From: The Department of Oral Microbiology, School of Dentistry, Karolinska Institutet, Stockholm, Sweden.

PROTEOLYTIC ACTIVITY OF DENTAL PLAQUE MATERIAL

I. ACTION OF DENTAL PLAQUE MATERIAL ON AZOCOLL, CASEIN AND GELATIN

by

P.-Ö. Söder

G. Frostell

As dental plaque material constitutes a very important etiologic factor in caries as well as in periodontal disease, much interest has been paid to the investigation of its properties. The present investigation will deal with only one of the properties of the dental plaque material — *its proteolytic activity* — which has been considered associated with periodontal disease as well as the breakdown of organic components of tooth tissues in dental caries.

The breakdown of azocoll, a cow hide powder preparation containing denatured collagen coupled to an azo dye (*Oakley, Warrack & van Heyningen,* 1946) has been used as an indicator of proteolytic activity. As the substrate is hydrolyzed the dye is released and may be determined spectrophotometrically. Other substrates, such as gelatin, casein, egg albumen, plasma proteins and native collagen, have been used to test for proteolytic activity in

extracts from dental plaque material or from suspensions of bacteria cultivated from such material. *Roth & Myers* (1952) reported proteolytic activity by microorganisms cultivated from periodontal pockets and described an azocoll plate method for studying the proteolytic activity of dental plaque material (*Roth & Myers,* 1955). They demonstrated hydrolysis of azocoll by 57 out of 60 samples of gingival scrapings (*Roth & Myers,* 1956). *Schultz-Haudt, Bibby & Bruce* (1954) demonstrated the presence of enzymes capable of breaking down azocoll in cell free filtrates from gingival deposits.

Schultz-Haudt & Scherp (1955) showed collagenase activity in mixed cultures of gingival bacteria. They used native collagen as substrate and measured its hydrolysis by the liberation of peptides containing hydroxyproline. *Lucas & Thonard* (1955) cultivated microorganisms from gingival pockets of twelve persons with periodontal disease and from the gingival sulci of six healthy persons. About half of the nine hundred and nine strains of the organisms isolated were capable of hydrolyzing azocoll. *Mergenhagen & Scherp* (1960) showed that the majority of broth cultures from human gingival scrapings could hydrolyse reconstituted collagen from rabbit skin, but a number of strains of oral bacteria failed to attack this substrate. *Gibbons & Mac Donald* (1961) succeeded in showing that oral strains of Bacteroides melaninogenicus possessed an enzyme capable of hydrolyzing native collagen as well as gelatin, azocoll, casein, egg albumen and plasma protein. This enzyme was of the same type as those produced by clostridia but less potent and was released from the cells when they were lysed. The collagenase activity of Clostridium welchii and Clostridium histolyticum has been investigated by a number of authors (*Oakley, Warrack & van Heyningen,* 1946; *Mandl, Mac Lennan & Howes,* 1953; *Mandl, Zipper & Ferguson,* 1958; *Keller & Mandl,* 1963).

The aim of the present study is to demonstrate the existance of proteolytic enzymes within dental plaque by screening and quantitative methods using azocoll, casein and gelatin either in solid or liquid substrate mixtures. Some of the experiments were performed under bacteriostatic conditions to prevent altering the bacterial flora balance. The enzyme activity is expressed in terms of a given quantity of dental plaque material.

MATERIAL AND METHODS

Dental plaque material was collected from 80 patients with and without periodontal disease. The patient was allowed to rinse the mouth with tap water and the surfaces of the teeth were sprayed with tap water and dried with air. The plaque material was collected carefully from the teeth with a blunt instrument and transferred to a small stainless steel spoon which was transported to the laboratory in a moist chamber to avoid any change in weight by loss of water (*Krasse,* 1954). The wet weight was determined, 4.0 ml distilled water was added to the material, and the suspension was homogenized in a Virchow glass mortar (*Frostell & Larje,* in print). Since the wet weights of the material obtained in this way varied considerably, sufficient distilled water was added to make the final concentration about 2 mg wet weight per ml.

A small aluminum pan, the weight of which was determined beforehand to the nearest 0.01 mg was filled with 1.00 ml of plaque suspension and kept at 105° C for 24 hours. The dry weight of the plaque material was determined on a Cahn electromagnetic balance to the nearest 0.01 mg (*Frostell & Larje,* in print).

Chemicals

Preparation of azocoll was made according to a method given by *Oakley, Warrack & van Heyningen* (1946). In some experiments commercial azocoll (California Corporation for Biochemical Research, Los Angeles, U.S.A.) was used. Nutrient gelatin from Difco Laboratories Incorporated, Detroit, Michigan, U.S.A. or (U.S.P. granular) from Fisher Sc. Co., New Jersey, U.S.A. was used. Merthiolate (Schmitt-Jourdan, Boulogne-sur-Seine, France) was used as bacteriostatic agent in some experiments.

Casein was produced from non-pasteurized skim milk at about 32° C by adding 1.0 N HCl drop by drop. The milk was stirred vigorously without mixing air into the solutions. The pH was adjusted to 4.5—4.7, and the milk was allowed to stand a few minutes. After that the pH was determined and the milk was cooled in tap water at 10° C. The precipitate sank to the bottom and the supernatant was poured off. Distilled water was added with con-

stant stirring until the initial volume was reached. 0.1 N sodium hydroxide was added drop by drop every other minute until the precipitate dissolved. The pH was not allowed to rise over 7. The casein was precipitated again with 1.0 N HCl at between 25—30° C. This procedure was repeated at least 3 times.

The precipitate was finally put in a cotton bag and pressed carefully. The casein was then stored in a freezing chamber in air tight folium to prevent air from entering.

A 1 per cent casein solution was prepared in the following way: One gram casein was dissolved in 20 ml distilled water and 4.0 ml 0.5 N NaOH and allowed to stand for 30 minutes. The pH was adjusted to 6.9 ± 0.1 with HCl, 50.0 ml 0.1 M phosphate buffer (pH 7.0) was added, and the volume brought to 100 ml with distilled water (a modification of the method of *Beloff & Peters,* 1945 and *Martin & Axelrod,* 1957). The solution was sterilized by heating to 80° C for 60 minutes three times at 24 hours' intervals.

Determination of the proteolytic activity

Azocoll plates were made by the procedure of *Mandl, Mac Lennan & Howes* (1953). 100 mg azocoll was suspended in 10 ml nutrient agar in a Petri dish. The material to be tested was placed onto the wells and incubated 18 hours at 37° C.

Gelatin assay plates were made according to a method described by *Clarke & Cowan* (1952). Nutrient agar containing 2 per cent gelatin, 0.025 per cent glucose and 0.1 M phosphate buffer was distributed in Petri dishes. After application of the test material the plates were incubated at 37° C for 18 hours. They were finally flooded with a 1 per cent solution of tannic acid to demonstrate the zone of gelatin hydrolysis.

Casein agar plates were prepared in the manner used for antibiotic assays (*Cheeseman,* 1963). The medium contained 1 per cent agar, 1 per cent casein and 0.01 per cent merthiolate as an antibacterial agent. The pH was adjusted to 5.7. The plaque suspensions were put into wells cut in the agar and the material was allowed to act for 48 hours at room temperature. Plates containing 0.1 per cent commercial trypsin (3 × chryst.) in the wells were used for comparison.

Caseinolytic activity was tested also in the following way: one ml of a plaque suspension was mixed with 5.0 ml of a 1 per cent casein solution and the mixture was kept at 37° C for different periods of time. The casein was precipitated with 0.3 M trichloroacetic acid (TCA) and the amount of aromatic amino acids released was determined spectrophotometrically at 280 mμ with a Beckman spectrophotometer model DU and expressed as tyrosine equivalents. In some experiments the released non-protein nitrogen (NPN) was determined by the micro-Kjeldahl method.

Finally, gelatinolytic activity was demonstrated by a viscosimetric method in mixtures consisting of 1.0 ml plaque extract and 3.0 ml of a 4 per cent gelatin solution (0.1 M Tris[hydroxymethyl]aminomethane [TRIS] — HCl buffer pH 8.1). The change in viscosity was calculated from the outflow times of the plaque-substrate mixture in Ostwald viscosimeters at 37° C.

EXPERIMENTS AND RESULTS

A zone appeared around the well cut in the azocoll assay plates where the plaque material was applied. The zones appeared slowly requiring about two days to develope clearly.

Gelatinolytic activity was marked by a zone of opacity around the well in the gelatin assay plates when the agar was floaded with 1 per cent tannic acid after 18 hours incubation.

In the casein-assay-plates a series of precipitation zones appeared around the wells in the agar when either a plaque suspension or trypsin solution was used (Fig. 1). A determination was made of the released NPN in these zones by cutting out small portions of agar and treating them with a small volume of TCA. The soluble nitrogen was determined by the micro-Kjeldahl method. In the precipitation zones produced by trypsin the non-protein-nitrogen was higher than in the adjacent portion of the agar. When the zone surrounding plaque material was analyzed, however, it was not possible to demonstrate any difference in NPN content between the zone and the surrounding agar.

These experiments with azocoll, gelatin and casein were repeated 5 times with material taken from 5 different patients. In the kinetic studies on casein hydrolysis (Fig. 2) portions of the reaction mixture were precipitated with 0.3 M TCA after 3, 6, 9,

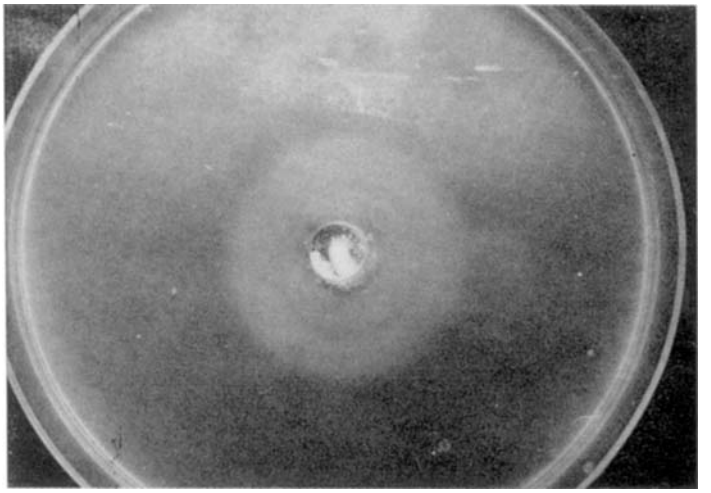

Fig. 1. Casein-agar plate, containing 1 per cent agar, 1 per cent casein and 0.01 per cent merthiolate, pH 5.7. The plaque suspension was put in the well in the center of the plate. The precipitation zones around the well result from the breakdown of casein.

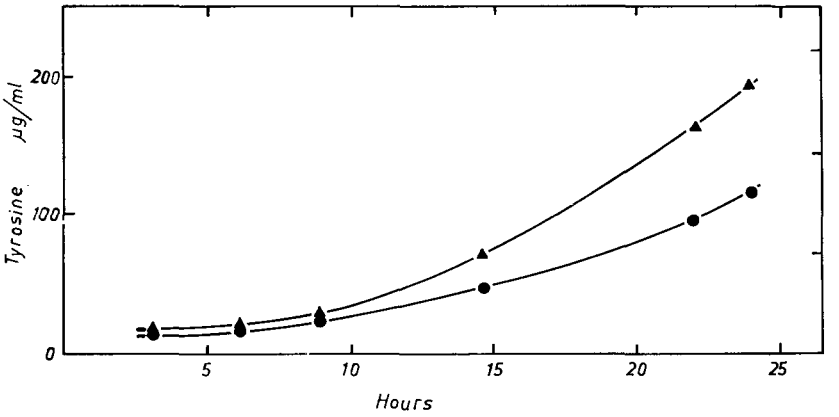

Fig. 2. The rate of hydrolysis of casein by plaque suspensions. 1.0 ml plaque suspension was mixed with 5.0 ml of a 1 per cent casein solution and after 3, 6, 9, 15, 22 and 24 hours at 37° C the casein was precipitated with 0.3 N TCA. The amount of aromatic amino acids was determined spectrophotometrically and expressed as tyrosine.

These are typical results from two different patients. The wet weight of the plaque material in experiment ▲—▲ was 1.90 and in experiment ●—● 2.00 mg/ml.

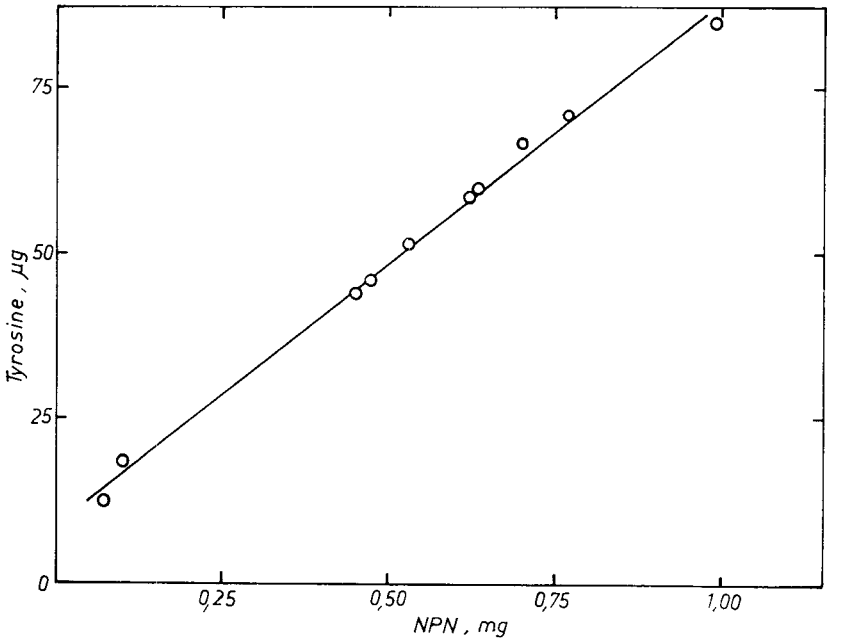

Fig. 3. The correlation between mg NPN determined by the micro-Kjeldahl method and µg tyrosine measured spectrophotometrically. The points on the curve correspond to the amounts of hydrolyzed products of casein not precipitated by TCA after different reaction times. 3.0 ml plaque suspension mixed with 6.0 ml 1.0 per cent casein.

15, 22 and 24 hours at 37° C and the amount of tyrosine determined. As can be seen from the figure the release of tyrosine was rather low during the first 10 hours of incubation, and increased slowly during the next 4 hours. In some experiments soluble nitrogen was also determined by the micro-Kjeldahl method and as can be seen from figure 3 there was a good correlation between the two methods of determination.

The soluble casein NPN formed after 18 and 24 hours enzyme reaction and in a non-enzyme control is shown in figure 4. These control tests revealed that about 1—1.5 per cent of the total casein content hydrolysed spontaneously. Material from different patients differed considerably in activity.

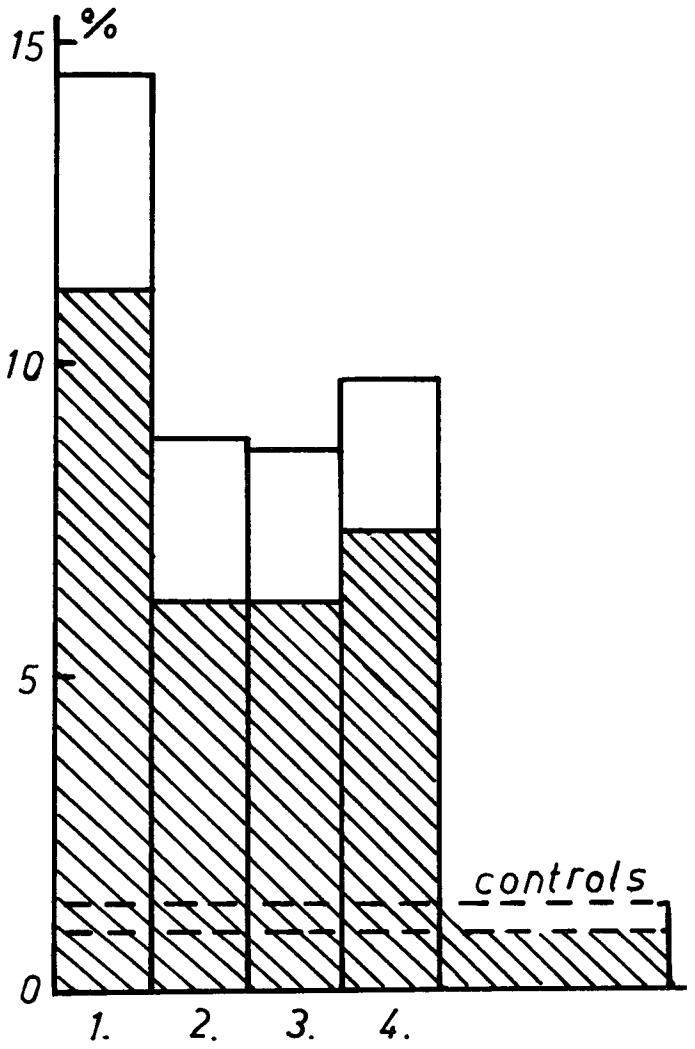

Fig. 4. The per cent of the total casein N not precipitated by TCA after 18 hours (shaded area) and 24 hours (clear area). The reaction mixture consisted of 1.0 ml plaque suspension plus 5.0 ml 1.0 per cent casein solution and was incubated at 37° C. The total N was determined by the micro-Kjeldahl method. No. 1, 2, 3, and 4 represent activity from different patients.

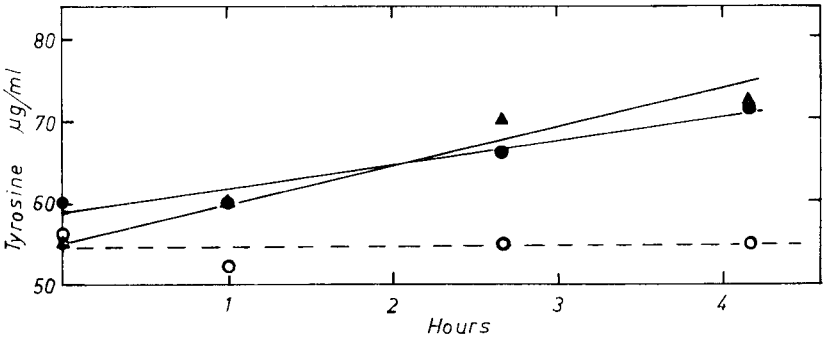

Fig. 5. The release of aromatic residues from casein expressed as µg tyrosine and followed over a 4 hour incubation at 37° C. The reaction mixture consisted of 1.0 ml plaque suspension and 20 ml of a 2.0 per cent casein solution. The total wet weights of the plaque material were 25.3 (▲--▲) and 10.1 mg (●--●). 0 = control.

The hydrolysis of casein during a 4 hour incubation period is shown in figure 5. Although the enzyme activity was weak, it was detectable in this period of time.

The effect of 0.01 per cent merthiolate on the caseinolytic activity in 5 plaque suspensions is shown in figure 6. The merthiolate caused about a 20 per cent reduction in enzyme activity measured as the release of TCA soluble nitrogen by the micro-Kjeldahl method. The activity was expressed in mg N released of the total after 18 hours incubation per mg dry weight of plaque material. The dry weights of the plaque were about 2.0 mg.

When gelatin was used as substrate a proteolytic activity could be demonstrated also (Fig. 7). The results were easily reproduced and it was easy to perform duplicate determinations with the same mixture. The error of the method was calculated from 25 duplicate determinations. This error (variation coefficient) was thus ± 4.9 per cent.

DISCUSSION

The preliminary screening experiments with assay-plates as well as the azocoll trials were useful for demonstration of proteolytic activity in the dental plaque, but gave no quantitative information of the amount of enzymes in the original sample.

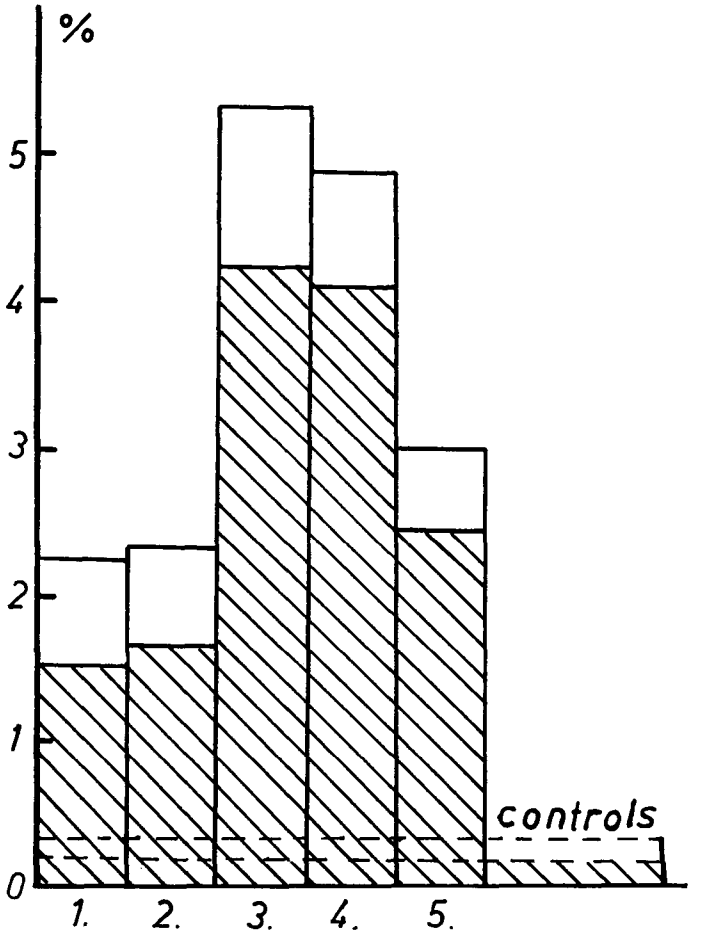

Fig. 6. The effect of 0.01 per cent merthiolate on enzyme action against casein. Plaque suspensions from 5 different patients were mixed with 1.0 per cent casein solution with merthiolate (shaded area) and without (clear area). The release of NPN was measured after 18 hours incubation at 37° C by the micro-Kjeldahl method and expressed as per cent N which was TCA soluble.

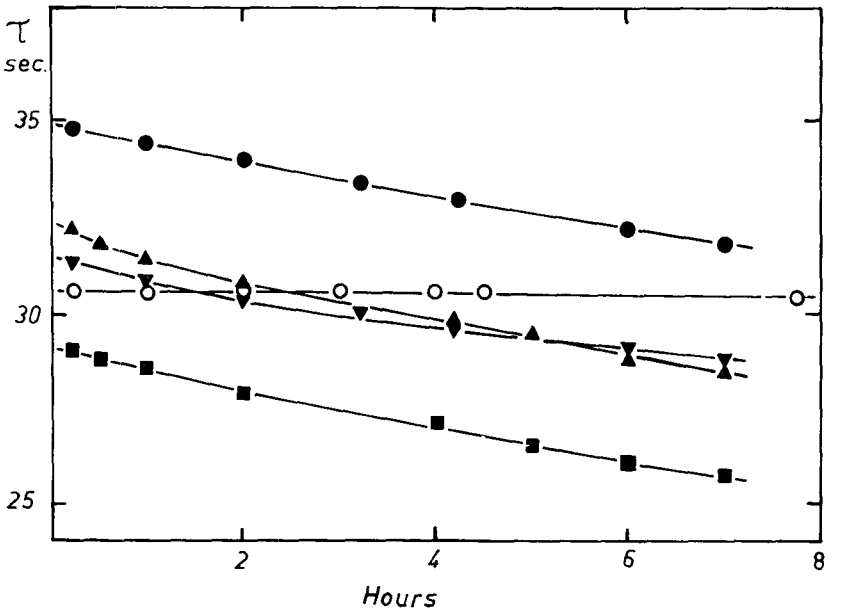

Fig. 7. The proteolytic activity of dental plaque material against gelatin. 1.0 ml of the plaque suspension was mixed with 3.0 ml of a 4 per cent stock gelatin solution. The outflow times in Ostwald viscosimeters with different standard outflow times were followed for 25 hours. (o- - - o = control).

Casein-agar-plate experiments revealed that proteolytic activity could be demonstrated over shorter periods of time. The method was very sensitive and was useful for demonstration of weak enzyme activity. The zones of precipitation, which are seen in figure 1, are similar to those produced by rennet in the same type of agar (*Cheeseman,* 1963).

Casein has been used often as a substrate for quantitative measurements of proteolytic activity (*Beloff & Peters,* 1945); however, it is not a very well defined substance and has a complex composition (*Lindqvist & Storgårds,* 1960). For this reason and because casein underwent spontaneous decomposition, it was not considered an appropriate substrate although it is very sensitive to weak enzymes (*Beloff & Peters,* 1945; *Martin & Axelrod,* 1957).

The curve in figure 2 may be partly due to the bacterial growth during the experiment and this possibility was one of the reasons why merthiolate was added later. However, the same type of curve was obtained when the plaque suspension was substituted by 1.0 ml of a papain solution (10^{-4} g/100 ml). The shape of the curve, also, may depend on the complexity of the casein molecule.

The decrease in enzyme activity found when merthiolate was present in the incubation mixture probably depended on the inhibition of bacterial growth, since it is shown that merthiolate does not interfere with proteolytic enzymes (*Lundblad*, 1952).

The trials with gelatin were convenient and precise. The gelatin was very stable and the method of measuring enzyme activity very sensitive. For these reasons this substrate was used for many of the later quantitative experiments on the proteolytic activity of dental plaque material together with several other natural and synthetic substrates. These experiments will be published in a future series of papers.

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SUMMARY

Dental plaque material was collected from persons with normal and diseased gingiva. The proteolytic activity in dental plaques was studied qualitatively by putting this material in wells cut in gelatin-agar, azocoll-agar and casein-assay-plates. The gelatin-agar-plates and the azocoll-plates were useful for screening tests on proteolytic activity. The casein-assay-plates were very sensitive and useful for demonstration of weak enzymes.

The proteolytic activity was determined quantitatively in casein solutions. The breakdown of casein was measured in two ways, 1) by determining the TCA-soluble aromatic amino acids spectrophotometrically and 2) by determining the TCA-soluble non-protein-nitrogen by the micro-Kjeldahl method.

Proteolytic activity against gelatin was demonstrated by measuring the outflow times of plaque extract-gelatin solutions in Ostwald viscosimeters. This method was very sensitive, convenient and precise for quantitative assay of proteolytic enzymes.

RÉSUMÉ

ACTIVITÉ PROTÉOLYTIQUE DE SUBSTANCE DE PLAQUE DENTAIRES

On a recueillé de la substance de plaques dentaires de personnes avec gencive normale et gencive cliniquement malade. L'activité protéolytique des substances enzymatiques de ces plaques dentaires a été étudiés quantitativement en mettant de cette substance dans des puits creusés dans la gélatine-agar, l'azocoll-agar et la caséine-agar. Les plaques de gélatine-agar et celles d'azocoll-agar étaient, cependant, moins sensibles que les plaques de caséine-agar. On a pu constater que, pour toutes ces méthodes, la substance des plaques a causé une protéolyse.

Quantitativement l'activité protéolytique a été déterminée dans une solution de caséine. Le degré lytique fut mesuré 1) en déterminant par spectrophotométrie les acides amino-aromatiques TCA-solubles, 2) en déterminant, par la méthode micro-Kjeldahl, la quantité d'azote non protéinique TCA-soluble.

L'activité protéolytique envers la gélatine fut démontrée en mesurant, à l'aide de viscosimètres Ostwald, le temps d'écoulement de solutions de substance de plaques mêlée avec de la gélatine. Cette méthode s'est trouvée très sensible, commode et précise pour des essais quantitatifs d'enzymes protéolytiques.

ZUSAMMENFASSUNG

PROTEOLYTISCHE AKTIVITÄT IN ZAHNBELAGMATERIAL

Dentales Plaquematerial wurde Personen mit normalen und mit klinisch kränklichem Zahnfleisch entnommen. Die proteolytische Aktivität solcher Plaques wurde quantitativ geprüft, indem mit solchem Material in Gelatine-Agar-Platten, Azocoll-Agar-Platten und Kasein-Platten geschnittene Kerben beschickt wurden. Die Gelatine-Agar-Platten und die Azocoll-Platten erwiesen sich als weniger empfindlich als die Kasein-Versuchsplatten bei Anwendung dieser Lysis Methoden.

Quantitativ wurde die proteolytische Aktivität in Kasein-Lösungen bestimmt. Die Zerstörung des Kaseins wurde auf zweierlei Art gemessen, 1) durch spektro-photometrische Bestimmung

der TCA-löslichen aromatischen Aminosäuren und 2) mittels Bestimmung des TCA-löslichen nicht-proteinen Stickstoffs nach dem Mikro-Kjeldahl Verfahren.

Proteolytische Aktivität gegen Gelatine wurde durch Messen der Ausflusszeiten von Lösungen von Plaquematerial gemischt mit Gelatine in Ostwald Viskosimetern festgestellt. Diese Methode erwies sich als sehr empfindlich, bequem und genau bei quantitativer Prüfung von proteolytischen Enzymen.

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Addresses:

P.-Ö. Söder,
Department of Oral Surgery,
School of Dentistry,
Karolinska Institutet,
Stockholm, Sweden.

G. Frostell,
Department of Oral Microbiology,
School of Dentistry,
Karolinska Institutet,
Stockholm, Sweden.