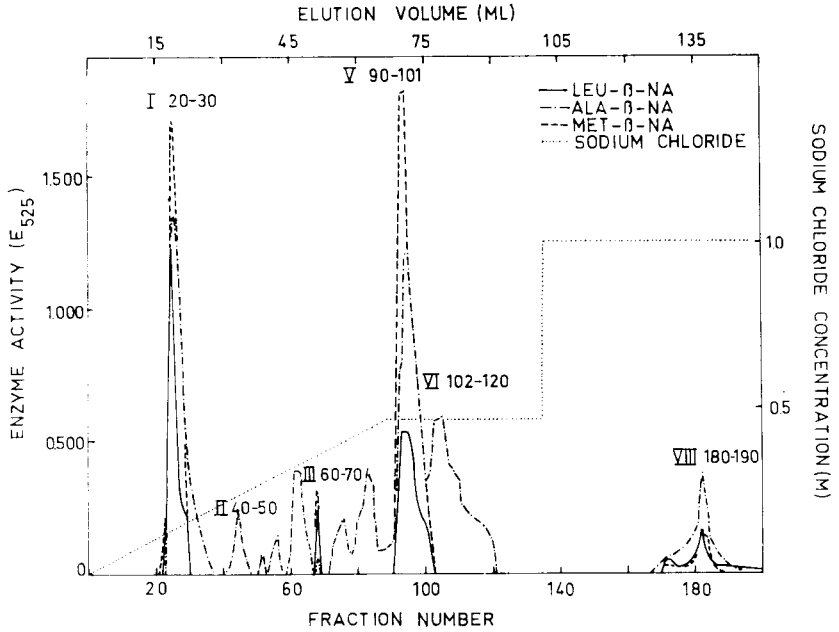

Fig. 6. Explanations as for Fig. 5.

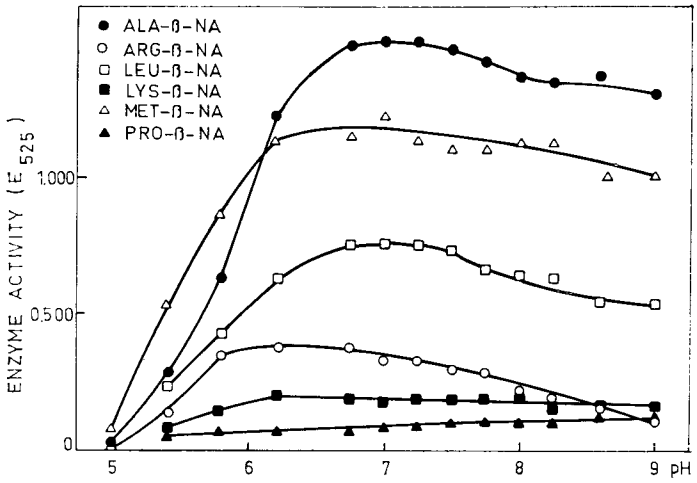

Fig. 7. Activity of salivary aminopeptidases (DEAE peak I) in universal buffer. Incubation time 6 hours.

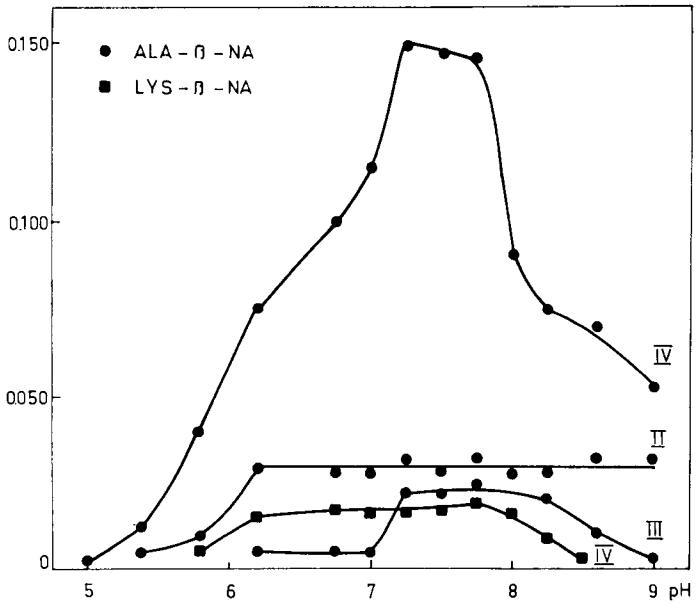

Fig. 8. Activity of salivary aminopeptidases (DEAE peaks II, III and IV) in universal buffer. Incubation time 24 hours.

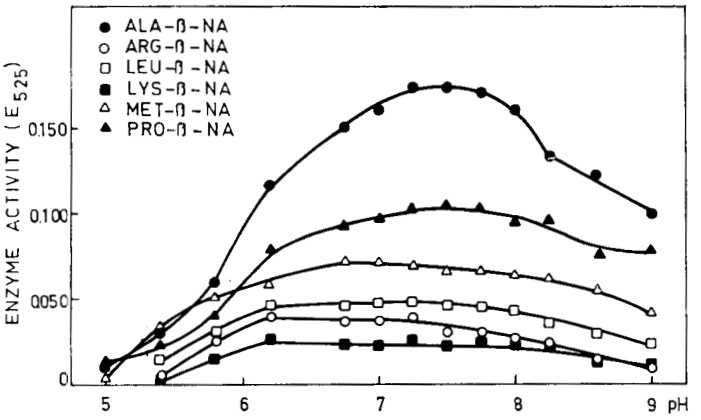

Fig. 9. Activity of salivary aminopeptidases (DEAE peak IV) in universal buffer. Incubation time 6 hours.

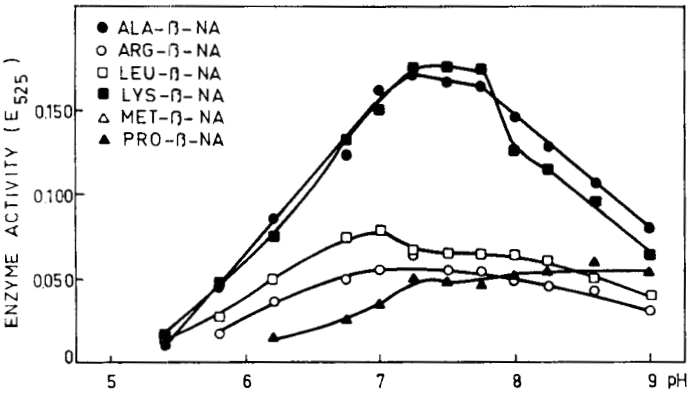

Fig. 10. Activity of salivary aminopeptidases (DEAE peak VI) in universal buffer. Incubation time 6 hours.

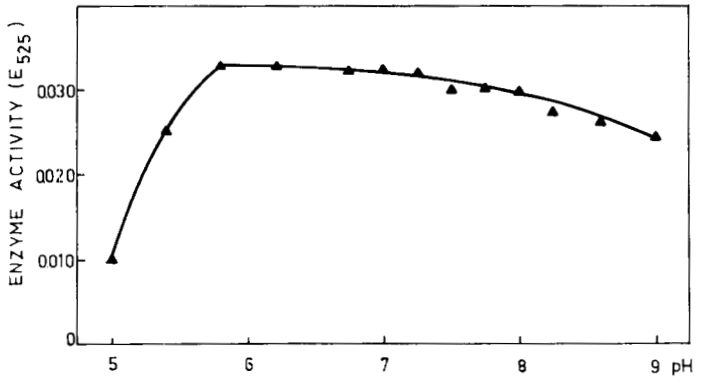

Fig. 11. Activity of salivary aminopeptidases (DEAE peak VII) in universal buffer. Incubation time 6 hours. Substrate: pro-β-NA.

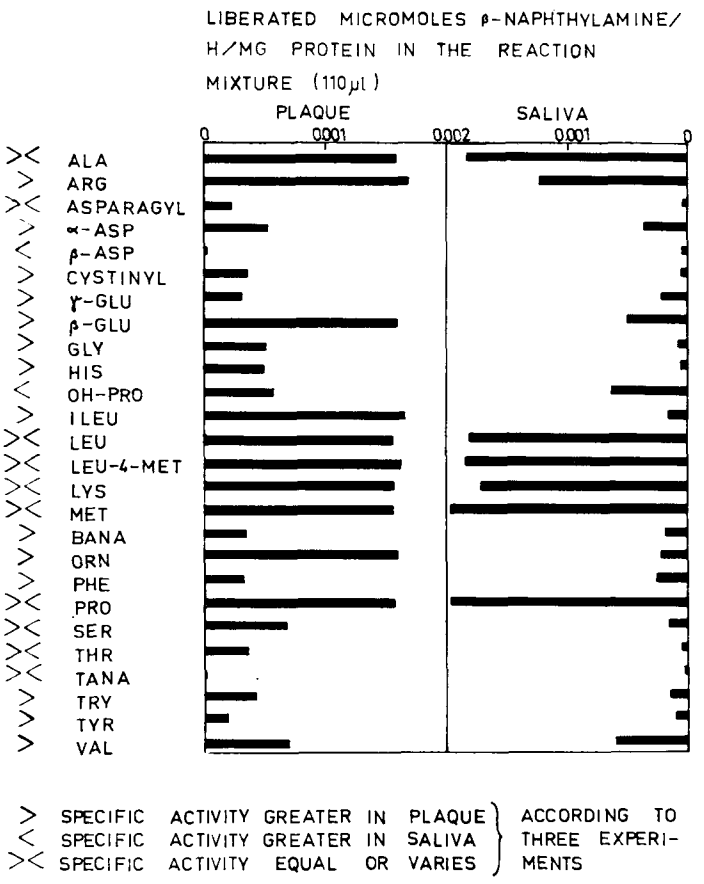

Fig. 12. Hydrolysis of various amino acid β-naphthylamides by plaque and salivary aminopeptidase-like enzymes. The activity is expressed as specific activities. The figure presents the results of one typical comparative experiment. Markings on the left show which of the two enzyme preparation possessed the largest activity in three repeated experiments, plaque (>), or saliva (<), or whether the two were equal or differed only slightly in a repeated test (><).

experiments were sufficiently high; accordingly the enzymes were nearly or entirely saturated with them. Further, the figures demonstrate that no aminopeptidase activity occurred below pH 5.

3. Affector studies

Tables I—VI show results of the affector studies. Only experiments performed on enzymes obtained in DEAE-chromatography

Table I.

Properties of salivary aminopeptidases (DEAE peak I, incubation time 6 hours): rates of hydrolysis as a function of metal ion, type of substrate, and type of inhibitor.[1])

Substrates		ala	arg	leu	lys	met	pro
Affector							
EDTA	a	97	85	88	94	72	0
	b	97	85	88	95	69	0
	c	90	83	88	70	70	0
Zn^{++}	a	92	70	77	64	68	17
	b	82	60	44	42	95	10
	c	35	0	17	6	23	10
Cu^{++}	a	70	77	59	37	49	11
	b	57	79	45	25	70	0
	c	7	45	0	0	0	0
$NaBH_4$	a	0	30	0	11	0	56
	b	0	40	0	18	0	49
	c	0	30	0	10	0	0
NEM	a	20	0	0	0	18	73
	b	20	0	0	0	10	46
	c	0	0	0	0	0	0
TPCK	a	20					
	b	20					
	c	0					
PCMB	a	0					
	b	0					
	c	0					
PMSF	a	0					
	b	0					
	c	0					

[1]) The values are all in comparative relation, 0 being assigned to the conditions giving no effect on the hydrolysis rate, other figures showing the percentage of activity destroyed during the reaction. Small letters a, b and c refer to the affector concentrations given in Methods, and ala, arg, etc. refer to the corresponding β-naphthylamides. — These remarks concern Tables II—VI as well.

Table II.

Properties of salivary aminopeptidases (DEAE peak V, incubation time 4 hours): rates of hydrolysis as a function of metal ion, type of substrate, and type of inhibitor.

Substrates		ala	arg	leu	lys	met	pro
Affector							
EDTA	a	51	40	80	40	32	0
	b	64	40	90	25	20	0
	c	44	40	90	25	20	0
Zn++	a	90	85	80	83	78	88
	b	82	85	80	75	61	84
	c	57	38	50	50	30	78
Cu++	a	80	70	90	67	52	70
	b	70	70	90	67	35	65
	c	26	30	40	33	10	35
$NaBH_4$	a	0	0	0	0	0	50
	b	0	0	0	0	0	35
	c	0	0	0	0	0	20
NEM	a	0	0	0	15	0	25
	b	0	0	0	15	0	20
	c	0	0	0	0	0	10
TPCK	a	25					
	b	30					
	c	25					
PCMB	a	35					
	b	35					
	c	30					
PMSF	a	35					
	b	25					
	c	15					

are included. At some enzyme peaks the activity was so low that no affector or other studies were possible. Concerning the posted Sephadex G-200 fractions the only thing worth noticing is that no iminopeptidase activity was inhibited by EDTA. However, in some experiments this activity could also be slightly activated by EDTA.

Table III.

Properties of salivary aminopeptidases (DEAE peak VI, incubation time 6 hours): rates of hydrolysis as a function of metal ion, type of substrate, and type of inhibitor.

Substrates		ala	arg	leu	lys	met	pro
Affector							
EDTA	a	100	70	80	72	86	0
	b	100	67	75	65	83	0
	c	100	65	75	65	80	0
Zn^{++}	a	100	78	85	100	93	84
	b	100	80	80	100	83	68
	c	100	67	70	100	74	52
Cu^{++}	a	100	85	88	80	72	56
	b	100	85	85	72	72	56
	c	50	60	80	48	45	40
$NaBH_4$	a	85	10	0	0	0	56
	b	85	15	0	0	0	52
	c	75	15	0	0	0	36
NEM	a	88	33	0	0	34	56
	b	80	26	0	0	30	44
	c	50	18	0	0	30	0
TPCK	a	100					
	b	100					
	c	85					
PCMB	a	100					
	b	100					
	c	100					
PMSF	a	100					
	b	90					
	c	80					

The effects of zinc and copper were studied because of their "heavy metal nature". In no case did these cations possess an activating effect. On the contrary, a marked inhibition was observed in most cases. As to the effect of PCMB, it is to be noted that the compound was tested only with ala-β-NA as substrate

Table IV.

Properties of salivary aminopeptidases (DEAE peak IV, incubation time 24 hours): rates of hydrolysis as a function of metal ion and type of inhibitor.

Substrate		ala
Affector		
EDTA	a	100
	b	100
	c	100
Zn++	a	100
	b	100
	c	100
Cu++	a	100
	b	100
	c	30
$NaBH_4$	a	70
	b	70
	c	70
NEM	a	70
	b	70
	c	65

Table V.

Properties of salivary aminopeptidases (DEAE peak VII, incubation time 6 hours): rates of hydrolysis as a function of metal ion and type of inhibitor.

Substrate		pro
Affector		
EDTA	a	0
	b	0
	c	0
Zn++	a	83
	b	78
	c	72
Cu++	a	67
	b	67
	c	55
$NaBH_4$	a	67
	b	67
	c	33
NEM	a	0
	b	0
	c	0

Table VI.

Properties of salivary aminopeptidases (DEAE peak VIII, incubation time 24 hours): rate of hydrolysis as a function of metal ion and type of inhibitor.

Substrate		pro
Affector		
EDTA	a	0
	b	0
	c	0
Zn++	a	67
	b	67
	c	42
Cu++	a	33
	b	30
	c	30
$NaBH_4$	a	50
	b	50
	c	40
NEM	a	30
	b	10
	c	6

and that almost all of the DEAE-peaks were inhibited by the PCMB concentrations used. The same was true for TPCK and PMSF, the effect of which were also tested on the hydrolysis of ala-β-NA by enzyme peaks obtained in Sephadex G-200 filtration. Their effects were, however, lower than that of PCMB.

Sodium borohydride affected hardly any of the enzyme activities. The only case in which it produced an inhibitory effect was in the hydrolysis of arg-β-NA by DEAE-peak I and of ala-β-NA by peak VII. The hydrolysis of pro-β-NA, however, differed, markedly from the other reactions, because all the observed iminopeptidase activities were inhibited by $NaBH_4$. NEM inhibited some reactions, but not all. All the iminopeptidase activities were also inhibited, but not as effectively as by $NaBH_4$.

4. Specific activities of aminopeptidases in saliva and plaque

Fig. 12 shows the results obtained in a typical experiment on salivary and plaque aminopeptidases. The plaque preparations

caused the fastest hydrolysis of most substrates. The activities directed toward these substrates in the oral cavity were thus regarded as having originated from the plaque.

DISCUSSION

The aim of this investigation was to elucidate the existence of aminopeptidase-like enzymes in human saliva. The chromatographic fractionation methods as used were sufficiently powerful to demonstrate a very rich aminopeptidase-like enzyme spectrum in the oral cavity. It is quite evident that this enzyme spectrum may vary considerably in quantity and in quality depending on the conditions in the mouth. Both the experiments described in this paper and those to be demonstrated in the immediately following ones have therefore been conducted on material collected from one single person. Later, comparative studies on material from different persons will be presented.

However, even in these studies on one and the same person the variability of the enzymic components of the human saliva was brought out, as appears from Fig. 12. It was impossible to obtain equal separations at different times. Some of these enzyme activities were so low that the incubation time had to be prolonged up to 24 hours. Therefore, particularly the results obtained in affector studies must not be considered absolute. The initial velocities were in many cases impossible to measure because of the small amounts of some enzymes available for studies.

1. Origin and fractionation of the aminopeptidases

DEAE-cellulose chromatography revealed the largest number of enzyme activities. The affector studies show, however, that some of the enzymes may have been fractionated in several portions, because the affector characteristics were very similar for many enzyme peaks. Various proteins may thus have combined with the enzyme molecules studied and may have determined their passage throught the column. Hence, it is difficult to determine the actual number of enzymes in the test material hydrolyzing, for instance, ala-β-NA. One the other hand, the rich bacterial flora known to be present in saliva, geve reason to expect an enzyme spectrum of equal complexity.

It might be expected that gel filtration should separate different enzyme species. Some protein molecules may, however, dissociate gradually to smaller fragments still possessing enzyme activity. Thus the amount of different aminopeptidases is again difficult to evaluate, but also the gel filtration studies show that they are fairly numerous. The majority of the enzymes observed showed a rather high stability under storage; after several months their activity was almost the same as immediately after fractionation. It is worth noticing that pro-β-NA was the perhaps most rapidly hydrolyzing of all the 26 different substrates. The partly denatured collagen molecules of the periodontal membrane might slowly be degraded by an iminopeptidase of this kind. Other substrates that were cleft as rapidly as pro-β-NA were ala-, leu-, lys-, arg-, and met-β-NAs; consequently these were selected as working substrates in this study.

When considering the structure of the substrates tested and their rates of hydrolysis, one notices that those bearing hydrophobic and aliphatic side chains were cleft more rapidly and by a larger number of enzymes than those possessing strongly basic or acidic side groups or an aromatic ring. This situation is represented in Fig. 12. It also appears from Fig. 12 that most of the enzymes observed originate from the plaque. In cases where the activity seemed to be higher in saliva, this activity may initially have originated from bacteria present in other parts of the oral cavity, which were not used as a source of plaque in these studies. Fig. 12 further shows that substrates in which the α-ammonium group was substituted for an aromatic residue, were hydrolyzed rather slowly. Marked differences between the plaque and salivary activities occurred in the hydrolysis of orn-β-NA and the hydroxyacid β-NAs. These were cleft most rapidly by plaque preparations, indicating that the corresponding enzymes may originate from the plaque.

2. Effect of pH

pH curves have sometimes been used to elucidate the number of different enzymes in a preparation. When using crude enzyme solution, however, this is not possible. One and the same enzyme may act at different rates on different substrates when the pH is changed. Therefore, the pH curves shown in Figs. 7–11 do not

necessarily reveal different enzyme species. Anyway, it seems clear that pro-β-NA is cleft by a specific enzyme a enzyme group, which catalyzes a rapid hydrolysis of this substrate at the alkaline site, as well.

The fact that all the enzymes tested were inactive below pH 5 may indicate both that these enzymes possess certain common structural and functional characteristics, and that there exists a number of unspecific aminopeptidase-like enzymes hydrolyzing most of the substrates used.

3. Affector studies

According to *Fasold et al.* (1961), sodium borohydride cleaves dithio bonds by oxidation, and NEM is known to be a potent sulphydryl inhibitor reagent (*Boyer,* 1957). The cases in which these two reagents have produced an effect could be understood if the compounds had reacted in the way described above. However, nearly all the enzyme peaks obtained in DEAE- and G-200-chromatography differed so much from each other in the presence of these compounds that no clear picture of the enzymes can be given. Even the specific iminopeptidase activity described above differed from one enzyme peak to the other. This could be caused by the greater affinity for these reagents of other and disturbing proteins present in the enzyme preparation.

PCMB also inhibits SH-enzymes by forming mercaptides with thiol groups. In fairly high concentrations, however, it inhibits the enzymes through reactions in which such groups are not involved (*Sohler et al.,* 1952). The influence of PCMB may also be explained as due to its denaturing action when destroying the tertiary structure of the protein molecule. This is the case, for instance, with muscle phosphorylase (*Madsen & Cori,* 1956). The effect of PCMB was studied only with ala-β-NA as substrate, and only the DEAE-pool VI contained an enzyme which was potently inhibited in fairly low concentrations. This inhibition is probably due to the denaturing effect of PCMB, because NEM did not inhibit the enzyme. The heavy metal cations Cu^{++} and Zn^{++} have apparently also acted as denaturing agents, because in many of the cases where they produced an inhibition, NEM had no effect.

EDTA inhibited all other hydrolyses except that of pro-β-NA. Whenever it had no unspecific anionic effects, EDTA may have

acted as a metal chelator. Any discrimination between these two effects is, however, impossible without further studies. The iminopeptidase apparently needs no metal ion activator. In this study TPCK was used as a deactivator of enzymes by histidine alkylations (*Shoellmann & Shaw,* 1962). The compound inhibited some of the hydrolyses by salivary aminopeptidases completely, but did not affect others. The EDTA-peak VI hydrolyzing ala-β-NA seems to contain an enzyme whose activity depends on the presence of an imidazole group at the active centre. This activity is also strongly inhibited by PCMB an PMSF. The latter was used because of its ability to inhibit trypsin and chymotrypsin-like enzymes, but not cholinesterase (*Fahrney & Gold,* 1963). It inhibited the hydrolysis of ala-β-NA, but the effect was lower than one would expect if the enzymes involved possess a serine residue at the active site.

A discrimination between the aminopeptidase-like enzymes is hardly possible on the basis of the presented affector studies because of the insufficient purity and the small amounts of enzyme preparations. However, for instance the activities directed toward ala-β-NA differed so greately in their sensitivity to heavy metals that the existence of several enzymes capable of splitting that substrate has in fact been demonstrated.

SUMMARY

The purpose of this investigation has been to separate aminopeptidase-like enzymes from human saliva and to characterize them. Using 26 different amino acid β-naphthylamides as substrates, it was demonstrated that saliva contains a complex enzyme spectrum. Both Sephadex G-200 and DEAE-chromatography were suited for their fractionation. All the enzymes investigated had a fairly high molecular weight, the lower limit being somewhat below 100,000. They are mostly produced by the plaque microorganisms. The substrates most rapidly cleaving were β-naphthylamides of L-proline, L-alanine, L-methionine, L-leucine, L-lysine, and L-arginine. Among the observed enzymes the compound hydrolyzing L-prolyl-β-naphthylamide differed from the others by virtue of its (their) fractionation pattern, substrate specifity, and behaviour in the presence of

EDTA— the hydrolysis of L-prolyl-β-naphthylamide, as opposed to that of all other substrates, was not inhibited by EDTA. Practically all the enzymes were inhibited by heavy metal cations and had their pH optimum close to neutrality. The substrate L-alanyl-β-naphthylamide was studied in more detail. Some of the enzymes hydrolyzing this substrate were sensitive to p-chloromercuribenzoate, phenylmethyl sulphonylfluoride and L-1-toluensulphonylamido-2-phenylethylchloromethyl ketone. These characteristics indicate the essentiality of histidine, of serine and perhaps of cysteine residues in the activities of these enzymes.

RÉSUMÉ

ETUDES SUR LES ENZYMES DE LA BOUCHE

I. FRACTIONNEMENT ET CARACTERISATION DES AMINOPEPTIDASES DE LA SALIVE HUMAINE

Le but de la présente étude a été de séparer des enzymes de type aminopeptidase à partir de la salive humaine et de caractériser ces enzymes. En utilisant 26 β-naphtylamides différentes d'acides aminés comme substrats, il a été possible de mettre en évidence que la salive contient un spectre enzymatique complexe. La chromatographie sur Sephadex G-200 et la chromatographie sur DEAE convenaient toutes deux au fractionnement de ces enzymes. Tous les enzymes étudiés ont un poids moléculaire assez élevé; la limite inférieure est un peu en dessous de 100000. Ils sont principalement produits par les micro-organismes de la plaque. Les substrats se dissociant le plus rapidement étaient les β-naphtylamides de l-proline, de l-alanine, de l-méthionine, de l-leucine, de l-lysine et de l-arginine. Parmi les enzymes observés, le composé hydrolysant la l-prolyl-β-naphtylamide différait des autres en vertu de son (ses) mode de fractionnement, de la spécificité du substrat et de la réaction en présence d'EDTA— l'hydrolyse de la l-prolyl-β-naphtylamide, contrairement à celle de tous les autres substrats, n'étant pas inhibée par l'EDTA. On peut dire qu'en fait tous les enzymes étaient inhibés par les cations de métaux lourds et que leur pH optimum était voisin de la neutralité. Le substrat que constitue la l-alanyl-β-naphtylamide a été étudié d'une manière plus détaillée. Quelques uns des enzymes hydrolysant ce substrat étaient sensibles au p-chloromer-

curibenzoate, au phénylméthylsulphonylfluorure et au l-1-toluensulphonylamido-2-phényléthylchlorométhylcétone. Ces caractères indiquent l'importance essentielle des résidus d'histidine, de sérine et peut-être de cystéine dans les activités de ces enzymes.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

I. DIE TRENNUNG UND CHARAKTERISIERUNG VON AMINOPEPTIDASEN IM MENSCHLICHEN SPEICHEL

Die Aminopeptidasen vom menschlichen Speichel wurden in dieser Untersuchung getrennt und charakterisiert. Bei Verwendung von 26 verschiedenen Aminosäure-β-naphthylamiden als Substrate hat man festgestellt, dass der Speichel ein kompliziertes Enzymspektrum hat. Sowohl Sephadex G-200 als DEAE-Chromatographie waren zur Trennung geeignet. Alle untersuchten Enzyme hatten ein relativ grosses Molekulargewicht, die untere Grenze war etwa 100.000 oder ein wenig darunter. Die untersuchten Aminopeptidasen werden zum grössten Teil in der Plaques gebildet und hydrolysieren am schnellsten die L-Prolin-, L-Alanyl-, L-Leucyl-, L-Methionyl-, L-Arginyl- und L-Lysyl-β-naphthylamide. Die L-Prolin-β-naphthylamid-spaltenden Enzyme unterscheiden sich untereinander am meisten in Trennung, Substratspezifität und im Verhalten zur Äthylendiamintetraessigsäure. Ausser der L-Prolin-β-naphthylamid-Hydrolyse wurden alle anderen untersuchten Hydrolysen durch Äthylendiamintetraessigsäure gehemmt. Alle untersuchten Aminopeptidasen werden gehemmt bei Anwesenheit von Schwermetallkationen und haben ihren optimalen pH-Wert etwa bei 7. Ein Teil von den L-Alanyl-β-naphthylamid-hydrolysierenden Enzymen war gegen p-Chlormercuribenzoat, Phenylmethylsulfonylfluorid und L-1-Toluensulfonamid-2-phenyläthylketon empfindlich. Für die Aktivität von diesen Enzymen können Histidin-, Serin- und Cysteinreste wichtig sein.

REFERENCES

Afonsky, D., 1961: Saliva and its relation to oral health, University of Alabama Press, Alabama.

Boyer, B. D., 1957: in The Enzymes, Vol. 1, 2nd ed., Academic Press, NY.

Biochemists' Handbook (ed. by Long, C.), 1961 p. 41, E. & F.N. Spon Ltd., London.

Chauncey, H. H., F. Lionetti, R. A. Winer & V. F. Lisanti, 1954: Enzymes of human saliva. The determination, distribution and origin of whole saliva enzymes. J. dent. Res. *33:* 321.

Eggers-Lura, E. H., 1944: Salivary Enzymes, Copenhagen, Ed. Munksgaard.

Eggers-Lura, E. H., 1949: Enzymes in Saliva, München, Carl Hanser Verlag.

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.

Fasold, H., G. Gundlach & H. Turba, 1961: Carboxymethylierung der Sulfhydryle in Chymotrypsin und Chymotrypsinogen nach Spaltung der Disulfidbrücken durch Natriumborhydrid. Biochem. Z. *334:* 255.

Layne, E., 1963: in Methods of Enzymology (ed. by Colowick, S. P. and N. O. Kaplan), Vol. III, p. 448, III Printing, Academic Press, NY.

Madsen, N. B. & C. F. Cori, 1956: The Interaction of Muscle Phosphorylase with p-Chloromercurobenzoate. I. Inhibition of Activity and Effect on the Molecular Weight. J. biol. Chem. *223:* 1055.

Mäkinen, K. K., 1966: Studies in oral enzymes. III. Fractionation of enzymes in human dental plaque hydrolyzing denatured hemoglobin. Acta odont. scand. *24:* 000.

Peterson, E. A. & H. Sober, 1962: Methods in Enzymology (ed. by Colowick, S. P. and N. O. Kaplan), Vol. V. Academic Press, NY.

Schoellmann, G. & E. Shaw, 1962: A new method for labeling the active centre of chymotrypsin. Biochem. Biophys. Res. Com. *7:* 36.

Sohler, M. R., M. A. Seibert, C. W. Kreke & E. S. Cook, 1952: J. biol. Chem. *198:* 281.

Sreebny, L. M., E. R. Kirch & R. G. Kesel, 1950: Location of the glycolytic enzymes in saliva. J. dent. Res. *29:* 506.

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