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ACTIVITY OF ARYLAMINOPEPTIDASES IN NORMAL AND INFLAMED HUMAN PERIODONTAL LIGAMENT

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INTRODUCTION

Earlier studies (*Paunio & Mäkinen*, 1969) concerning the activity of arylaminopeptidases in human periodontal ligament have been carried out on nonmarginal material obtained apart from the area of inflammation. It is clear, however, that correspondly enzyme activity determinations from the marginal part of the ligament, close to the focus of inflammation, would yield valuable information, particularly as different aminopeptidase-like enzymes have been suggested to be involved in the inflammatory processes. Due to a lack of information about the activity of such enzymes in the marginal part of the ligament, this study was undertaken.

There are, however, histochemical studies on gingival aminopeptidase activity (*Quintarelli*, 1962; *Paunio & Mäkinen*, 1970). These papers have simply demonstrated the enzyme activity in the tissues only and no conclusions about the possible changes in the activity of aminopeptidases in inflammation could be made on the basis of the results obtained.

MATERIALS AND METHODS

The periodontal ligament material was obtained from about 700 extracted human teeth which were stored at −2°C after extraction. The samples were

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handled and divided into diseased and control material as described earlier (*Paunio & Mäkinen*, 1969). The marginal part (the epithelial attachment and the area ca. 2 mm below it) and the remaining non-marginal part (the area below the marginal area, excluding, however, the most apical region of the root) were investigated in this study. Samples representing the whole ligament material (marginal and non-marginal areas) were used when studying the effect of age on the enzyme activity only.

Chemicals. All chemicals used in this study were the same as described previously (*Mäkinen*, 1966, 1969; *Paunio & Mäkinen*, 1969).

Other methods. The determination of arylaminopeptidase activity and protein concentration, as well as the methods used in column cromatography, have been described in earlier publications (*Mäkinen*, 1966, 1969). The azo-dye-binding property of various tissue proteins was also taken into consideration in the determination of the enzyme activity (*Paunio, Brusiin & Mäkinen*, 1970).

The effect of the following compounds on the rate of the enzyme reactions was tested: EDTA, sodium p-chloromercuriabenzoate (PCMB), and phenylmethanesulphonyl fluoride (PMSF). The concentrations of these compounds in the reaction mixtures were as follows: 0.1×10^{-4} M, 0.2×10^{-4} M, and 1.0×10^{-4} M. In addition, the effect of Ca^{++} and Zn^{++} ions (added in chlorides) was studied at the concentrations of 0.6×10^{-4} M, 1.2×10^{-4} M, and 3.0×10^{-4} M.

RESULTS

1. *Effect of age.* The effect of age on the rate of the hydrolysis of some *N*-L-aminoacyl-2-naphthylamines catalyzed by unfractionated enzyme preparations is presented in Table I. It was seen that arylaminopeptidase activity of the control samples decreased with increasing age. This type of correlation was not found in the diseased material, except for the hydrolysis of N-L-methionyl-2-naphthylamine. In samples representing the diseased material, the enzyme activity was lowest in the material representing the oldest age group.

2. *Arylaminopeptidase activity in various parts of the ligament.* Table II shows the results obtained when studying the arylaminopeptidase activity with unfractionated enzyme preparations separately derived from marginal and non-marginal parts of diseased an normal periodontal ligaments. The diseased material representing marginal samples displayed lower rates of hydrolysis than the control material, while in the preparations derived from

Table I.

Effect of age on the arylaminopeptidase activity of human periodontal ligament. 2-NA = 2-naphthylamine. Ala = alanine, arg = arginine, leu = leucine, lys = lysine and met = methionine

Control tissue	Age group (years)	Enzyme activity (μmoles of 2-NA per min and per mg protein $\times 10^4$)				
		Ala-2-NA	Arg-2-NA	Leu-2-NA	Lys-2-NA	Met-2-NA
	70—80	180	85	97	85	127
	60—69	183	90	121	95	147
	30—40	182	90	107	87	151
Diseased tissue	60—80	173	79	101	82	136
	30—40	191	81	112	89	145
	20—29	203	96	119	95	163

Table II.

Arylaminopeptidase activity in human periodontal ligament material of various types, tested in 0.005 M tris-HCl buffer, pH 7.0. Substrate concentration: 0.166 mM. 2-NA = naphthylamine. (M = marginal, N = non-marginal)

2-NA of	Enzyme activity (μmoles 2-NA per min and per mg protein $\times 10^4$)			
	Diseased M	Control M	Diseased N	Control N
L-Alanine	160	190	158	148
L-Arginine	74	104	69	70
Glycine	10	14	10	8.0
L-Hydroxyproline	1.7	1.9	4.3	2.7
L-Isoleucine	6.5	7.8	9.7	7.3
L-Leucine	60	70	80	68
L-Lysine	64	93	70	58
L-Methionine	89	141	123	111
L-Ornithine	17	18	15	14
L-Phenylalanine	29	39	31	28
L-Tyrosine	10	12	10	8.4

the non-marginal part of the ligaments the rates of hydrolysis were somewhat higher in the diseased material than in the control material. In the non-marginal samples the differences in the enzyme activity between the diseased and control material were not so clear as in the marginal samples.

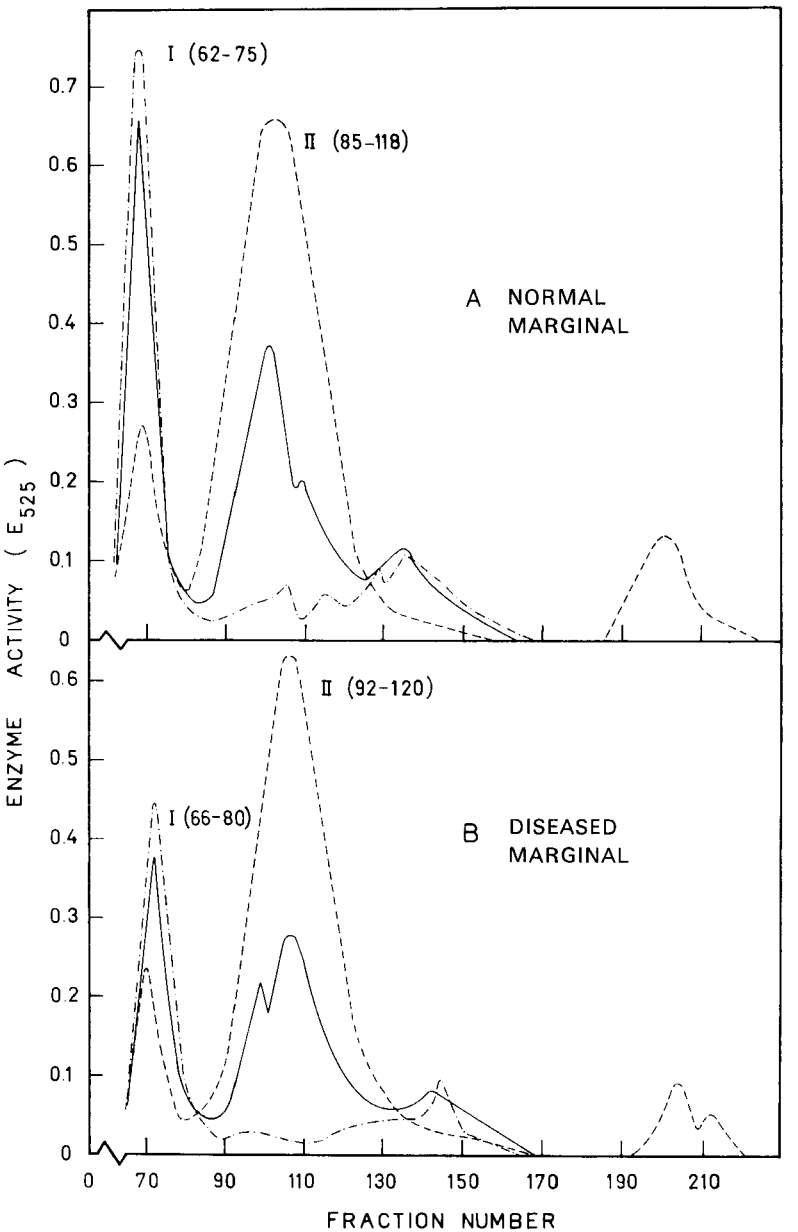

Fig. 1. Molecular exclusion chromatography of arylaminopeptidases of human periodontal ligament (marginal part) acting on *N*-L-methionyl-2-naphthylamine (——), *N*-L-alanyl-2 naphthylamine (- - -) and *N*-L-arginyl-2-naphthylamine (-.-.-). A: experiment with normal material; B: experiment with diseased material. For both experiments the following details imply: Column: Sephadex G-200 (65 cm × 2.5 cm); elution buffer: 0.01 M tris-HCl, pH 7.0; sample: 1.3 ml; temperature: +4°C; fraction volume: 1.5 ml; hydrostatic pressure: 15 cm.

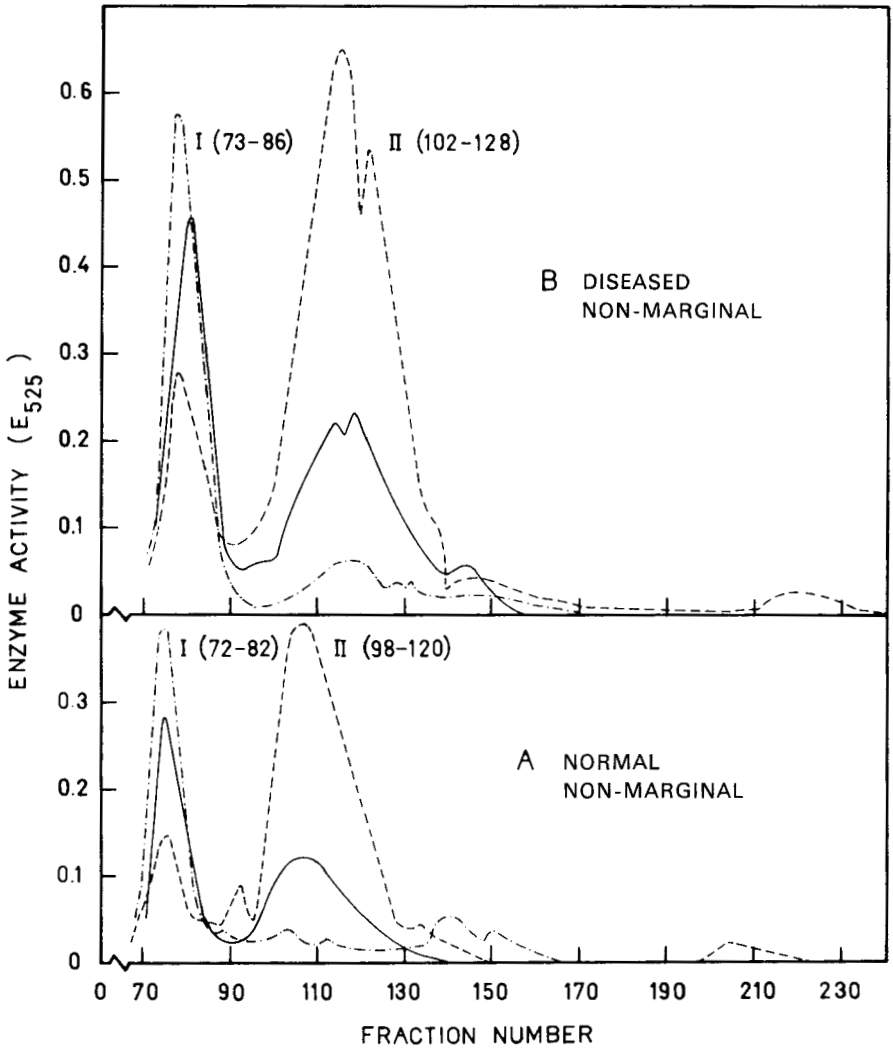

Fig. 2. Molecular exclusion chromatography of arylaminopeptidases of human periodontal ligament (non-marginal part). All experimental details and symbols and their meaning are the same as in Fig. 1.

The marginal samples contained the following amounts of protein (in mg per ml): diseased, 1.3; control, 1.2. The corresponding values for the non-marginal samples were as follows: diseased, 0.7; control, 0.8.

3. *Fractionation and characterization of arylaminopeptidases.* The results from molecular exclusion cromatography on Sephadex® G-200 gel

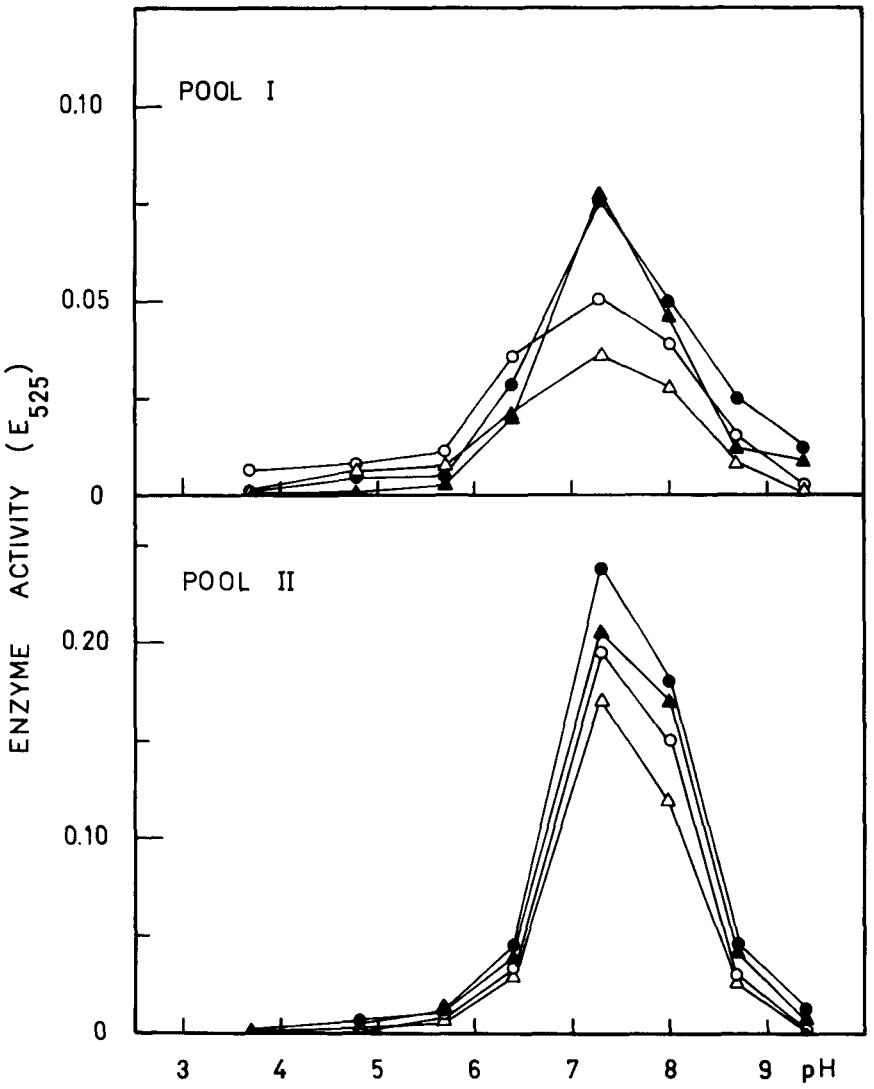

Fig. 3. Effect of pH on the rate (in $10^{-7} \times$ M min^{-1}) of the hydrolysis of *N*-L-alanyl-2-naphthylamine catalyzed by eight different enzyme preparations obtained as shown in Figs. 1 and 2. ●—●, Control marginal; O—O, Control non-marginal; ▲—▲, Diseased marginal; △—△, Diseased non-marginal.

with all four types of enzyme preparations are shown in Figs. 1 and 2. The following results require consideration:

a) In general, two main enzyme peaks with arylaminopeptidase activity were encountered in the chromatograms; b) In the first enzyme peak obtained

Table III.

The effect of some enzyme affectors on the rate of the hydrolysis of N-L-alanyl-2-naphthylamine (0.166 mM) catalysed by different arylaminopeptidase preparations obtained from human periodontal ligament (Figs. 1 and 2). The effect is given in % inhibition caused by EDTA, PCMB, PMSF and Zn^{2+} ions, or percentage activation caused by Ca^{2+} ions, as compared to the effect observed without added affector (water instead). (M = marginal, N = non-marginal)

		Sephadex G-200 peak I				Sephadex G-200 peak II			
Affector	Concentration (M)	Diseased M	Control M	Diseased N	Control N	Diseased M	Control M	Diseased N	Control N
EDTA	0.1×10^{-4}	67	65	70	75	87	89	90	89
	0.2×10^{-4}	77	81	80	82	93	95	91	93
	1.0×10^{-4}	89	93	95	91	97	98	99	95
PCMB	0.1×10^{-4}	47	43	54	41	22	20	20	17
	0.2×10^{-4}	53	52	71	59	25	21	27	20
	1.0×10^{-4}	66	68	87	70	38	41	50	44
PMSF	0.1×10^{-4}	50	45	40	38	27	25	31	30
	0.2×10^{-4}	61	63	67	62	34	31	39	40
	1.0×10^{-4}	87	92	94	89	54	54	67	56
Zn^{2+}	0.6×10^{-4}	47	55	60	61	66	60	73	77
	1.2×10^{-4}	69	65	77	74	80	86	90	87
	3.0×10^{-4}	90	92	98	97	94	97	99	99
Ca^{2+}	0.6×10^{-4}	8	12	4	15	5	10	16	15
	1.2×10^{-4}	10	11	13	17	7	13	16	16
	3.0×10^{-4}	20	15	18	20	11	19	17	18

with marginal material the rate of the hydrolysis of N-L-arginyl-2-naphthylamine and N-L-methionyl-2-naphthylamine was lower in the diseased material than in the control material; c) In samples representing diseased non-marginal material both enzyme peaks displayed higher enzyme activity than in the control material, in spite of the opposite protein concentrations.

The enzyme peaks obtained were studied for some enzymic properties: the effect of substrate concentration, temperature, pH and affectors. The results obtained from all these experiments are summarized here. All experiments were caried out with N-L-alanyl-2-naphthylamine (0.166 mM) and with altogether eight enzyme preparations obtained from experiments shown in Figs. 1 and 2.

1. Effect of enzyme and substrate concentration. Plots of the rate of the hydrolysis against enzyme concentration yielded linear curves, and the

Michaelis-Menten plots of the rate versus the substrate concentration yielded almost typical hyperbolic curves under the conditions employed. 2. The effect of pH on the rate of hydrolysis of the substrate used showed that the optimum pH in all cases was close to 7.3, when tested in the following buffer solutions: 0.025 M $\beta\beta$-dimethylglutarate buffer, 0.05 M tris-HCl buffer, and 0.025 M glycinate buffer. Typical bell-shaped curves were obtained indicating two successive ionizations (Fig. 3). 3. The effect of temperature on the rate of the reactions was approximately the same with all preparations: the enzyme activity decreased gradually when tested in temperatures ranging from 20°C to 50°C. 4. The effect of some enzyme affectors is shown in Table III. The strongest reduction in the rate of the hydrolysis was obtained in the presence of EDTA, but PCMB and PMSF also inhibited the enzyme reactions.

No significant differences in the inhibition patterns were found between the marginal and non-marginal samples in the first enzyme peak. This concerns the second enzyme peak as well, although there were differences between the pattern of inhibition given by these two enzyme peaks. For example, the inhibition caused by PCMB and PMSF was stronger in the first enzyme peak than in the second one. In all samples Zn^{2+} ions caused potent reduction in the rate of hydrolysis of N-L-alanyl-2-naphthylamine, while Ca^{2+} ions exerted slight activation, regardless of which of the two enzyme peaks was studied.

DISCUSSION

The decrease of the arylaminopeptidase activity of the periodontal ligament with increasing age is evidently a natural change of the aging tissue. Tissue changes concerning the diseases of the periodontium have sometimes been compared with the normal wound healing process. Consequently, in periodontal diseases, the wound healing process does not reach its final stage (*Schultz-Haudt & Lundqvist*, 1962; *Paunio*, 1969).

There are end products of microbial metabolism and various tissue proteins in the focus area and its immediate environment, capable of reacting to the azo-dye, used in arresting the enzyme reactions (*Mäkinen & Mäkinen*, 1969; *Paunio, Brusiin & Mäkinen*, 1970). Because the differences in enzyme activities observed in the present study were not caused by the action of the factor mentioned, they can only be ascribed to the different amount of the enzyme protein in the material studied. No qualitative changes were seen to occur between the arylaminopeptidase activity in diseased and normal tissue.

In the focus area (in inflamed gingiva and gingival pocket) and its close proximity the inflammatory reactions are prominent. On the other hand, in locations more distant from the focus area (in the middle and apical parts of the periodontium) the healing process is at a more advanced stage. It has been found that the amount of RNA in the healing wound first decreases (inflammation stage), after which it again increases (synthetic stage) (*Tsanev*, 1963). These changes can perhaps be compared with results obtained in the present study: the decrease in the synthesis of the enzyme proteins in the marginal area and the increase in the non-marginal area.

All the enzymic properties of the arylaminopeptidases revealed were the same, regardless of the nature of the tissue. These common properties included the effect of substrate concentration, temperature, pH, and certain chemical compound on the action of the enzymes as well as the fractionation patterns on Sephadex G-200 gel. Hence, it could be concluded that the substrates studied in this paper (particularly *N*-L-alanyl-2-naphthylamine) are mostly hydrolysed by the same enzymes in normal and diseased tissue. No specific inflammatory peptidases were observed, although their presence could have been demonstrated by other substrates or in other conditions.

If it is assumed that specific inflammatory peptidases or proteinases are considered enzymes being active after formation from inactive precursors, the enzymes revealed in the present and the previous (*Paunio & Mäkinen*, 1969) studies would then be constitutive. This is evident because the enzymes present in inflammed ligaments have been found to be the same as in normal ligament material. It is known that inflammation induces the formation of specific proteolytic enzymes or causes their activation (*Beloff & Peters*, 1945; *Ungar*, 1947). Because tissue injury and bacterial inflammation in principle result in basically similar biochemical processes in the inflamed area, it could be questioned whether a part of the enzymes studied in the present paper would still represent so called inflammatory hydrolases, which are liberated from the tissue in extraction, by buffer. However, in the present experiments, their concentration in the samples must have been too small to permit their detection in the presence of a large number of constitutive enzymes. One exception, is, however, most likely aminopeptidase B. It is considered a peptidase active in various inflammatory processes (*Hopsu, Mäkinen & Glenner*, 1966). It probably caused most of the enzymic hydrolysis of *N*-L-arginyl- and *N*-L-lysyl-2-naphthylamine reported in this paper. Because the working substrate was *N*-L-alanyl-2-naphthylamine, which is not hydrolyzed by aminopeptidase B, the involvement of this enzyme in the periodontium remains to be investigated. However, it has been shown to

occur in gingiva and dental pulp (*Mäkinen & Paunio*, 1970; *Mäkinen, Brummer & Scheinin*, 1970).

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SUMMARY

Arylaminopeptidase activity in normal and diseased human periodontal ligament was studied with several *N*-L-aminoacyl-2-naphthylamines as substrates of which the hydrolysis of *N*-L-alanyl-2-naphthylamine was studied more closely. When whole ligament material was studied, the enzyme activity in control samples decreased with increasing age, while this type of a correlation was not observed in the diseased material exept for the hydrolysis of one substrate. When the ligaments were divided into marginal and non-marginal material, the diseased material representing marginal samples displayed lower rates of hydrolysis than the control material. In the apical part the situation was the reverse. All enzyme properties investigated (fractionation pattern on Sephadex G-200 gel, effects of temperature, pH, substrate concentration, and chemical compounds) suggest that the differences observed were due to different amounts of the same enzymes present in the ligaments, rather than the involvement of diverse enzymes in normal and diseased tissue. The enzymes revealed with *N*-L-alanyl-2-naphthylamine were not considered to belong to so called inflammatory peptidases or proteinases.

RÉSUMÉ

ACTIVITÉ DES ARYLAMINOPEPTIDASES DANS LE DESMODONTE HUMAIN NORMAL ET ENFLAMMÉ

L'activité des arylaminopeptidases dans le desmodonte normal ou enflammé a été étudiée en utilisant comme substrats plusieurs *N*-L-aminoacyl-2-naphtylamines, l'hydrolyse de la *N*-L-alanyl-2-naphtylamine faisant en particulier l'objet d'une étude plus approfondie. Lorsque le matériel étudié provenait de desmodontes entiers, l'activité enzymatique diminuait dans les échantillons témoins lorsque l'âge augmentait, mais, dans le matériel enflammé, il n'a pas été constaté de corrélation de ce type, sauf en ce qui concerne l'hydrolyse d'un des substrats. Lorsque les desmodontes ont été

partagés et répartis en deux groupes: matériel marginal et matériel non-marginal, on a trouvé pour le matériel marginal enflammé que les niveaux de l'hydrolyse étaient plus bas que pour le matériel témoin. Dans la partie apicale, la situation était inverse. Toutes les propriétés enzymatiques étudiées (mode de fractionnement sur gel Sephadex G-200, actions de la température, du pH, de la concentration du substrat, et des produits chimiques) indiquent que les différences observées étaient dues aux différences dans les quantités des mêmes enzymes présentes dans les desmodontes, plutot qu' à l'action d'enzymes différentes dans le tissu normal et enflammé. Les enzymes mises en évidence au moyen de la *N*-L-alanyl-2-naphtylamine n'ont pas été considérées comme appartenant aux peptidases ou protéinases »inflammatoires».

ZUSAMMENFASSUNG

DIE ARYLAMINOPEPTIDASEAKTIVITÄT DER KLINISCH GESUNDEN BZW. ENTZÜNDETEN WURZELHAUT

Unter Anwendung mehrerer *N*-L-Aminoasyl-2-Naphthylamine als Substrate wurde die Arylaminopeptidaseaktivität extrahierter Zähne bei klinisch gesunden bzw. entzündeten Parodontien untersucht.

Die Spaltung des *N*-L-Alanyl-2-Naphthylamins wurde dabei einer eingehenden Untersuchung unterzogen. An Hand der Kontrollproben wurde im Material aus dem gesamten Wurzelhautgebiet mit steigendem Alter der Patienten ein Absinken der Enzymaktivitäten festgestellt. Die betr. Korrelation war — von einem Substrat abgesehen — in Proben aus dem antzündeten Material nicht zu beobachten Nach Trennung der Wurzelhaut in einen marginalen und einen nichtmarginalen Teil wurde beim Material aus dem marginalen Teil in den entzündeten Proben eine schwächere Enzymaktivität als in den Kontrollproben, beim Material aus dem nichtmarginalen Gebiet jedoch ein umgekehrtes Verhältnis der Enzymaktivität wahrgenommen.

Sämtliche untersuchten Enzymeigenschaften (Fraktionierung mit Sephadex G-200 sowie die Wirkung von Temperatur, Substratkonzentration, pH-Wert und einigen chemischen Verbindungen) weisen in die Richtung, dass die beobachteten Unterschiede der Enzymaktivitäten wahrscheinlich eher von der unterschiedlichen Menge gleicher Enzyme als von vollkommen verschiedenen Enzymen in klinisch gesundem und krankem Gewebe herzuleiten sind. Die *N*-L-Alanyl-2-Naphthylaminspaltenden Enzyme wurden weder zu den sogen. Entzündungspeptidasen noch zu den Entzündungsproteinasen gezählt.

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