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STUDIES ON ORAL ENZYMES

II. FRACTIONATION AND CHARACTERIZATION OF AMINOPEPTIDASES IN HUMAN DENTAL PLAQUE

by

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INTRODUCTION

The microorganisms in the dental plaque are generally thought to play a role in dental caries or in periodontal disease. It is also known that saliva contains a large number of bacteria. They have mostly been thought of as being detached from the plaque or gingival debris. Consequently, mere saliva has often been utilized in investigations concerning the role of bacteria in the diseases mentioned above. *Krasse* (1954) has shown, however, that Streptococcus salivarius comprises only a small percentage of the facultative streptococci present in the dental plaque, while this organism comprises a large proportion of the facultative streptococci present in saliva and on the tongue. Later *Gibbons et al.* (1964) have confirmed *Krasse*'s findings, and, in addition, they have shown that the primary ecological site of Bacteroides melaninogenicus in the oral cavity is the gingiva crevice area.

Accordingly, the dental plaque needs not necessarily be the source of all oral bacteria. However, even the bacteria originating outside the plaque, may, nevertheless, exist within it. Hence the plaque and its bacteria might be considered as the final factor

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being related to dental caries and periodontal diseases. The enzymes of the plaque, whether extracellular, or produced by microorganisms in their normal metabolism, or released from dead and broken bacteria, may play a role in the development of the named diseases. This is why, instance, the studies of *Gibbons et al.* (1961) on the degradation of collagenous substrates by Bacteroides melaninogenicus are of great value in attempts to throw light on enzymes in the oral cavity. These investigators found that the native collagen is degraded by a collagenase of the bacteria.

The more precise nature of the various enzymes of the dental plaque is, however, not sufficiently known. This paper describes some characteristics of the plaque aminopeptidase-like enzymes.

MATERIALS AND METHODS

The reagents and methods used in this study have been described (*Mäkinen,* 1966) except for a few slight alterations. The test material was bacterial plaque, obtained from the same person as the saliva. The material was collected from unbrushed teeth every morning for about a fortnight and stored at + 1° C in 5 ml plastic containers sealed tightly with covers. When sufficient material had been collected (200—400 mg fresh weight), the contents of the containers were weighed and suspended all together in 2 ml of cold (+ 1—+ 4° C) 0.154 M NaCl solution. The suspension was centrifuged (23500 × g, 15 min.) and the clear supernatant was used as raw material in column chromatography. The protein concentration of the enzyme preparation varied between 0.5 and 1.0 mg/ml.

As regards the affector studies, the number of compounds tested was increased in that the effects on the hydrolysis of pro-β-NA of the following compounds were studied: Ca^{++}, Mg^{++}, Co^{++}, Mn^{++}, Ni^{++}, Pb^{++}, Hg^{++} (all as chlorides, except Hg^{++} which was used as nitrate), F^-, Cl^-, CN^-, SCN^-, ICH_3COO^-, citrate (all as sodium salts), E-600, DFP and 1.10-phe.[1]) The affector concentrations in the reaction mixtures were: (a) 0.2×10^{-3} M, (b) 0.1×10^{-3} M, (c) 0.2×10^{-4} M, and (d) 0.1×10^{-4} M for Ca^{++}, Mg^{++}, Co^{++}, Mn^{++}, Zn^{++}, F^-, Cl^-, CN^-, SCN^-, ICH_3COO^-, citrate, and 1.10-phe: (a) 0.1×10^{-4} M, (b) $0.2 \times$

10^{-5} M, (c) 0.2×10^{-6} M, and (d) 0.2×10^{-7} M for Hg^{++}: (a) 0.2×10^{-4} M, (b) 0.1×10^{-4} M, and (c) 0.2×10^{-5} M for Pb^{++} and Ni^{++}. All other reagents and concentrations were as stated in Part I of this work (*Mäkinen,* 1966).[1]) The chemicals mentioned above were purchased from E. Merck AG (Darmstadt, Germany), except E-600, which was a product of Koch-Light Laboratories Ltd. (Colnbrook, England), and DFP, a product of Sigma Chemical Company (St. Louis, Mo., USA). DFP was dissolved in and diluted with dry isopropanol to desired concentrations. All other compounds were used as water solutions or suspensions.

RESULTS

1. Fractionation oi the enzymes.

Figs. 1 and 2 show the results obtained in gel filtration on Sephadex G-200 columns. When compared to the fractionation patterns obtained with saliva (*Mäkinen,* 1966), the following points are to be noted. First, the enzymes splitting ala-, leu-, met-, arg-, and lys-β-NA were in both cases fractionated in a roughly similar way. Three different enzyme peaks could be distinguished, and the substrates containing hydrophobic side chains (ala-, leu-, and met-β-NA) were also cleft by some additional enzymes, fractionated after the three main enzyme peaks. Gel filtration studies on plaque aminopeptidases conducted on Sephadex G-100 gel gave the same results as the studies on saliva, that is, most enzyme activities were found in fractions containing Blue Dextran. Pro-β-NA was the most rapidly hydrolyzing substrate in the G-100 fractionation. Enzymes hydrolyzing pro-β-NA were also fractionated in the same manner; two different enzyme peaks were observed. DEAE-fractionation of aminopeptidases gave similar qualitative results with plaque as with saliva: ala-, leu-, met-, lys-, and arg-β-naphthylamides were hydrolyzed by three main peaks. Instead, the iminopeptidase activity of plaque came up only in one or two clear peaks, whereas several peaks could be distinguished with saliva.

[1]) The abbreviations are as stated in Part I of this work with three additions: E-600 = diethyl p-nitrophenyl phosphate; DFP = di-isopropyl fluorophospate; 1.10-phe = 1.10-phenantroline.

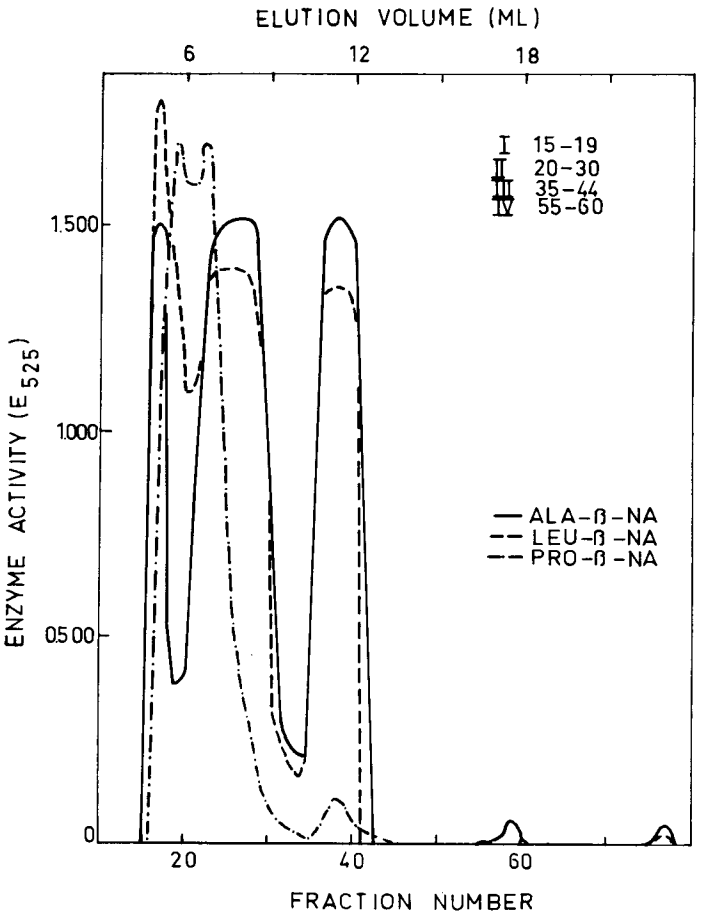

Fig. 1. Sephadex G-200 chromatogram of plaque aminopeptidases. Column: 56×0.7 cm; sample: 1 ml of a protein solution (preparation described in methods); elution: 0,05 M TRIS-HCl buffer, pH 7.0; flow rate: 0,08 ml/min; hydrostatic pressure: 10 cm; temperature: +4°C; fraction volume: 0.3 ml. Blue Dextran was fractionated into tubes 16—19. Incubation time 24 hours.

Some quantitative differences were observed between the plaque and the saliva fractionation patterns obtained with DEAE cellulose. In plaque, the last pro-β-NA hydrolyzing peak, which detached from the column with the aid of almost 1 M NaCl, was the most active.

2. Effect of pH.

The results of tests concerning the pH optimum for the hydrolysis of the six substrates by various enzyme peaks closely

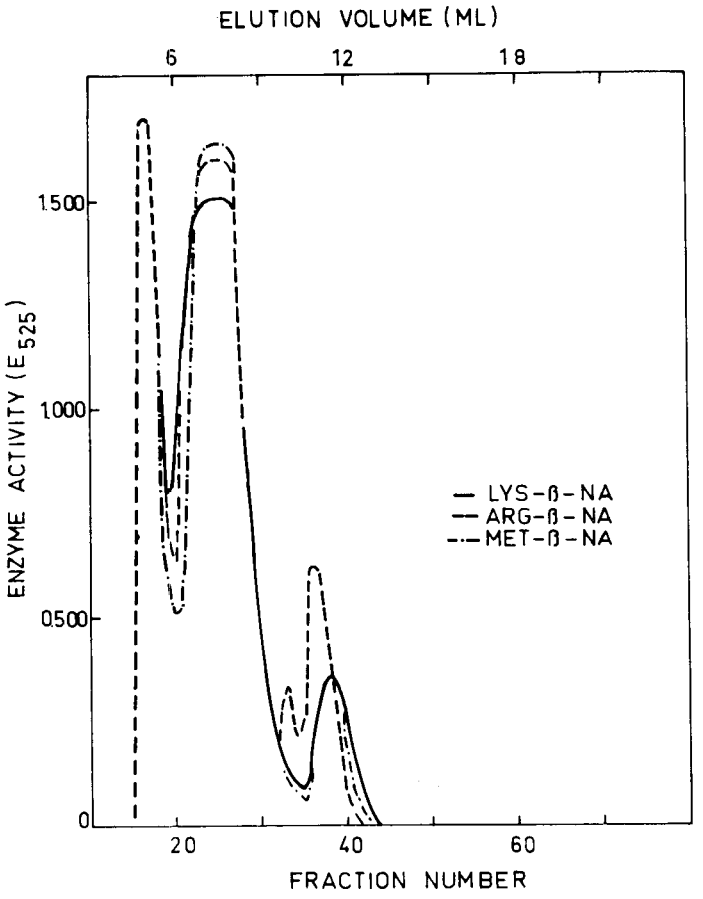

Fig. 2. Explanations as for Fig. 1.

resembled those described for saliva. For this reason, only a few examples are presented here. Figs. 5—7 show that the effect of pH on the activity of the tested enzymes is similar to that on the salivary aminopeptidases and that the pH optimum for the reactions is close to 7.

3. Affector studies.

As a rule, the results of the affector studies were very similar to those obtained with salivary aminopeptidase-like enzymes. This means that in the concentrations used, both Zn^{++} and Cu^{++} in-

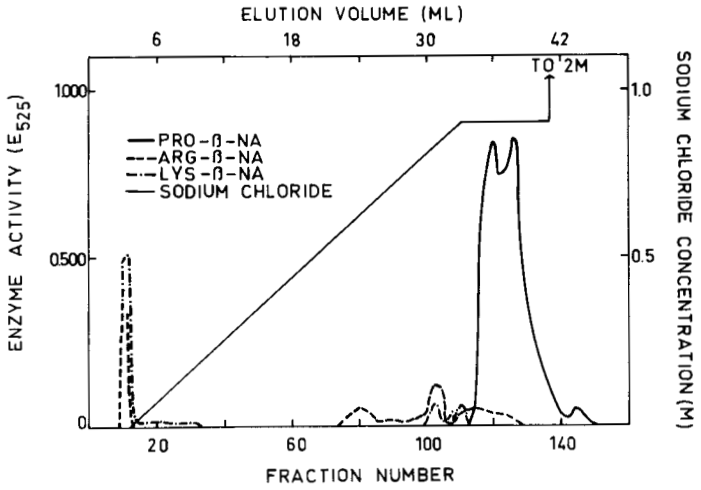

Fig. 3. DEAE chromatogram of plaque aminopeptidases. Column: 18×1.0 cm; sample: 5 ml of a protein solution (the initial 1.5 ml of preparate had been transferred into 0,005 M TRIS-HCl buffer, pH 7.0, by allowing it to pass through a Sephadex G-25 column (12×1.5 cm) with the aid of the buffer. The collected volume was 5 ml and the solution was marked with Blue Dextran. Elution: 0.005 M TRIS-HCl buffer, pH 7.0, containing a sodium chloride gradient from 0.005 to 0.9 M, then increasing the concentration abruptly to 2 M; flow rate: 0.05 ml/min.; hydrostatic pressure: 120 cm; temperature: +4°C; fraction volume: 0.3 ml. Incubation time 24 hours.

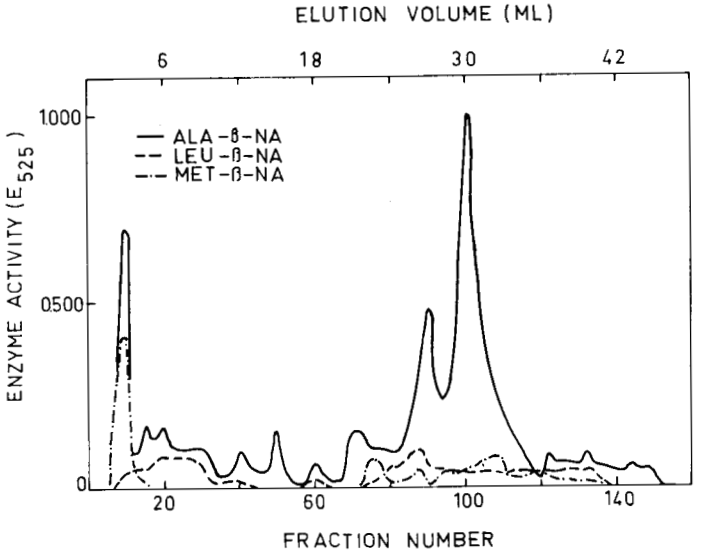

Fig. 4. Explanations as for Fig. 3.

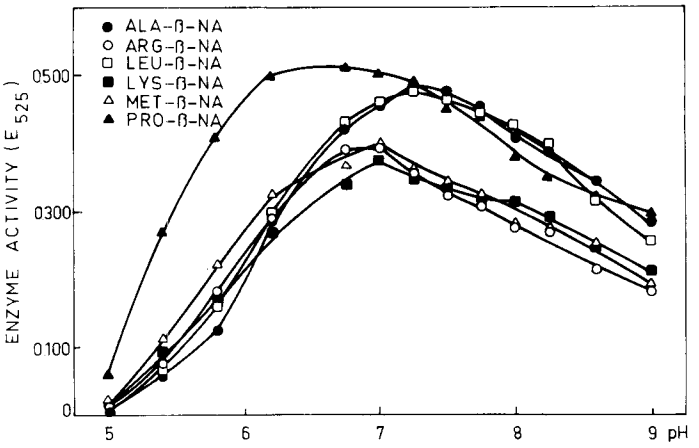

Fig. 5. Activity of plaque aminopeptidases (Sephadex G-200 peak I) in universal buffer. Incubation time 24 hours.

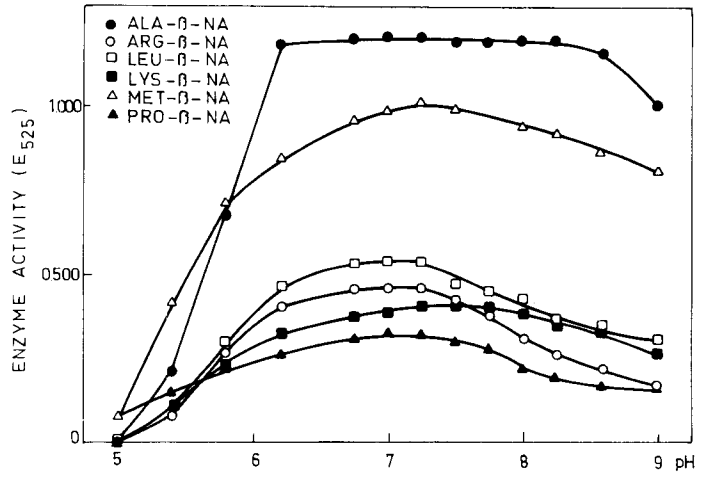

Fig. 6. Activity of plaque aminopeptidases (Sephadex G-200 peak II) in universal buffer. Incubation time 24 hours.

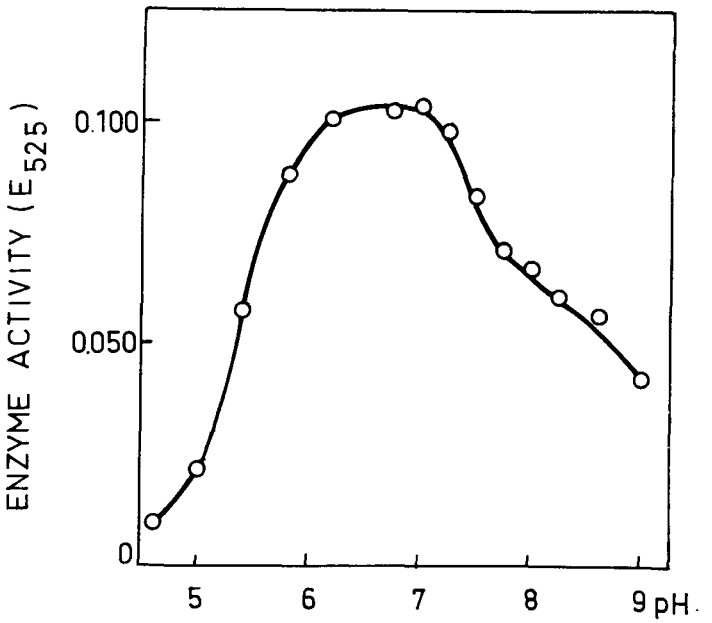

Fig. 7. Activity of pro- β-NA hydrolyzing enzyme(s) in universal buffer. The enzyme preparation was obtained by pooling the fractions 115—140 obtained in DEAE chromatography.

activated all the reactions to a great extent, and that EDTA inhibited all other hydrolyses except the one caused by iminopeptidases. $NaBH_4$ and NEM inhibited the iminopeptidase activity in precisely the same way as with saliva, but they were almost entirely without effect in the case of other substrates. PCMB, TPCK, and PMSF also had similar effects on both plaque and salivary enzyme activities. It is to be noted that the numerous enzyme peaks hydrolyzing, for instance, ala-β-NA, and obtained both with plaque and salivary aminopeptidases on DEAE-columns, do not necessarily correspond to each other. The affector characteristics of these low peaks were, however, of the same nature for both materials. Table I gives additional data on the iminopeptidase group. It shows that no activator has been found for these enzymes. Most of the heavy metal cations tested proved inhibitory in concentration between 0.2×10^{-4} and 0.2×10^{-5} M. Hg^{++} and PCMB had no effect in the low concentrations used.

Table 1

Properties of plaque aminopeptidases (DEAE peak VII, incubation time 24 hours). Rates of hydrolysis as a function of metal ion and type of inhibitor. In each column the values show the percentage of enzyme activity destroyed during the incubation, 0 being assigned to the conditions giving no effect on the rate of hydrolysis. Substrate: pro-β-NA.

Affector concentrations (M)	0.12×10^{-3}	0.1×10^{-3}	0.2×10^{-4}	$0.1—10^{-4}$
EDTA	0	0	0	0
Ca^{++}	0	0	0	0
Mg^{++}	0	0	0	0
Co^{++}	0	0	0	0
Mn^{++}	0	0	0	0
Zn^{++}	32	27	10	10
F^-	0	0	0	0
Cl^-	0	0	0	0
CN^-	0	0	0	0
SCN^-	0	0	0	0
ICH_3COO^-	0	0	0	0
citrate	0	0	0	0
1.10-phe	10	15	10	0
E-600	0	0	0	0
DFP	0	0	0	0
PCMB	0	0	0	0

	0.2×10^{-4}	0.2×10^{-4}	0.2×10^{-5}	0.2×10^{-6}	0.2×10^{-7}
Pb^{++}	15	15	10		
Ni^{++}	15	15	10		
Hg^{++}		0	0	0	0

DISCUSSION

The experiments on aminopeptidases demonstrated in these two papers have brought out many and striking similarities between the enzymes in the saliva and those in plaque. Actually, it must be mostly the same enzymes which occur in both. This

hypothesis is supported both by the results obtained in fractionation, and by the affector and other studies presented, as well as by the related evidence in favour of the supposition that most of these enzymes originate with plaque microorganisms (*Mäkinen,* 1966). Accordingly, the discussion of their common characteristics is not repeated here.

However, there is also a difference between the two groups of fractionation patterns. The figures illustrating aminopeptidase activity in plaque do not reveal so many small enzyme peaks hydrolyzing substrates containing CH_3-terminal amino acid side chains as those describing the corresponding activity in saliva. This is apparently due to the much lower protein concentration in the plaque material used in the column chromatography. Accordingly, the concentrations of aminopeptidases must have been low, as well, and the enzymes could not be observed even after 24 hours of incubation. The results obtained support the hypothesis that in many cases the more easily obtained saliva can be used as raw material in studies of the plaque aminopeptidases.

For the iminopeptidase-like enzymes no specific activator was found. The actual number of these enzymes has not been determined in this study, but many experiments indicate that there exists only one main type, which is fractionated in several peaks in a way described (*Mäkinen,* 1966). However, in this connection the term iminopeptidase group is used. The group was found to be quite active and stable in the presence of many reagents commonly used as enzyme inhibitors; neither PCMB, E-600, nor DFP had any effect. The latter compounds are considered very potent inhibitors for enzymes containing an active serine (*Gladner et al.,* 1958). Iodoacetate had no effect, either. This compound is an SH-reagent, though not quite specific (*Dixon et al.,* 1964). The heavy metals Co^{++} and Mn^{++} had no effect, nor had Hg^{++} when used in low concentrations. On the other hand, Ni^{++}, Cu^{++}, Zn^{++}, and Pb^{++} were slightly inhibitory. These metals evidently broke down a portion of the tertiary structure of the enzyme molecule. These results indicate that the iminopeptidase group possess neither an active sulphydryl group nor any active serine residue.

The low inhibitory effect of 1.10-phe can be explained as an unspecific effect, not as the action of a metal chelator. None of

the other anions tested (SCN^-, CN^-, Cl^-, F^-, citrate) had any effect on the hydrolysis of pro-β-NA.

When discussing the affector characteristics of the iminopeptidase group, it must be remembered, that the compounds used may have failed to react because of an improper spatial structure, the nature of the active site allowing only the substrate molecule to enter the site of catalysis. Such a structure would be rather stable against many extraneous factors.

The iminopeptidase activity has been found to be fairly stable also toward prolonged storage. The enzymes involved were almost fully active after a six months storage at — 20° C, although the preparation had been thawed and frozen several times.

SUMMARY

The aminopeptidases of the human dental plaque have been fractionated and characterized. L-alanyl-, L-leucyl-, L-lysyl-, L-arginyl-, L-methionyl-, and L-prolyl-β-naphthylamides were used as substrates and the enzymes hydrolyzing them were fractionated on Sephadex- and DEAE-columns. The results closely resembled those obtained with salivary aminopeptidases demonstrated in the previous paper of this series. The same is true of the studies of the pH optimum and the affector characteristics. The hydrolysis of L-prolyl-β-naphthylamide was studied in more detail. The reaction was slightly inhibited by the heavy metal cations Zn^{++}, Pb^{++} and Ni^{++}; Hg^{++} and p-chloromercuribenzoate were without any effect when used in somewhat lower concentrations than the above affectors. Organophosphorus compounds had no effect, either, nor had iodoacetate or a group of anions commonly present in the oral cavity. Finally, the results indicate that the aminopeptidases occurring in the human dental plaque are mainly the same enzymes as those in human saliva.

RÉSUMÉ

ETUDES SUR LEZ ENZYMES DE LA BOUCHE

II. FRACTIONNEMENT ET CARACTERISATION DES AMINOPEPTIDASES SUR UNE PLAQUE DENTAIRE HUMAINE

Les aminopeptidases de la plaque dentaire humaine ont été fractionnées et caractérisées. Les β-naphtylamides de l-alanine,

de l-leucine, de l-lysine, de l-arginine, de l-méthionine et de l-proline ont été utilisées comme substrats, et les enzymes les hydrolysant ont été fractionnés sur colonnes de Sephadex et de DEAE. Les résultats ressemblaient fortement à ceux obtenus avec les aminopeptidases salivaires (voir l'article précédent de cette série). Cela est également vrai en ce qui concerne l'effet du pH optimum et les caractéristiques des effecteurs. L'hydrolyse de la l-prolyl-β-naphtylamide a été étudiée d'une manière plus détaillée. La réaction était légèrement inhibée par les cations de métaux lourds Zn^{++}, Pb^{++} et Ni^{++}; Hg^{++} et le p-chloromercuribenzoate restaient sans effet lorsqu'on les utilisait à une concentration légèrement inférieure aux effecteurs ci-dessus. Les composés organophosphorés étaient aussi sans action, ainsi que l'iodoacétate et qu'un groupe d'anions fréquemment présents dans la cavité buccale. Enfin, les résultats indiquent que les aminopeptidases existant dans la plaque dentaire humaine sont dans l'ensemble les mêmes enzymes que dans la salive humaine.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

II. DIE TRENNUNG UND CHARAKTERISIERUNG VON AMINOPEPTIDASEN IM MENSCHLICHEN PLAQUE

Die Aminopeptidasen von menschlicher Plaques wurden getrennt und charakterisiert. Als Substrate wurden L-Alanyl-, L-Leucyl-, L-Methionyl-, L-Arginyl-, L-Lysyl- und L-Prolin-β-naphthylamide verwendet. Die Enzyme wurden durch Sephadex- und DEAE-Säulen getrennt. Die erhaltenen Ergebnisse hatten grosse Ähnlichkeit mit den früheren Untersuchungsergebnissen vom menschlichen Speichel. Dasselbe betrifft die pH-Optimum- und Affektoruntersuchungen der Aminopeptidasen. Die Hydrolyse von L-Prolin-β-naphthylamid wurde genauer untersucht. Sie wird leicht durch Zn^{++}-, Pb^{++}- und Ni^{++}-Ionen gehemmt. Hg^{++} und p-Chlormercuribenzoat hatten gar keine Wirkung, wenn sie etwas verdünnter als die letzteren Affektoren verwendet wurden. Auch organische Phosphorverbindungen, Jodacetat und einige im Mund gewöhnlich vorkommende Anionen hatten keine Wirkung aus der Hydrolyse vom L-Prolin-β-naphthylamid. Die Ergebnisse zeigen, dass die Aminopeptidasen von Speichel und Plaques in der Hauptsache dieselben Enzyme sind.

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