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STUDIES ON ORAL ENZYMES

IV. FRACTIONATION AND CHARACTERIZATION OF VARIOUS HYDROLYTIC ENZYMES IN HUMAN SALIVA

by

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INTRODUCTION

Certain relations between the enzymes and the state of oral health have been suggested. Knowledge of the role and activity of salivary enzymes is, however, too meager to permit the use of enzyme assays for diagnostic purposes, although nearly all types of the enzyme activities commonly estimated in biological materials have also been studied in saliva.

In this paper some characteristics of a number of different enzymes present in saliva are described, including esterase-like enzymes hydrolyzing various α- and β-naphthylesters of carboxylic acids, and enzymes having activity toward phosphate esters, glucosides, sulphates, and carboxypeptidase-like enzymes hydrolyzing N-carbo-β-naphthoxy-DL-phenylalanine. The main purpose has been to demonstrate the complex and varied enzyme spectrum of human saliva. This has been done by studying the fractionation patterns of the enzymes involved.

The literature contains several descriptions of the salivary enzymes in question. *Mahler et al.* (1955) have shown the ability

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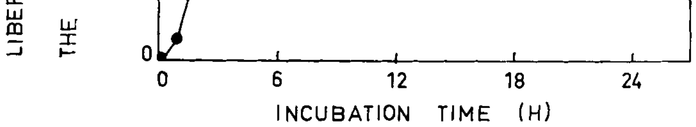

Fig. 1. Progress curve of the hydrolysis of L-lysine methylester by unfractionated whole saliva preparation in universal buffer, pH 7.0.

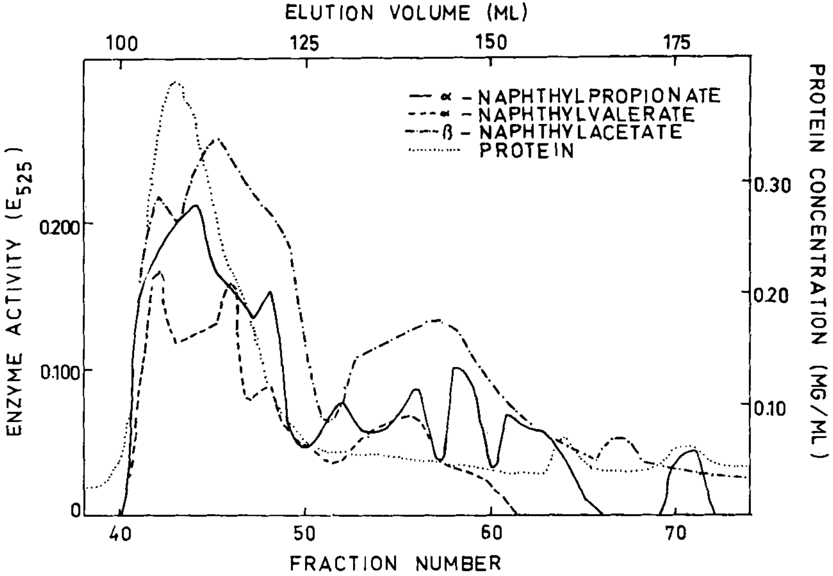

Fig. 2. Sephadex G-100 chromatogram of human saliva. Column: 96×1.5 cm; 6 ml of a protein solution whose preparation is described earlier; elution: 0.05 M TRIS-HCl buffer, pH 7.0; flow rate: 0.3 ml/min.; hydrostatic pressure: 20 cm; temperature: + 2°C; fraction volume: 2.5 ml. Blue Dextran was fractionated into tubes 42—44. Incubation time: 24 hours.

of whole saliva to hydrolyze aromatic esters of various carboxylic acids. *Pincus* (1950) has obtained a sulphatase-like enzyme from pure cultures of proteus types of microorganisms from carious material. Phosphorolytic enzymes have been studied by many investigators. *Dentay & Rae* (1949), and *Helman & Mitchell* (1954) have used disodium salt of phenyldihydrogen phosphoric acid in estimating the phosphatase activity in saliva. According to *Dentay & Rae* (1949) only a very small amount of phosphatase comes from lactobacilli. *Fitzgerald* (1952) has demonstrated that several strains of lactobacilli, streptococci, micrococci, and aerobacter possessed phosphatases. According to *Carter et al.* (1955) there exists a relationship between caries activity and the phosphatase concentration in saliva. High lactobacilli and streptococci counts were associated with high concentration of the enzyme. *Citron* (1945) has shown that Actinomyces israeli produces an alkaline phosphatase in the saliva. The main source of these enzymes seems to be bacteria and cellular elements in the saliva, which is certainly true of all the enzymes described in this paper. Recently, esterolytic enzymes of human parotid saliva have been studied by *Paunio et al.* (1966). At least five different activities were found to hydrolyze β-naphthyllaurate and four to hydrolyze triolein.

MATERIALS AND METHODS

The materials and methods used in this study, including the processes of collecting and preparing the saliva and all the chromatographic procedures, were principally the same as described in the first three papers in this series (*Mäkinen*, 1966, a, b, c).*) Some additions, however, should be noted.

For the determination of phosphatase activity sodium and calcium α-naphthylaphosphate was used. For sulphatase-like enzymes 6-bromo-β-naphthylsulphate potassium salt was used. 6-Bromo-β-naphthyl-α-D-glucoside was used for α-glucosidases, 6-bromo-β-carbonaphthoxycholine iodide for cholinesterases, and

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*) The abbreviations used are the same as in the previous papers in this series, with three additions: BPANE = N-benzoyl-DL-phenylalanine-β-naphthylester: CNP = N-carbo-β-naphthoxy-DL-phenylalanine: CNC = 6-bromo-β-carbonaphthoxycholine iodide.

N-carbo-β-naphthoxy-DL-phenylalanine for carboxypeptidase-like enzymes. The glucosidase and cholinesterase substrates were products of *Dajac Laboratories* (*The Borden Chemical Company, Philadelphia, Pen., USA*). Other substrates were purchased from Mann Chemical Laboratories, Inc. (New York, USA). The substrates used to determine esterase-like activities were N-benzoyl-DL-phenylalanine-β-naphthylester (*Nutritional Biochemicals Cor-*

Table I.

Ability of unfractionated whole saliva preparation to hydrolyze various naphthylesters. The values are in comparative relation to the hydrolysis of α-naphthylvalerate, noted as 100. Incubation time 24 hours.

Substrate	Hydrolysis rate
BPANE	1.8
α-naphthylpropionate	83
α-naphthylvalerate	100
α-naphthyllaurate	13
β-naphthylacetate	68
β-naphthyllaurate	9.0
β-naphthylpalmitate	3.0
β-naphthylstearate	1.0

Table II.

Ability of unfractionated whole saliva preparation to hydrolyze phosphatase, sulphatase, α-glucosidase and cholinesterase substrates as a function of pH. The values are in comparative relation to the hydrolysis of 6-bromo-β-naphthyl-α-D-glucoside at pH 7·0, noted as 100. Incubation time 24 hours.

Substrate	Hydrolysis rate		
	pH 4.5	pH 7.0	pH 9.0
Sodium α-naphthylphosphate	62	61	61
Calcium α-naphthylphosphate	49	60	44
6-bromo-β-naphthylsulphate	5.2	41	15
6-bromo-β-naphthyl-α-D-glucoside	7.2	100	70
6-bromo-β-carbonaphthoxycholine iodide	42	7.3	19

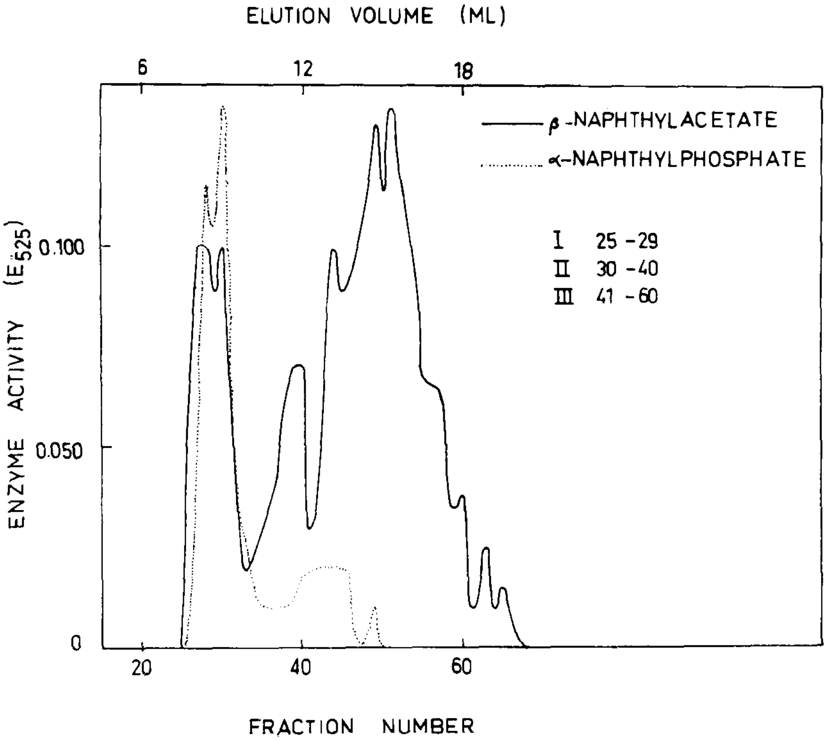

Fig. 3. Sephadex G-200 chromatogram of human saliva. Column: 56×0.7 cm; sample: 0.8 ml protein solution whose preparation is described earlier; elution: 0.05 M TRIS-HCl buffer, pH 7.0; flow rate: 0.12 ml/min.; hydrostatic pressure: 10 cm; temperature: + 4°C; fraction volume: 0.3 ml. Blue Dextran was fractionated into tubes 24—26. Incubation time: 24 hours.

poration, Cleveland, USA), β-naphthylacetate, -laurate, -palmitate, and -stearate, and α-naphthylpropionate, -valerate, and laurate, and L-lysine methylester (*Sigma Chemical Company, St. Louis, USA*). Sodium taurocholate (*E. Merck AG, Darmstadt, Germany*) was used in studying possible lipase-like enzymes in saliva.

When naphthylesters were used as substrates, the enzyme activities were estimated as described before (*Mäkinen*, 1966, c). For the determination of the hydrolysis of L-lysine methylester, the method suggested by *Siegelman et al.* (1962) was used with a single alteration: the reagent volumes were reduced to one tenth.

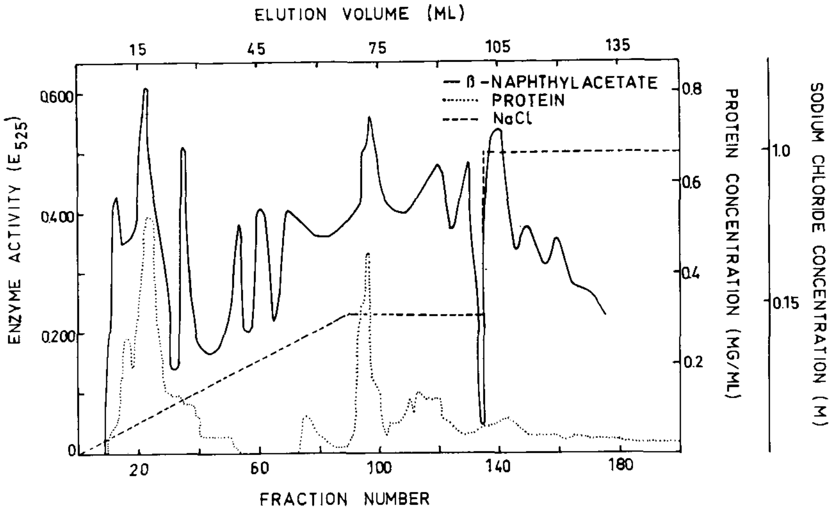

Fig. 4. DEAE-chromatogram of saliva. Explanations are the same as described in Mäkinen, 1966 a, Fig. 5.

RESULTS

1. Hydrolysis of various esterase substrates by unfractionated saliva enzyme. Tables I and II show the results obtained when various naphthylesters were used as substrates for unfractionated whole saliva preparations. Table I shows that all the naphthylesters used were hydrolyzed at least to some extent. In repeated experiments α-naphthylvalerate was always the substrate cleft most rapidly and the ratios between the rates of hydrolysis of the other substrates were also the same as shown in the table illustrating one separate test. Sodium taurocholate, used as 1.6 $\times 10^{-2}$ M in the reaction mixture, did not activate the reactions. On the contrary, a more or less strong inhibition was observed. Table II shows how the hydrolysis rates varied as a function of pH when phosphatase, sulphatase, glucosidase and cholinesterase substrates were used. As a rule the reactions proceeded most rapidly at neutral pH, except in the case of CNC, where the hydrolysis was faster at pH 4.5. Fig. 1 shows progress curve for the hydrolysis of L-lysine methylester. According to the results, human saliva contains enzymes capable of hydrolyzing CH_3-units from other groups.

2. Fractionation of various hydrolytic enzymes of saliva. Figs. 2—6 present results obtained in chromatographic experiments on saliva. These curves were selected from several others as illustrations of the varied and complex enzyme spectrum of preparations of human saliva, collected at different times. For instance, Figs. 3 and 5, showing fractionation patterns of enzymes hydrolyzing calcium α-naphthylphosphate, are not identical, though two main peaks are to be seen in both the fractionations hydrolyzing this substrate. Fig. 4 shows that at least ten different enzymes hydrolyzing β-naphthylphosphate can be found, using DEAE chromatography. About as many different activities were found to hydrolyze the α-glucosidase substrate and CNP (Figs. 5 and 6). α-Naphthylvalerate was the substrate most easily hydrolyzed, as can be seen from the chromatograms. Thus, the most proper esterase substrate for saliva preparations was the one bearing an aliphatic side chain of five carbon atoms, and the susceptibility to the hydrolysis was lowered when the number of carbon atoms in the side chain of the substrate molecule decreased or increased from five.

3. Affector studies. The rate of hydrolysis of β-naphthylacetate

Table III.

Effect of certain enzyme inhibitors on the hydrolysis of β-naphhthylacetate by pooled enzyme fractions obtained in Sephadex G-200 filtration. The figures show the percentage of the activity destroyed during the incubation time (24 hours)

Affector	Peak I	Peak II	Peak III
PMSF 0.2×10^{-3} M	100	100	100
0.1×10^{-3} M	100	50	100
0.2×10^{-4} M	100	10	60
PCMB 0.2×10^{-4} M	100	100	100
0.2×10^{-5} M	100	100	100
0.2×10^{-6} M	60	100	100
TPCK 0.2×10^{-3} M	100	100	100
0.1×10^{-3} M	60	40	100
0.2×10^{-4} M	0	0	0

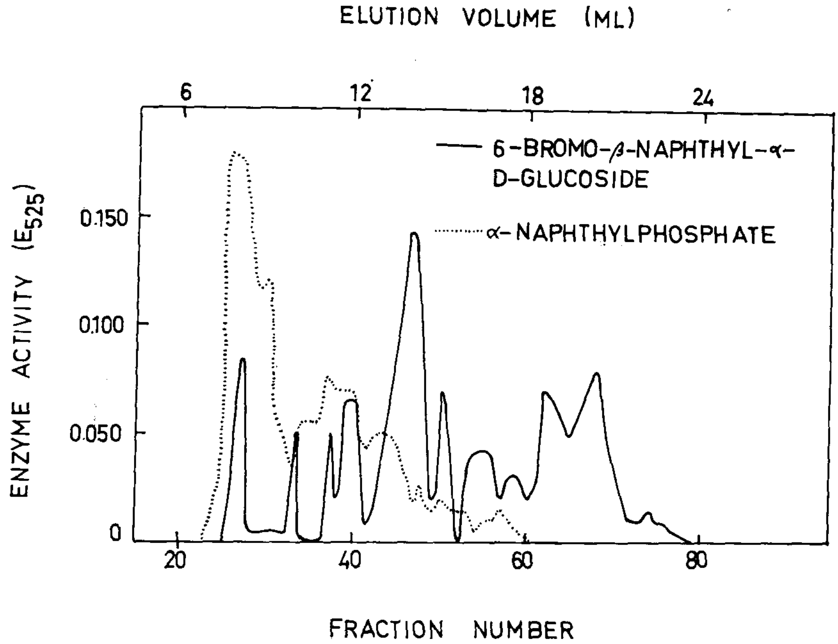

Fig. 5. Sephadex G-200 chromatogram of human saliva. Explanations as for Fig. 3.

by pooled fractions obtained in Sephadex G-200 filtration was tested in the presence of various enzyme inhibitors. The results are shown in Table III. The purpose of these affector studies was to throw light on the possibly trypsin- or chymotrypsin-like nature of the enzymes involved. Therefore PMSF and TPCK were tried as inhibitors. The results show that each enzyme peak contained esterases sensitive to these compounds, thus indicating that the enzymes may function in a way similar to the two proteases mentioned above. PCMB effectively inhibited the enzymes splitting β-naphthylacetate in the concentrations used.

DISCUSSION

The complexity of the enzyme spectrum of human saliva has been emphasized in earlier investigations (*Mäkinen,* 1966, a, b, c). One of the purposes of this paper has been to show how the variability of human saliva influences its fractionation patterns.

One of the facts revealed by these studies is that human saliva contains quite a few esterase activities hydrolyzing various naphthylesters, for instance, β-naphthylacetate. Evidently most of them correspond to unspecific enzymes capable of hydrolyzing, for instance, amino acid naphthylamides, as well. The fractionation patterns show that it is very difficult to discriminate between true salivary esterases and peptidases, even in saliva from one single person. The situation would be still more complex if mixed saliva collected from different persons with different oral hygiene were to be used. When using such a sensitive method for the estimation of enzyme activities as that used in these investi-

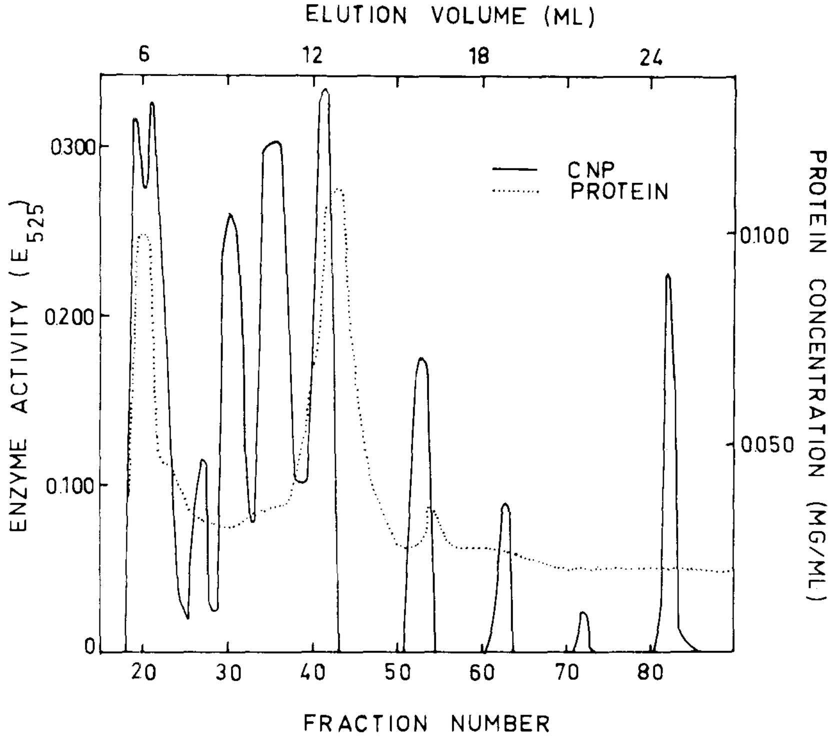

Fig. 6. Sephadex G-200 chromatogram of human saliva. Column: 55×0.7 cm; sample: 1 ml protein solution (preparation described earlier); elution: 0.05 M TRIS-HCl buffer, pH 7.0; flow rate: 0.11 ml/min.: hydrostatic pressure: 10 cm; temperature: + 3°C; fraction volume: 0.3 ml. Blue Dextran was fractionated into tubes 18—21. Incubation time: 24 hours.

gations, many small and accidental activities will, of course, also appear in the chromatograms.

Figs. 3 and 5 show fractionation patterns of enzymes hydrolyzing separate substrates using Sephadex G-200 columns. These curves are similar in form although separate saliva preparations were used as raw material. Accordingly, the phosphatase group present in saliva may be more stable than many others. No stability was, however, observed with the enzymes hydrolyzing CNP fractionated at different times. To some extent the same is true of the various glucosidase-like enzymes as well. The group of enzymes which acts on 6-bromo-β-naphthyl-α-D-glucoside may be quite considerable and varied.

It is to be noted that the cholinesterase substrate was not used explicitly in order to test the true cholinesterase in the mouth, but merely as a substrate of special structure in the study of the general ability of saliva to hydrolyze molecules of that kind. The pH optimum obtained for the hydrolysis of CNC was clearly at the acidic site, which proves that, in the mouth, CNC is cleft principally by other esterases than the commonly known cholinesterases, the pH optimum of which is close to, or above neutrality.

No evidence of the existence in human saliva of true lipases was obtained, if the fact that taurocholate failed to activate the hydrolysis of naphthylesters bearing long aliphatic chains is used as a criterion. *Paunio et al.* (1966) have not been able to prove the existence of lipases in human saliva. The occurrence of true lipases in the oral cavity seems to be very restricted. If any such activity should appear, its source may well be found in the oral tissues.

The data obtained in this and the previous investigations show that the oral cavity comprises a very complex and varied biochemical system. Many kinds of enzymic reactions may take place in the oral cavity, and the number and nature of various metabolites and end products washing the teeth and other oral tissue surfaces, and penetrating into crevices between them, is considerable. Thus the problems to be solved in this study differs essentially from those meeting in many other studies of tissues or bacterial cells.

SUMMARY

Various naphthylesters and L-lysine methylester were used as substrates in studies of esterase-like enzyme activities in human whole saliva. The substrates α-naphthylvalerate, α-naphthylpropionate and β-naphthylacetate were rapidly hydrolyzed, whereas only a slow hydrolysis of substrates bearing longer aliphatic side chains was observed. Sodium taurocholate had no activating effect on the hydrolysis of the naphthylesters, which indicates that human saliva does not contain true lipases. It was found that α-naphthylphosphates were cleft at nearly the same rate at pH 4.5, 7.0 and 9.0, whereas pH 4.5 was the most favourable for the hydrolysis of 6-bromo-β-carbonaphthoxycholine. The enzymes sulphatase and glucosidase were highly active at pH 7. The fractionation of the saliva on Sephadex- and DEAE-columns revealed about ten enzyme activities cleaving β-naphthylacetate as well as several enzymes hydrolyzing other esterase substrates. The hydrolysis of β-naphthylacetate was found to be sensitive to phenylmethyl sulphonylfluoride and to L-1-tosylamido-2-phenylethylchloromethyl ketone, which indicates that the esterases involved were of trypsin- or chymotrypsin-like nature. The enzyme spectrum of human saliva was found to be sensitive both qualitatively and quantitatively.

RÉSUMÉ

ÉTUDES SUR LES ENZYMES DE LA BOUCHE

IV. FRACTIONATION ET CARACTÉRISATION DES ENZYMES HYDROLYTIQUES DANS LA SALIVE HUMAINE

Divers naphtylesters et le méthylester de l-lysine ont été utilisés comme substrats pour l'étude des activités estérasiques existant dans la salive humaine mixte. On a observé une hydrolyse rapide des substrats suivants: valérate d'α-naphtyle, propionate d'α-naphtyle et acétate de β-naphtyle, tandis que, pour les substrats portant de longues chaînes latérales aliphatiques, on n'a pu observer qu'une lente hydrolyse. Le taurocholate de sodium n'avait pas d'action activatrice sur l'hydrolyse des naphtylesters, ce qui indique que la salive humaine ne contient pas de lipases véritables. La dissociation des α-naphtylphosphates s'est produite à peu près à la même vitesse aux pH 4,5, 7,0, et 9,0, alors que le pH 4,5 était le plus favorable pour l'hydrolyse de la 6-bromo-β-carbonaphtoxy-choline. La sulfatase et la glucosidase étaient extrêmement actives au pH 7. Le fractionnement de la salive sur colonnes Sephadex et DEAE a mis en évidence environ dix activités enzymatiques dissociant l'acétate de β-naphtyle, et plusieurs enzymes hydrolysant d'autres esters. L'hydrolyse de l'acétate de β-naphtyle s'est révélée sensible au phénylméthylsulfonylfluorure, et à la 1-1-tosylamido-2-phényléthylchlorométhylcétone, ce qui indique que les estérases entrant en jeu sont du type de la trypsine ou de la chymotrypsine. Le spectre enzymatique de la salive humaine était influencé tant du point de vue qualitatif que du point de vue quantitatif.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

IV. DIE TRENNUNG UND CHARAKTERISIERUNG VON VERSCHIEDENEN HYDROLASEN IM MENSCHLICHEN SPEICHEL

Zur Untersuchung von Esterase- und anderen Enzymen im menschlichen Gesamtspeichel wurden verschiedene Naphthylester und L-Lysylmethylester als Substrate verwendet. α-Naphthylvalerat, α-Naphthylpropionat und β-Naphthylacetat wurden schnell hydrolysiert, dagegen war die Spaltung von Substraten mit einer längeren aliphatischen Seitenkette langsam. Natriumtaurocholat aktivierte die Hydrolyse von Naphthylestern nicht, so dass eine echte Lipase im Speichel nicht nachgewiesen werden konnte. α-Naphthylphosphate wurden fast genau so schnell bei pH 4,5, 7,0 und 9,0 gespalten. 6-Brom-β-carbonaphtoxycholin wurde dagegen am schnellsten bei pH 4,5 hydrolysiert. Die Sulfatase- und Glucosidaseaktivität var am grössten in der Nähe vom pH-Wert 7. Die Trennung vom Speichel durch Sephadex- und DEAE-Säulen brachte etwa zehn β-Naphthylacetat-spaltende Enzyme, zum Vorschein ebenfalls mehrere, die andere Substrate spalten. Die Hydrolyse vom β-Naphthylacetat war gegen Phenylmethylsulfonfluorid und L-1-Toluensulfonylamido-2-phenyläthylchlormethylketon empfindlich. Das zeigt, dass diese Enzyme einen Trypsin- und Chymotrypsinähnlichen Charakter haben könn-

ten. Das Enzymspektrum vom menschlichen Speichel schien sehr empfindlich, sowohl gegen quantitative als auch qualitative Veränderungen zu sein.

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