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STUDIES ON ORAL ENZYMES

VI. HYDROLYSIS OF PERIODONTAL COLLAGEN BY PLAQUE ENZYME EXTRACT

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INTRODUCTION

It is a commonly accepted idea that the primary etiological factor of periodontal disease is an irritation caused by microorganisms in the gingival crevice. The microorganisms may produce their effect upon the periodontal membrane through their enzymes. Such oral enzyme activities have been studied intensively (*Dewar,* 1958; *Lucas & Thonard,* 1954; *Omata & Hampp,* 1961; *Scherp & Schultz-Haudt,* 1955 and 1964; *Schultz-Haudt et al.,* 1954 and 1953), and in particular Bacteroides melaninogenicus is thought to play an important role in the ethiology of the periodontal diseases (*Macdonald & Gibbons,* 1962; *Sawyer et al.,* 1962).

If enzymes actually are involved in the development of the above-mentioned disease, it would be sensible to believe that several enzymes exist which are responsible for the breakdown of the collagenous material. It is perhaps easier to understand a situation in which small changes in pH and in temperature

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around the tissue molecules — as already suggested for example by *Schultz-Haudt* (1964) — denature the collagen molecules, and when the proteolytic enzymes present in the oral cavity are then able to disintegrate them. There are certainly other factors also leading to a partial denaturation of collagen, of which various small mechanically-produced injuries — caused by a dentist for example — are no less important. So far nobody has convincingly shown that oral enzymes actually hydrolyze peptide or other chemical bonds present in the periodontal membrane. This paper describes some preliminary results obtained when studying the effect of oral enzymes on connective tissues.

MATERIALS AND METHODS

1. Reagents

The reagents used in this study were the same as in the previous papers of this series except for a few additions. The plaque was collected and its enzymes extracted in the same way as earlier (*Mäkinen,* 1966 b). The collagen used as substrate was obtained as follows.

The material was collected from extracted teeth. The teeth were stored at — 20° C before their preparation. They were first washed for three hours with 0.5 M EDTA solution, the pH of which was adjusted to 7 with KOH, in order to help the later detaching of the periodontal membrane. The membrane was removed carefully from the central part of the root and avoiding mixing with the periodontal membrane of the material from the apical and coronary area. The detached bulk was homogenized for 60 seconds in 4 ml cold 0.5 M acetic acid using the Ultra-Turrax top drive homogenizer at a speed of 20000 r.p.m. (Janke et Kunkel KG, Stauffen i. Br., Germany). The resulting mixture was then agitated for 15 hours at 4° C. It was then centrifuged (15 minutes, 23500 × g at 4° C) and the soluble collagen, at this stage still together with some other contaminating proteins, was precipitated with NaCl (15 % precipitation). The mixture was allowed to stand in the cold overnight. The precipitate was then dissolved in cold 0.5 M acetic acid and a 10 % NaCl precipitation was performed. The same precipitation was carried out two more times, centrifuging and dissolving the sediment every time

as described above, and allowing the mixtures to stand overnight.

The resulting precipitate was divided into three portions and washed three times with 0.05 m β,β-dimethylglutaric acid buffer, pH 6.0, 7.0 and 8.0, and finally suspended the fourth time in the same buffers at a concentration of about 1 mg/ml. This suspension of soluble collagen was used as an enzyme substrate in some experiments. The purity of this collagen was tested by estimating the nitrogen and hydroxyproline content of the preparation and by electrophoresis. The ratio between the nitrogen and hydroxyproline contents was 1.55 as an average of four determinations. In the electropherogram typical fractions formed by a mild denaturation of soluble collagen were to be seen.

The material obtained after dissolving the soluble collagen from the previous step in 0.5 M acetic acid was used as an enzyme substrate when studying the hydrolysis of insoluble collagen. The hydroxyproline test showed that this material was almost pure collagen. In addition, there were evidently some carbohydrate-protein complexes (to maintain the structure of collagen). If there is elastin in the periodontal membrane, it is to be found in this mass, which is insoluble in 0.5 M acetic acid.

2. Estimation of hydrolysis of collagen

The hydrolysis of the substrate was estimated by an ninhydrin procedure and based on the measurement of the amino groups liberated in the hydrolysis of peptide bonds as suggested by *Yemm & Cocking* (1955). In detail, the whole procedure was as follows:

The enzyme reactions were carried out in a micro mixture containing the following substances: (a) 100 μl 0.05 M β,β-dimethylglutaric acid buffer, (b) 20 mg (wet weight) unsoluble collagen (or 25 μl soluble collagen suspension), (c) 25 μl plaque extract, (d) 25 μl water or affector solution, 5 μl of toluene was added in the very first experiments, but because the results were the same in the presence or in the absence of it, it was not used later. The mixtures were then incubated, usually for 24 hours at 37° C under continuous agitation. The mixtures were then centrifuged and from each tube two samples of 25 μl were taken for the determination of the hydrolysis of collagen. The liberation of groups measurable with ninhydrin was finally determined colori-

metrically in the tubes stained with ninhydrin at 570 mμ with a Beckman B spectrophotometer.

The following control tubes were prepared every time and handled in the same way as the ordinary samples:

(a) Mixtures containing only substrate and buffer (water instead of enzyme).

(b) Mixtures containing only enzyme and buffer (water instead of substrate).

(c) Mixtures containing only enzyme, buffer and affector (water instead of substrate).

(d) Mixtures containing only substrate, affector and buffer (water instead of enzyme).

With these mixtures it was possible to control all "background" reactions. Thus, any increase in absorbance in the ninhydrin reaction was considered as being most likely due to the liberation of amino acids or peptides during the hydrolysis, after taking into account the color reactions caused by the control mixtures. Because it is known that the colorimetric reaction is not entirely specific for amino acids, since color with ninhydrin is produced by ammonia and many amino compounds (including peptides and proteins), the hydrolysis of collagen was determined also by thin layer chromatography. Here the solvent phase was freshly prepared n-butanol: acetic acid: water (200 : 50 : 50). For color development 300 mg ninhydrin was dissolved in 97 ml n-butanol after which 3 ml acetic acid was added. Free amino acids were used as a reference. The spots containing glycine and alanine (in this system glycine and alanine appeared in the same spot together with some peptides) were detached from the plates and the color complex extracted in 3 ml of metanol. The color intensity of the resulting solutions, obtained after spinning down the silica gel, was measured colorimetrically at 570 mμ. For thin layer chromatography the samples were obtained from the same reaction mixtures as for the test tube determinations, and 50 or 75 μl was applied from each tube on a 0.35 mm thick silica gel plate (silica gel G according to Stahl).

3. Determination of phosphorus

The amount of phosphorus in the reaction mixtures was determined by an ultramicro adaptation of the method of Fiske and

Subbarow, using the Beckman Model 150 Ultramicro Analytical System. 20 μl of the clear supernatant from each sample was used in the test.

RESULTS

Table I shows the results obtained in a typical experiment. It shows that a marked increase occurs in the number of chemical groups measurable with ninhydrin, particularly at pH 7 and 8 when the insoluble collagen was incubated with plaque enzymes. The presence of SH-groups (in cysteine and dithiothreitol) greatly accelerates the reaction. This indicates the necessity of SH-groups for the activity of the enzymes involved. EDTA seemed to inhibit the reaction, indicating that some metalloenzymes may

Table I

Liberation of groups measurable with ninhydrin in the hydrolysis of insoluble collagen from the periodontal membrane by plaque enzyme extract. The figures mean absorbancies obtained in the determination of the color produced by ninhydrin (test tube reaction) after substracting all background reactions at each pH.

Affector	pH 6	pH 7	pH 8
H_2O	0.080	0.045	0.090
1.4×10^{-3} M EDTA	0	0	0.050
1.4×10^{-3} M cysteine	0	0.190	0.200
1.4×10^{-3} M dithiothreitol*)	0	0.285	0.180

*)) Cleland's reagent (Calbiochem, Lucerne, Switzerland)

Table II

Liberation of groups measurable with ninhydrin in the hydrolysis of soluble collagen from the periodontal membrane by plaque enzyme extract. Other explanations as in Table I.

Affector	pH 7.0
H_2O	0.010
1.4×10^{-3} M EDTA	0
1.4×10^{-3} M cysteine	0.035
1.4×10^{-3} M dithiothreitol	0.070

also be involved, if the effect of it is not of unspecific nature. A demonstrable hydrolysis occurs also without any affector. Table II shows that in addition soluble collagen is hydrolyzed and that the affector characteristics of this reaction are similar to those for the previous reaction.

Some of these results were checked by thin layer chromatography. The plates showed most easily the various amino acid transformations found to take place in the reaction mixtures. It was, however, possible to measure colorimetrically the intensity of the spots on the plates containing glycine and alanine. After subtracting all the background reactions it was found that the concentration of free alanine and glycine and some peptides was increased in the reaction mixtures during the incubation. Table III shows a typical result. The figures in tables I—III are not comparable with each other because different amounts of ninhydrin and sample were used in each.

Fig. 1 shows a progress curve using periodontal collagen as the substrate and plaque extract as an enzyme preparation. The hydrolysis of the substrate was measurable with ninhydrin after about ten hours. The curves in separate experiments did not all have linear parts such as that in Fig. 1. The same figure also shows the liberation of phosphate from the substrate. The progress curves illustrating this reaction usually reached a horizontal level after about 40 hours, indicating that the enzyme prepara-

Table III

Liberation of groups measurable with ninhydrin in the hydrolysis of insoluble collagen from the periodontal membrane by plaque enzyme extract. The figures are obtained from an experiment by thin layer chromatography. The color intensity of the glycine and alanine containing spots has been measured.

Affector	pH 6	pH 7	pH 8
H_2O	0.020	0.020	0.040
1.4×10^{-3} M EDTA	0	0	0.020
1.4×10^{-3} M cysteine	0	0.050	0.080
1.4×10^{-3} M dithiothreitol	0	0.075	0.060

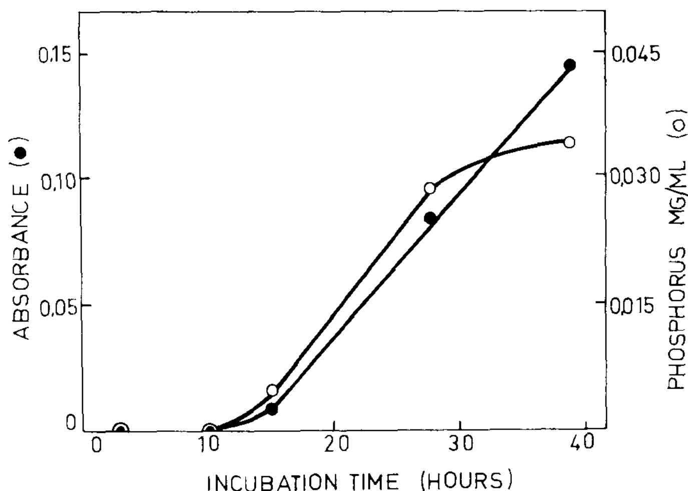

Fig. 1. Progress curves showing the liberation of groups measurable with ninhydrin and the liberation of phosphorus from human periodontal "native" collagen by human plaque enzyme extract.

tion used was unable to hydrolyze phosphate ester bonds after that time.

Table IV gives the results obtained when the phosphorus content of the reaction mixtures was determined at pH 6, 7 and 8. Most rapid liberation of phosphorus occurred at pH 7, and at each pH the reaction was accelerated in the presence of SH-groups, whereas EDTA had nearly no activating effect.

The content of hydroxyproline in the reaction mixtures was

Table IV

Liberation of inorganic phosphate (expressed as mg of elemental phosphorus per ml) from the insoluble periodontal collagen during a 24 hours incubation with the plaque enzyme extract.

Affector	pH 6	pH 7	pH 8
H_2O	0.010	0.020	0.011
1.4×10^{-3} M EDTA	0.010	0.014	0.013
1.4×10^{-5} M cysteine	0.015	0.020	0.019
1.4×10^{-3} M dithiothreitol	0.015	0.028	0.012

estimated by the method suggested by *Stegeman* (1958) and *Woessner* (1961). In this study it was observed that the concentration of the hydroxyproline decreased during a 24 hours incubation time, when compared with the concentration at the moment zero. Evidently the hydroxyproline formed in the reaction mixtures, and that already existing before the incubation had started was degraded to other amino acids by the enzymes of the plaque preparation. However, some experiments conducted on thin layer plates showed clear blue spots in the same level as proline standard (stained with isatin). These spots were not observable in tests performed with background mixtures. Unfortunately, the blue color was not very stable.

DISCUSSION

When one tries to elucidate the possible hydrolytic action of salivary enzymes on teeth and periodontal membrane, it is at first more useful to leave out of consideration the origin of this activity and simply to see the simple system tissue versus saliva and plaque. The hydrolysis must first be demonstrated and only after this is it sensible to unravel the origin of this activity.

It is difficult to imagine the surface of the gingival crevice without projecting chemical groups of which some might have been produced by local irreversible or reversible "microdenaturations" caused by heat, changes in pH and in the chemical composition of the environment, or by mechanical measures. Owing to the very rich enzyme spectrum present in the oral cavity, possibilities always exist for enzymatic hydrolysis of the chemical bonds of the partly denatured molecules in the connective tissue, or molecules of the granulation tissue which would be more susceptible to the action of enzymes. Whether these enzyme hydrolyses actually are the primary, secondary, or other reactions leading to periodontal disease or dental caries, is another problem which must be studied separately. These studies describe, however, one way which may be used when trying to elucidate the etiology of periodontal disease and of dental caries, i.e. that of using more or less native material as substrates for the oral enzymes. A second one was described in the previous papers of this series, i.e. the use of a larger number of synthetic model sub-

strates in order to examine the nature of the enzyme activities present in saliva and plaque. Both kinds of study should be carried out simultaneously.

As to the studies presented in this paper, it is important to mention that several practical difficulties exist when one wishes to demonstrate the described hydrolysis. The most important points are as follows:

1. Proper and careful handling of enzyme preparations.

2. The use of suitable activators for the enzymes involved.

3. The use of sufficiently sensitive analytical methods and instruments.

4. The accurate control of various "background" reactions; the possible spontaneous hydrolysis of the substrate, the possible breakdown of the used enzyme preparation in the presence and absence of the used activators, the transamination and further reactions of cysteine used as activator, and the retardation of the bacterial growth.

5. The use of substrates and enzyme preparations obtained from persons with sufficiently altered collagen, i.e. having a reduced amount of cross links and other stable chemical bonds.

6. To follow all the suggestions and precautionary measures given by *Yemm & Cocking* (1955) about the ninhydrin method.

The results obtained in this study show the enzymatic liberation of chemical groups demonstrable with ninhydrin and the liberation of phosphate from the periodontal collagen. The thin layer plates show, in addition, the liberation of alanine and glycine and/or some peptides from the substrate. As to the possible nature of the enzyme(s) involved, it is not at all certain that a collagenase would be the key catalyst in the breaking of the peptide bonds. Certain "broadspectrum" proteolytic enzymes are quite as likely. The substrates used in this study are "natural" and the enzymic reaction is accelerated in the presence of SH-groups. On the other hand, there are data indicating that cysteine does not inhibit collagenase acting against certain synthetic substrates as it does when collagen or gelatin is the substrate (*Heyns & Legler*, 1959). There is still more evidence for the noncollagenolytic nature of the observed hydrolysis, i.e. the fact that no free amino acids result in the hydrolysis of collagen or gelatin by col-

lagenase (*Seitfer et al.*, 1961). In these experiments free amino acids were evidently formed from the substrate during the reaction. It is, however, self-evident that the free amino acids might have originated from peptides like proline-glycine-alanine — produced first by a collagenase — by aminopeptidases or imidodipeptidases present in the crude enzyme preparation. Thus it seems likely that the described hydrolysis of periodontal collagen is caused by several hydrolytic enzymes.

The liberation of phosphate from the collagenous substrate is to be expected to some extent, if one considers that the material used contained some carbohydrates with phosphate bonded to them, or that the periodontal collagen molecules themselves contained phosphate links between polymers. The liberation might be caused by esterases in the enzyme preparation. It is also not unexpected, that the reaction has proceeded most rapidly at pH 7 because it has been shown (*Mäkinen*, 1966 d) that a marked hydrolysis of naphthylphosphates by oral enzymes also occurred just near neutrality. Thus the enzymes hydrolyzing phosphate ester bonds of the oral connective tissues need not necessarily be classical acidic or alkaline phosphatases, but merely enzymes acting rapidly also at pH 7.

Veis & Schlueter (1964) and *Schlueter & Veis* (1964) have suggested that the dentine collagen system contains a set of phosphate cross-linkages and also some carbohydrate cross-linking. The material used in this study contained no dentin. It is therefore suggested that these phosphate esters may be involved in diester cross-link formation also in the periodontal collagen and that esterase-like enzymes in the dental plaque are able to liberate at least some of this phosphate. In addition, it is worth noticing that in studies simultaneously conducted with these presented here, we have so far not been able to demonstrate the enzymatic liberation of phosphorus from human enamel and dentin. Therefore, the phosphorus present in the collagenous substrate used in these studies might not be affected in the same way as in the enamel and dentine.

SUMMARY

Insoluble and soluble collagen from the human periodontal membrane has been isolated and used as a substrate for crude

plaque enzyme extract. The hydrolysis of peptide bonds was determined by a ninhydrin procedure in test tubes and on thin layer plates. Evidently free glycine and alanine together with some peptides were found to form as a result of the hydrolysis of collagen. The plaque enzyme preparation also liberated phosphate from the substrate, indicating that the periodontal collagen may contain phosphate cross-linkages. Both peptide and phosphate ester bonds were cleaved most rapidly near pH 7, and the reactions were somewhat accelerated by SH-groups, whereas EDTA usually produced an opposite effect.

RÉSUMÉ

ÉTUDES SUR LES ENZYMES DE LA BOUCHE

VI. L'HYDROLYSE DU COLLAGUÈNE PERIODONTALE AVEC EXTRACT D'ENZYME DE LA PLAQUE

Du collagène soluble et insoluble provenant du desmodonte humain a été isolé et utilisé comme substrat pour un extrait enzymatique de plaque non préparée. L'hydrolyse des liaisons peptidiques a été mise en lumière en utilisant la ninhydrine, en tubes à essai et en couches minces. De la glycine et de l'alanine, manifestement libres, avec quelques peptides, se sont formés comme résultat de l'hydrolyse du collagène. L'extrait enzymatique de plaque a aussi libéré du substrat un phosphate, ce qui semble indiquer que le collagène desmodontal contiendrait des liaisons croisées phosphatées. La dissociation des liaisons peptidiques et des liaisons des phosphates se faisait le plus rapidement aux environs du pH 7, les réactions se trouvant quelque peu accélérées par les groupes SH, tandis que l'EDTA avait en général l'effet contraire.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ENZYME IN DER MUNDHÖHLE

VI. DAS HYDROLYSIS DES PERIODONTALEN COLLAGEN VOM DENTALEN PLAQUE HERVORGEBRACHT

Lösliches und unlösliches Collagen wurde aus menschlichem periodontalen Membran isoliert und als Substrat für rohes Plaque Enzym Extrakt verwandt. Die Hydrolyse der Peptidverbindungen

war mit einer Ninhydrin-Prozedur und auf Dünnschichtplatten determiniert. Es stellte sich heraus, dass sich offenbar bei der Collagenhydrolyse freies Glysin und Alanin zusammen mit einigen Peptiden bildeten. Auch das Plaque Enzym Präparat legte Phosphate des Substrates frei, was darauf deuten kann, dass im periodontalen Membran Kreuzverbindungen von Phosphat enthalten sind. Sowohl Peptid- als auch Phosphatesterverbindungen spalteten sich am schnellsten bei einem pH-Wert, der etwa bei 7 lag, und die Reaktionen wurden durch SH-Gruppen etwas beschleunigt, wogegen EDTA durchgängig die entgegengesetzten Effekte verursachte.

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