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ARYLAMINOPEPTIDASE ACTIVITY IN DENTAL PULP

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INTRODUCTION

Very little information is available on the chemistry of pulp, although it is evident that pulp is an important storehouse of a number of enzymes, undergoes active respiration, and displays all the functions of a vital tissue (*Zipkin*, 1968). The aim of this paper is to provide information on the arylaminopeptidase activity of swine and human dental pulp tissue. Special attention was given to the presence of aminopeptidase B-like enzyme in the pulp tissue. It was reasonable to expect this type of activity, as a broad distribution of aminopeptidase B in animal tissue has already been shown (*Mäkinen & Hopsu-Havu*, 1967 a; *Hopsu, Mäkinen & Glenner*, 1966 a; *Mäkinen*, 1968; *Mäkinen & Raekallio*, 1969), as well as the red blood cells constituting one of the synthesis sites (*Mäkinen & Mäkinen*, 1970).

This study is the first of a series of papers where the effect of various chemical compounds, used in endodontics, is studied with regard to the activity of the aminopeptidase B-like enzymes. These affector studies were motivated by an earlier finding (*Hopsu, Mäkinen & Glenner*, 1966 b) that aminopeptidase B (or other similar enzymes) may be responsible for the liberation of mediators, like bradykinin, in the course of the inflammation process. Any information about the inhibition or activation of such enzymes must be explored to understand the biochemistry of inflammation.

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MATERIALS AND METHODS

Chemicals. All chemicals and their sources were the same as described in earlier publications (*Mäkinen*, 1968, 1969 a).

Preparation of pulpal enzyme preparations. Swine dental pulps were obtained from a local slaughter house. The pulp was removed from the extracted teeth after killing the animals. The extraction was carried out 10 min after killing and the pulps were removed within 120 min. The pulps were homogenized for 5 min by grinding the material by hand in a mortar at +4°C. Glasswool was added to the tissue samples to render more effective homogenization. Appr. 5 g of swine pulp tissue was handled at a time in this way. The resulting crushed mass was then suspended in 10 ml of cold (+4°C) tris-HCl buffer, pH 7.2. The suspension was centrifuged for 20 min at 19.000 rpm (43.500 × g) in cold. The clear supernatant fluid was used in subsequent experiments.

Human pulp was obtained from teeth extracted mainly for orthodontic reasons and was generally handled as the swine pulp. However, the pulps were stored for 1—3 weeks at —20°C before use.

Swine serum was obtained from the same source as the pulp. Whole blood samples were centrifuged in cold (+4°C) for 10 min at 13.500 rpm (22.000 × g). The mass containing the red blood cells was suspended in cold water to accomplish disruption of cells (appr. 10 ml water per 2 g wet weight cell mass). The resulting mixture was stirred with a glass rod for appr. 10 min. The stroma was centrifuged as above, and the clear and red protein samples were investigated.

RESULTS

Preservation of enzyme activity. Crude enzyme preparations derived from swine dental pulp were stored at +4°C and —20°C and the enzyme activity against *N*-L-arginyl-2-naphthylamine and *N*-L-alanyl-2-naphthylamine was followed (the freezed preparation was thawed and refrozen during the test at three day intervals). The activity of the crude enzyme preparations was not essentially lowered during 14 days at +4°C. The sample stored at —20°C lost its activity gradually to one third during the same time interval. It is thus possible that pulpal arylaminopeptidases may lose some of their activity during histochemical enzyme assays involving the use of a cryostat.

Effect of sodium chloride on arylaminopeptidase activity. Table I shows the results when the effect of 0.2 M NaCl on the enzymic hydrolysis of a number of aminoacyl-2-naphthylamines was studied. The sodium chloride

Table I.

Effect of 0.2 M NaCl on the rate of the hydrolysis of some N-L-aminoacyl-2-naphthylamines (2-NA) by two enzyme preparations derived from swine dental pulp, as compared to the rate measured in the absence of added NaCl (water instead). Tested in 0.05 M phosphate buffer, pH 7.0, at 0.166 × 10^{-3} M substrate concentration. Aminopeptidase B-like enzyme refers to the preparation obtained by pooling fractions 95—105 (Fig. 1 A) and the crude preparation refers to centrifuged swine dental pulp homogenate

Substrate	Rate (M per min and per mg protein × 10^3)			
	Aminopeptidase B-like enzyme		Crude preparation	
	0.2 M NaCl	H_2O	0.2 M NaCl	H_2O
N-L-Alanyl-2-NA	0.36	0.45	7.35	9.81
N-L-Arginyl-2-NA	5.20	1.67	26.23	7.26
N-L-Leucyl-2-NA	0.23	0.23	6.47	6.44
N-L-Lysyl-2-NA	2.75	0.80	13.48	6.36

concentration of 0.2 M was used because aminopeptidase B is most active at NaCl concentrations ranging from 0.15 to 0.2 M (*Mäkinen*, 1969 b). It was found that the hydrolysis of *N*-L-arginyl- and *N*-L-lysyl-2-naphthylamine was accelerated by 0.2 M NaCl, an indication of the presence of an aminopeptidase B-like enzyme.

The activity of the same enzyme preparations against other *N*-L-aminoacyl-2-naphthylamines was also tested, but only the substrates listed in Table I were hydrolyzed at a considerable rate. However, during prolonged incubation other substrates were also seen to have hydrolyzed at low rate (as to the other substrates, see the list in Table II).

Ion exchange and molecular exclusion chromatography. Fig. 1. shows the results of ion exchange chromatography of swine dental pulp arylaminopeptidases acting on *N*-L-arginyl- and *N*-L-alanyl-2-naphthylamine. The fractionation pattern of the enzymes in Fig. 1 A closely resembles that obtained earlier with rat livers and erythrocytes, i.e. an aminopeptidase B-like enzyme is eluted out of the column between two enzymes hydrolyzing other *N*-L-aminoacyl-2-naphthylamines (*Hopsu, Mäkinen & Glenner*, 1966a; *Mäkinen & Mäkinen*, 1970). All other enzymic properties investigated with the aminopeptidase B-like enzyme were approximately the same as earlier reported for liver enzyme (the experimental details will not be given here). These properties were: the effect of pH on the rate of the reaction, and the effect of various enzyme inhibitors and activators. Hence the enzyme

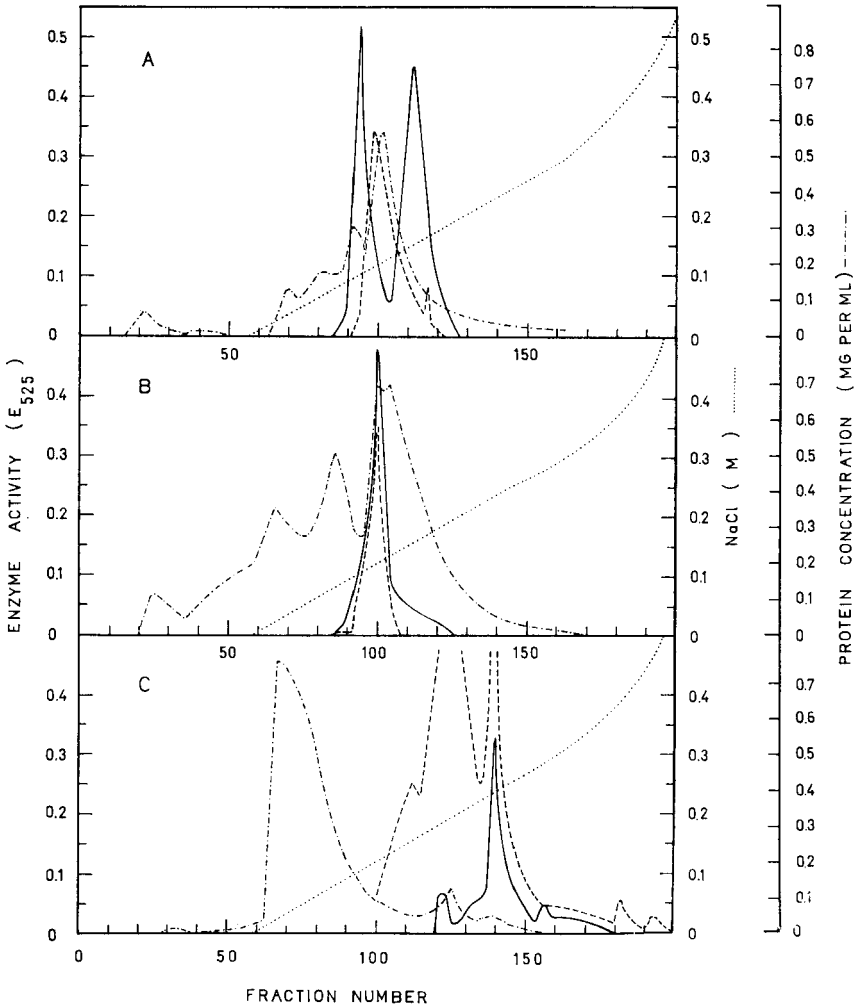

Fig. 1. Fractionation of arylaminopeptidases of swine dental pulp (A), serum (B) and red cells (C) on DEAE cellulose (*Schleicher & Schüll*, 230—270 mesh) columns. For all fractionations the following experimental details imply. Column: 1.6 × 23.0 cm; elution buffer: 0.01 M tris-HCl, pH 7.0, containing a NaCl gradient from 0 M to 0.75 M; mixing volume: 200 ml + 200 ml. Fraction volume: 1.5 ml; temperature: +2°C. ———, *N*-L-methionyl-2-naphthylamine; - - - -, *N*-L-arginyl-2-naphthylamine.

preparation made by pooling fractoins 95—105 (Fig. 1 A) is in the following termed aminopeptidase B, which is either identical or closely resembling rat liver and red cell aminopeptidase B). Fig. 1 further shows that swine pulp tissue contains arylaminopeptidases which seem not to occur in nor-

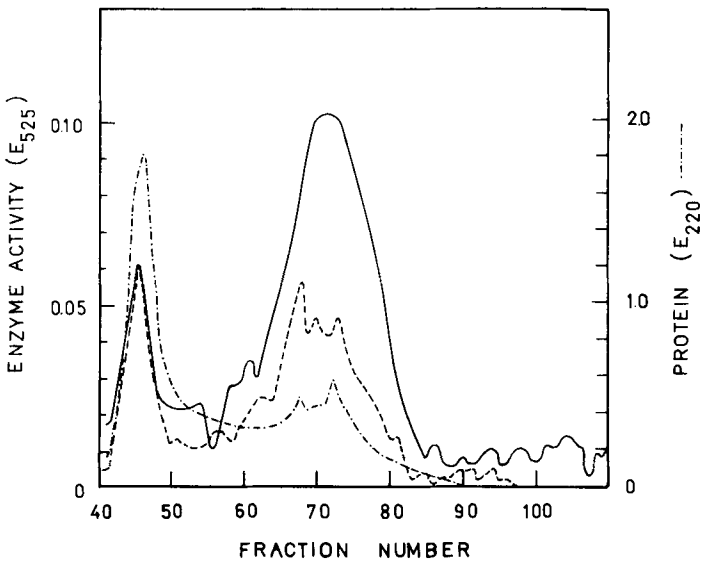

Fig. 2. Molecular exclusion chromatography of human dental pulp homogenate. Column: Sephadex G-200 (98.5 × 1.5 cm); void volume, 58 ml; Sample: 1.0 ml of centrifuged homogenate (see the Materials and Methods section); Elution buffer: 0.05 M tris-HCl buffer, pH 7.15; fraction volume: 1.5 ml; temperature: +2°C. Hydrostatic pressure: 15 cm. ———, *N*-L-arginyl-2-naphthylamine; - - - -, *N*-L-alanyl-2-naphthylamine; -.-.-, protein concentration (E_{220}). Due to the low amount of protein applied on the column, no positive result was obtained in attempts to assay protein at 280 mμ.

mal swine serum and red cells. Fig. 2 shows molecular exclusion chromatography of arylaminopeptidases of human dental pulp. Essentially similar results were obtained with swine pulp. The second enzyme peak was seen to contain an aminopeptidase B-like enzyme. This was tested as earlier described in this paper. The substrate specificity of the resultant two arylaminopeptidase peaks is shown in Table II.

Effect of DMSO, eugenol, phenol and camphor. The effect of some chemical compounds, often used in the field of endodontics, on the arylaminopeptidase activity of swine and human dental pulp was investigated. In Table III the results with a crude enzyme preparation derived from swine pulp (and serum) are presented. Essentially similar results were obtained with human dental pulp enzymes.

In Fig. 3 the effect of DMSO and eugenol on aminopeptidase B-catalyzed hydrolysis of *N*-L-arginyl-2-naphthylamine is shown. The highest rate of the hydrolysis was attained at 0.25 M DMSO. Eugenol inhibited the aminopeptidase B-like enzyme slightly at rather low concentrations. The tested concen-

Table II.

Human dental pulp arylaminopeptidase substrate specificity. The value, 100, was assigned to that substrate hydrolyzed at the highest rate. The substrate concentration was 0.166 × 10^{-3} M in each case. A relative rate of hydrolysis of zero was obtained for the following N-L-aminoacyl-2-naphthylamines (2-NA): α-N-L-aspartyl-2-NA, β-N-L-aspartyl-2-NA, β-N-L-glutamyl-2-NA, N-L-threonyl-2-NA, N-L-tosylarginyl-2-NA, and N-L-valyl-2-NA. I and II refer to the enzyme preparations obtained by pooling fractions 43—48 (I) and 66—86 (II) in the experiment presented in Fig. 2. The relative activity is different from that shown in the peaks of Fig. 2. This is due to the differences in the preservation of the enzymes

Substrate	Enzyme	
	I	II
N-L-Alanyl-2-NA	100	96
N-L-Arginyl-2-NA	56	71
N-L-Asparagyl-2-NA	23	
N-L-Cystine-di-2-NA	9	18
α*N*-L-Glutamyl-2-NA	12	96
N-L-Glycyl-2-NA	10	76
N-L-Histidyl-2-NA	12	
N-L-Hydroxyprolyl-2-NA	7	
N-L-Isoleucyl-2-NA	20	35
N-L-Leucyl-2-NA	91	100
N-L-Lysyl-2-NA	25	32
N-L-Methionyl-2-NA	78	
N-DL-Benzoylarginyl-2-NA	15	
N-L-Ornityl-2-NA	12	
N-L-Phenylalanyl-2-NA	60	35
N-L-Prolyl-2-NA	2	
N-L-Seryl-2-NA	3	
N-L-Tryptophyl-2-NA	8	
N-L-Tyrocyl-2-NA	6	

trations of eugenol were extended to approximately 0.1 mM only. This was due to the low solubility of eugenol into water (approximately 100 mg per 100 ml at 30°C, or approximately 110 mg per 100 ml at 40°C).

DISCUSSION

It is evident that aminopeptidase B occurs very widely in animal tissues. Its existence in the liver (*Hopsu, Kantonen & Glenner,* 1964; *Hopsu, Mäkinen & Glenner,* 1966 a), skin (*Mäkinen & Raekallio,* 1969; *Hopsu-Havu & Jansen,* 1968) and other tissues of rats (*Hopsu, Kantonen & Glenner,* 1964; *Mäkinen & Hopsu-Havu,* 1967 a) has already been demonstrated. It has

Table III.

Effect of some chemical compounds (0.166×10^{-3} M) on the rate of the arylaminopeptidase-catalyzed hydrolysis of some N-L-aminoacyl-2-naphthylamines (2-NA) by enzyme preparations obtained from swine dental pulp and serum, as compared to the rate measured in the absence of the compounds (water instead). Tested in 0.05 M phosphate buffer, pH 7.0, at 0.166×10^{-3} M substrate concentration. The enzyme preparations were unfractionated samples of pulp homogenate and serum obtained as described in the Materials and Methods section

Substrate	Rate (M per min and per mg protein × 10^3)					
	H_2O	DMSO	Eugenol	Camphor	Phenol	
N-L-Alanyl-2-NA	4.41	6.05	0	3.81	4.12	Pulp homogenate
N-L-Arginyl-2-NA	19.90	30.00	10.50	15.25	19.65	
N-L-Leucyl-2-NA	2.66	4.25	0	1.74	2.35	
N-L-Lysyl-2-NA	10.57	22.15	7.16	7.05	9.95	
N-L-Methionyl-2-NA	3.91	7.45	2.41	3.00	3.38	
N-L-Alanyl-2-NA	8.55	8.15	8.50	7.28	8.42	Serum
N-L-Arginyl-2-NA	3.49	3.28	3.50	2.75	3.10	
N-L-Leucyl-2-NA	5.47	5.12	5.89	4.79	5.48	
N-L-Lysyl-2-NA	3.87	3.57	4.25	3.08	4.05	
N-L-Methionyl-2-NA	1.65	1.62	2.30	1.28	2.30	

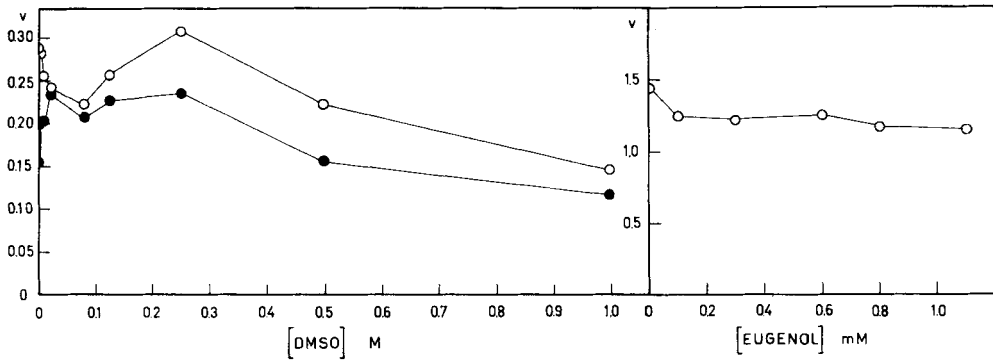

Fig. 3. Effect of DMSO and eugenol on the rate v (in $10^1 \times$ M min^{-1} of the hydrolysis of *N*-L-arginyl-2-naphthylamine (○—○) and *N*-L-lysyl-2-naphthylamine (●—●) by partially purified aminopeptidase B (the enzyme preparation was obtained by pooling fractions 95—105 in Fig. 1). For both Figs. the enzyme preparation was obtained from different chromatographic fractionations; hence the difference in v in A and B.

also been identified in the livers of the guinea pig and cat (*Hopsu, Mäkinen & Glenner*, 1966 c), in the livers of human foetuses (*Mäkinen*, 1968), in rat erythrocytes (*Mäkinen & Mäkinen*, 1970), and in human gingival tissue (*Paunio & Mäkinen*, data to be published). The enzymic and protein properties of aminopeptidase B have already been described (*Hopsu, Mäkinen & Glenner*, 1966 d; *Mäkinen & Hopsu-Havu*, 1967 b, c; *Mäkinen*, 1968; *Mäkinen, Euranto & Kankare*, 1969; *Euranto, Kankare & Mäkinen*. 1969; *Mäkinen*, 1969 b). It is not known whether all the tissues mentioned contain a tissue specific molecular form (isoenzyme), but there is evidence that at least rat liver and red blood cells have their own molecular form of aminopeptidase B (*Mäkinen & Mäkinen*, 1970).

From the finding that aminopeptidase B can be synthetized in red blood cells, it probably occurs as well in dental pulp (although there would not be any pulp-specific enzyme) and in gingival tissues, perhaps also in inflammatory exudates in the periodontal diseases, exerting its function in these tissues or tissue fluids. It is also possible that aminopeptidase B plays a role in normal healing, as in that following tooth extraction. It is important to recall that there is no apparent aminopeptidase B in normal serum (*Mäkinen & Hopsu-Havu*, 1967 a; *Mäkinen & Raekallio*, 1969). The results obtained in this laboratory, partly described in this paper, indicate that an aminopeptidase B-like enzyme is indeed present in swine and human dental pulp. However, no tissue specific molecular form of aminopeptidase B in pulp could be positively demonstrated.

Of the compounds tested for their effect on the pulpal arylaminopeptidase activity, only eugenol (2-methoxy-4-allylphenol) proved to be inhibitory, but the degree of inhibition in most cases was rather low at the concentrations used. However, the amount of this compound coming into contact with oral tissue during dental medication may be considerably higher than that here used and thus more effective inhibition may actually result. As to the mechanism of inhibition, no explanation can be given on the basis of the experiments carried out. However, it is commonly known that certain derivatives of benzene, particularly the phenols, react readily with protoplasmic constituents. The antiseptic and toxic actions of the phenols increase with the number of hydroxyl groups, and the addition of alkyl radicals leads to a quantitative variation in pharmacologic actions.

Camphor [1,7,7-trimethylbicyclo(2.2.1)-2-heptanone] and dimethyl sulphoxide proved inoccuous for the arylaminopeptidases studied. Rammler (1967) provided a preliminary report of his investigations into the effect of DMSO on several enzyme systems, and he stated that the inclusion of hydrolytic enzymes in such a study is relevant because DMSO may alter the con-

centration of water, which is an essential constituent of hydrolytic reactions. In the paper cited DMSO was found to be rather harmless for enzyme proteins. The observation that DMSO activates aminopeptidase B may result in the higher reactivity of anions in DMSO than in alcohol or aqueous systems (*MacGregor*, 1967). Because DMSO has a higher dielectric constant than alcohols and does not solvate anions by hydrogen bonding, it may be an activator for aminopeptidase B, which requires chloride ions for maximum activity.

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SUMMARY

The enzymic hydrolysis of some *N*-L-aminoacyl-2-naphthylamines was studied with enzyme preparations obtained from swine and human dental pulp. Molecular exclusion and ion exchange chromatography followed by detailed biochemical analysis showed that pulp tissue contains aminopeptidase B. It is not known whether this enzyme is derived from red cells or pulp tissue itself. The effect of DMSO, eugenol, phenol and camphor on the activity of pulpal arylaminopeptidases was tested. Only eugenol inhibited strongly some of the enzymes involved.

RÉSUMÉ

ACTIVITÉ ARYLAMINOPEPTIDASIQUE DANS LA PULPE DENTAIRE

L'hydrolyse de quelques *N*-L-aminoacyl-2-naphtylamines a été étudiée au moyen de préparations enzymatiques provenant de la pulpe dentaire humaine et de la pulpe dentaire du porc. Par chromatographie sur échangeurs d'ions et par exclusion moléculaire, suivies d'analyses biochimiques détaillées, il a été démontré que le tissu pulpaire contient l'aminopeptidase B. On ignore si cet enzyme provient des globules rouges ou du tissu pulpaire lui-même. L'action du DMSO, de l'eugénol, du phénol et du camphre sur l'activité des arylaminopeptidases pulpaires a été étudiée. Seul l'eugénol (0,166 mM) inhibait fortment les enzymes entrant en jeu.

ZUSAMMENFASSUNG

DIE AKTIVITÄT VON ARYLAMINOPEPTIDASE IN DER ZAHNPULPA

Es wurde die enzymatische Hydrolyse einiger *N*-L-Aminoacyl-2-Napthylamine mittels Enzympräparaten untersucht, die aus der Zahnpulpa des Schweines und vom Menschen gewonnen wurden. Molekulare Siebung und Ionenaustauschchromatographie mit anschliessender detaillierter biochemischer Analyse zeigten, dass Pulpengewebe Aminopeptidase B enthält. Es ist unbekannt, ob dieses Enzym von Erythrozyten oder von Pulpengewebe selbst stammt. Es wurde der Effekt von DMSO, Eugenol, Phenol und Kampfer auf die Aktivität von Arylaminopeptidasen der Pulpa geprüft. Nur Eugenol behinderte die beteiligten Enzyme stark.

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