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STUDIES ON ORAL ENZYMES

V. OCCURRENCE AND PROPERTIES OF AMINO- PEPTIDASE-LIKE ENZYME ACTIVITIES IN GUINEA-PIG SALIVA

by

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INTRODUCTION

There is little information about salivary enzymes in the animal species: by carrying out a systematic and comparative enzyme analysis on human beings and animals, certain conclusions might perhaps be drawn, that would lead to a better understanding of oral biochemistry and of the mechanism of the development of dental caries and periodontal disease.

In this paper some enzyme activities present in guinea-pig saliva are described. It is seen that certain quantitative and qualitative differences exist between the salivary enzyme spectra of human beings and of guinea-pigs.

This paper gives results from experiments concerning the ability of centrifuged guinea-pig saliva to hydrolyze various naphthylamides and -esters, including studies on the pH optimum of some reactions: affector studies concerning the hydrolysis of ala- β -NA are also presented.*)

*) The same abbreviations are used as in Mäkinen, 1966 a--d.

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MATERIALS AND METHODS

This study was carried out by following largely the same methods and using the same chemical reagents as in the investigations on human saliva or plaque (Mäkinen, 1966, a, b, c, d). The only exceptions were the way of collecting the test material, and its use in the enzyme determinations.

In collecting the saliva the animals were as little disturbed as was possible. The animals have thus been spared for possible further studies. The saliva was collected evenly from six two-year-old guinea-pigs of both sexes by introducing the tip of a small and soft plastic spatula into the mouth of the animal. The tip of the spatula was clipped comblike to increase the area that came in contact with the mouth. After the animal had chewed the spatula a few seconds, it was dipped into 2 ml of cold (+ 1—

Table I

Ability of centrifuged guinea-pig saliva to hydrolyze various amino acid β -naphthylamides. The activity is expressed as μ moles of β -NA liberated per hour per mg protein in the reaction mixture (110 μ l) (test conditions: 0.014 M universal buffer, pH 7.0, +37°C).

Substrate	Activity	Substrate	Activity
Ala-	0.0015	Leu-4-methoxy	0.0001
Arg-	0.0004	Lys-	0.0001
Asparagyl-	0.0001	Met-	0.0015
α -Asp-	0.0002	BANA	0
β -Asp-	0.0002	Orn-	0.0002
Cystine-di-	0	Phe-	0.0015
γ -Glu-	0.0001	Pro-	0
β -Glu-	0	Ser-	0.0001
Gly-	0.0001	Thr-	0.0001
His-	0.0003	TANA	0
Try-	0.0003	OH-pro-	0
Ileu-	0.0001	Tyr-	0.0002
Leu-	0.0001	Val-	0

Table II

Ability of centrifuged guinea-pig saliva to hydrolyze various esterase substrates. The values are in comparative relation to the value 100 given to the hydrolysis of α -naphthylvalerate. Incubation time 24 hours.

Substrate	Hydrolysis rate
BPANE	0
α -naphthylpropionate	70
α -naphthylvalerate	100
α -naphthyllaurate	8.3
β -naphthylacetate	33
β -naphthyllaurate	0
β -naphthylpalmitate	0
β -naphthylstearate	0
Sodium α -naphthylphosphate	
Calcium α -naphthylphosphate	
6-bromo- β -naphthylsulphate	0
6-bromo- β -naphthyl- α -D-glucoside	2.4
6-bromo- β -carbonaphoxycholine iodide	0

+ 4° C) 0.154 M NaCl solution and shaken gently. The sodium chloride solution was kept in an ice bath. The spatula was dried, and the procedure was repeated till a sufficient amount of saliva had been collected. This took only a few minutes per animal, because the methods used for estimating the enzyme activities were very sensitive and did not need large amounts of enzymes. The saliva was then either frozen as described earlier (Mäkinen, 1966, a), or used immediately. The resulting suspension was centrifuged (23500 \times g, 15 min.) in cold (+ 1° C) and the sediment was discarded. The clear, undiluted supernatant was used as enzyme preparation.

RESULTS

1. Hydrolysis of amino acid β -naphthylamides. Table I shows the results obtained when testing the ability of centrifuged guinea-pig saliva to hydrolyze various amino acid β -naphthylamides. Ala-,

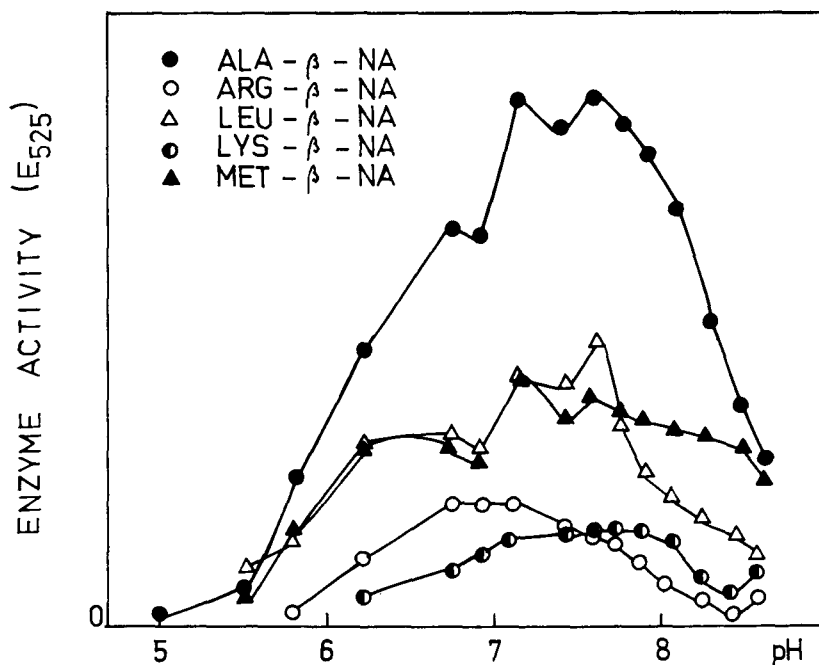


Fig. 1. Effect of pH on the hydrolysis of various amino acid β -naphthylamides in universal buffer. Incubation time 24 hours.

met-, and phe- β -NAs were hydrolyzed much more rapidly than any of the other substrates. Even guinea-pig saliva collected at different times, was shown to be liable to certain qualitative and quantitative changes. Table I presents only one typical experiment, not mean values of several tests.

2. Hydrolysis of esterase substrates. Table II shows the results obtained when the hydrolyzing ability of centrifuged guinea-pig saliva was tested on various esterase substrates. As can be seen from the table, α -naphthylvalerate was the substrate most rapidly cleft. Thus, the situation was the same as with human saliva. Distinct lipase substrates were hydrolyzed at a low rate, or not

at all. As with human saliva, BPANE was also cleft very slowly or not at all in certain experiments.

3. pH Optimum of the hydrolysis of β -NAs. The pH optimum of the hydrolysis of five different amino acid β -naphthylamides is shown in Fig. 1. Because of the heterogeneity of the enzyme preparation, several peaks appear in the curves. Substrates bearing a CH_3 -terminal side chain, all gave similar pH curves. The same applies to arg- and lys- β -NA bearing basic side chains. It also appears that a large amount of the total hydrolytic activity toward these substrates occurs above pH 7.

4. Affector studies. The effect of certain common enzyme inhibitors on the hydrolysis of ala- β -NA are shown in Table III. As can be seen from the table, only EDTA produced an inhibitory effect. The results indicate that the corresponding enzyme (or rather enzymes) hydrolyzing ala- β -NA, is rather stable against many enzyme inhibitors, and that it may require a metal cation to be fully activated.

Table III

Effect of various enzyme inhibitors on the hydrolysis of ala- β -NA by centrifuged guinea-pig saliva. The reported values are relative, 0 being assigned to the conditions not affecting the rate of hydrolysis. The figures show the percentage of the activity destroyed during the incubation time (24 hours).

Affector		Effect
EDTA	0.2×10^{-3} M	75
	0.1×10^{-3}	75
Zn ++	0.2×10^{-3}	0
	0.1×10^{-3}	0
TPCK	0.2×10^{-3}	0
	0.1×10^{-3}	0
PMSF	0.2×10^{-3}	0
	0.2×10^{-3}	0
PCMB	0.2×10^{-4}	0
	0.2×10^{-5}	0
NEM	0.2×10^{-3}	0
	0.1×10^{-3}	0

DISCUSSION

The main purpose of this study was to demonstrate various aminopeptidase activities in guinea pig saliva. When comparing the results with those obtained with human saliva and plaque (Mäkinen, 1966, a, b, c, d), at least two differences are to be noted:

1. Only ala-, met-, and phe- β -NA were hydrolyzed noticeably by guinea-pig saliva. Phe- β -NA was cleft very slowly by the enzymes in human saliva.
2. Arg-, lys-, and pro- β -NA were not hydrolyzed as rapidly by guinea-pig saliva as by human saliva.

The discovery, that guinea-pig saliva lacks iminopeptidase activities similar to those found in human saliva, was probably the most remarkable results of this study. In this connection, it is worth noting that other investigations made in this laboratory have shown that dog saliva does not possess iminopeptidases, or else it has very little activity toward pro- β -NA (Larmas, 1966). Thus, it could be supposed that the iminopeptidase-like enzymes described earlier (Mäkinen, 1966, a, b), play a role in the breakdown of collagenous material, for instance, the periodontium, thus starting the development of periodontitis. However, it should be noted that accidental slow breakdowns could be observed in repeated tests also with the substrates reported as not being hydrolyzed by guinea-pig saliva in Table I.

Concerning the esterase activities, the hydrolysis of α -naphthylvalerate was remarkably rapid as compared to that of other substrates used. α -Naphthylvalerate was also the ester hydrolyzed most rapidly by preparations from human saliva. Dog's saliva seems to form an exception: the studies of Larmas, made in this laboratory, have demonstrated that the ester hydrolyzed most quickly by dog saliva is β -naphthylacetate (Larmas, 1966). As in the previous study (Mäkinen, 1966, d), the cholinesterase substrate was not necessarily used to demonstrate cholinesterase, but only in order to elucidate the general ability of the test material to split substrates of that kind. Guinea-pig saliva did not hydrolyze it, thus differing even here from preparations of human saliva.

Fig. 1, showing the effect of pH on the hydrolysis rate of five different amino acid β -naphthylamides, demonstrates that the saliva preparations used contained several different enzymes hydrolyzing those substrates. A distinct enzyme or enzyme group is responsible for the breakdown of basic amino acid β -naphthylamides and the substrates bearing hydrophobic CH_3 -terminal side chains. It is to be expected, that guinea-pig saliva does not possess as rich an enzyme spectrum as human saliva. Such a supposition was confirmed by the affector studies. Only one of the commonly potent enzyme inhibitors tested produced an effect on the hydrolysis of ala- β -NA: therefore a single main enzyme may be responsible for the hydrolysis of that substrate in guinea-pig saliva. Even this activity differed from that present in human saliva. The latter always contained enzymes splitting ala- β -NA which were sensitive to zinc cations. The guinea-pig enzyme was not affected by zinc.

SUMMARY

The ability of centrifuged guinea-pig saliva to hydrolyze various amino acid β -naphthylamides and naphthylesters has been studied in order to trace possible differences between animal and human saliva. Guinea-pig saliva was found to hydrolyze rapidly only three of the 26 different amino acid β -naphthylamides tested, that is, L-alanine-, L-methionine-, and L-phenylalanine- β -naphthylamides. These substrates were hydrolyzed most rapidly at, or slightly above, pH 7. The absence of iminopeptidase activity in guinea-pig saliva is remarkable. The rates of hydrolyses by guinea-pig and human saliva of esterase substrates were the same: α -naphthylvalerate was hydrolyzed most rapidly. The enzyme splitting L-alanyl- β -naphthylamide was inhibited in the presence of 0.2×10^{-3} and 0.1×10^{-3} M EDTA, but it seemed unaffected by other enzyme inhibitors. Many qualitative and quantitative differences were observed between the aminopeptidase activities in guinea-pig and human saliva.

RÉSUMÉ

ETUDES SUR LES ENZYMES DE LA BOUCHE

V. OCCURRENCE ET PROPRIÉTÉS DES ACTIVITÉS DES ENZYMES AMINOPEPTIDATIQUES DANS LA SALIVE DES COCHONS D'INDE

L'hydrolyse de diverses β -naphthylamides d'acides aminés et de divers naphthylesters par la salive centrifugée de cobaye a été étudiée par l'auteur dans le but de mettre en évidence des différences éventuelles entre la salive animale et la salive humaine. Il est apparu que la salive du cobaye n'hydrolysait rapidement que trois des 26 β -naphthylamides d'acides aminés qui ont été employées, les β -naphthylamides de l-alanine, de l-méthionine et de l-phénylalanine. L'hydrolyse de ces substrats se faisaient le plus rapidement à un pH égal ou légèrement supérieur à 7. L'absence d'activité imino-peptidasique dans la salive du cobaye est frappante. L'hydrolyse des esters par la salive du cobaye et par la salive humaine se faisait à la même vitesse; l'hydrolyse du valérate d' α -naphthyl était la plus rapide. L'enzyme dissociant l'l-alanyl- β -naphthylamide était inhibé en présence de $0,2 \times 10^{-3}$ et de $0,1 \times 10^{-3}$ M d'EDTA, mais ne semblait pas influencé par d'autres inhibiteurs. De nombreuses différences qualitatives et quantitatives ont été observées entre les activités aminopeptidasiques de la salive du cobaye et de la salive humaine.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

V. ÜBER DEM VORKOMMEN UND DEN EIGENSCHAFTEN VON AMINOPEPTIDASEÄHNLICHEN ENZYMAKTIVITÄTEN IM SPEICHEL VOM MEERSCHWEINCHEN

Vom zentrifugierten Speichel des Meerschweinchen wurde bei der Untersuchung die Fähigkeit zur Hydrolyse von verschiedenen β -Naphthylamiden und Naphthylestern der Aminosäuren ermittelt. Die Untersuchung diente zum Nachweis von Unterschieden zwischen tierischem und menschlichem Speichel. Der zentrifugierte Speichel vom Meerschweinchen hydrolysierte schnell nur drei von insgesamt 26 verschiedenen β -Naphthylamiden und

zwar L-Alanyl, L-Methionyl- und L-Phenylalanyl- β -naphthylamide. Diese Substrate wurden am schnellsten bei pH 7 oder ein wenig darüber gespalten. Die Veränderung der Imino-peptidaseaktivität war ein bedeutendes Resultat. Die Hydrolysegeschwindigkeit von Esterasesubstraten war beim Meerschweinchen und Menschen dieselbe. Am schnellsten wurde α -Naphthylvalerat gespalten. Durch Wirkung vom $0,2 \times 10^{-3}$ und $0,1 \times 10^{-3}$ M Äthylendiamintetraessigsäure wurden die L-Alanyl- β -naphthylamidspaltenden Enzyme gehemmt. Die anderen untersuchten Enzym-inhibitoren hatten keine Wirkung. Es wurden viele qualitative und quantitative Unterschiede in der Aktivität von Aminopeptidasen im Speichel vom Meerschweinchen und Menschen beobachtet.

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