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CHARACTERISTICS OF THE HYDROLYSIS OF 4-PHENYLAZOBENZYLOXYCARBONYL-L-PROLYL-L-LEUCYL-GLYCYL-L-PROLYL-D-ARGININE (A COLLAGENASE SUBSTRATE) BY ENZYME PREPARATIONS DERIVED FROM CARIOUS DENTINE

by
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INTRODUCTION

The activity of collagenase can be measured by the breakdown of collagen (*Gallop, Siffer & Meilman*, 1957) or the synthetic substrate carbobenzoxy-glycyl-prolyl-glycyl-glycyl-prolyl-alanine (*Grassmann & Norwig*, 1960). Collagenase is also assayed by the increase in TCA-soluble nitrogen (*MacLennan, Mandl & Howes*, 1953). The release of bound dye has been used to estimate activity against denatured collagen (*Oakley, Warrack & van Heyningen* 1946). According to *Wunsch* and *Heidrich* (1963) collagenase activity is more conveniently determined by 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine which is cleaved by collagenase into a chromophore (4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucine) and a tripeptide (glycyl-L-prolyl-D-arginine).

Collagenolytic activity in oral tissues has been identified by *Bennick* and *Hunt* (1967), and *Gibbons* and *MacDonald* (1961) demonstrated the liberation of a collagenolytic enzyme in *Bacteroides melaninogenicus*, a microorganism which is said to be important in the etiology of gingival disease. The aim of this paper is to present data concerning collagenase-like enzyme activity in human carious dentine, using the above mentioned chromophore-acylpentapeptide as substrate.

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MATERIALS AND METHODS

1. *Chemicals.* The collagenase substrate, 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine, and 4-phenylazobenzyloxycarbonyl L-prolyl-L-leucine, as well as phenylmethanesulphonyl fluoride, N-ethylmaleimide, benzethonium chloride (benzyldimethyl{2-[2-(p-1, 1, 3, 3-tetramethylbutylphenoxy)ethoxy]-ethyl}ammonium chloride) and domiphen bromide [(β-phenoxyethyl)dimethyldodecylammonium bromide] were purchased from Mann Research Laboratories, Inc. (New York, N.Y., USA). Dequalinium chloride (decamethylenebis- 4-aminoquinaldinium chloride) was a product of Trawend Werk (Konstanz, W.-Germany). Dithiothreitol and sodium p-chloromercuribenzoate were obtained from Calbiochem (Los Angeles, Calif., USA). All other reagents were obtained from E. Merck AG (Darmstadt, W.- Germany).

2. *Enzyme preparations and determination of enzyme activity.* The making of enzyme preparations from human carious dentine is described elsewhere in detail (*Mäkinen*, 1968; *Mäkinen, Larmas & Scheinin*, 1969; *Mäkinen*, 1969). It may be mentioned, however, that these enzyme preparations were aqueous extracts of carious dentine made by treating powdered carious dentine with cold buffer. The resulting enzyme preparations contained 0.4—0.5 mg protein per ml. The collagenase activity was in principle tested as suggested by *Wunsch* and *Heidrich* (1963). The spontaneous hydrolysis of the substrate was constantly followed and found to be negligible. Appropriate blanks were constantly included in all determinations.

The various chemical compounds investigated for their effect on the collagenase-like activity of carious dentine were dissolved and treated as earlier described (*Mäkinen*, 1969).

3. *Thin layer chromatography.* The thin layer chromatographic experiments were carried out with the Desaga devices (C. Desaga GmbH, 69 Heidelberg, Germany) on 0.35 mm thick Kieselgel G layers. The development was conducted with n-butanol-acetic acid-water mixture (3:1:1, by vol.), and the amino acids were detected with a modified ninhydrin reagent of *Moffat* and *Lytle* (1959). Otherwise the instructions given by *Randerath* (1964) were followed. The chromatograms were scanned with the Chromoscan recording and intergrating densitometer equipped with a thin layer attachment (Joyce, Loebl & Company Limited, Gateshead, England), as described in more detail elsewhere (*Mäkinen*, 1969). The reaction mixtures to be analyzed were incubated for 10 h. in small test tubes sealed with Parafilm®. After the time involved the mixtures were placed in an iced water bath and aliquots of 50 μl were applied (in approximately 5 μl amounts at a time, using an electric fan in evaporating) on plates coated with 0.35 mm thick Kieselgel G layer.

4. *Amino acid analysis.* The amino acid analyses were performed on a Beckman Unichrom Amino Acid Analyzer according to the instructions given by the manufacturer (Beckman Instruction Manual for Beckman Unichrom Amino Acid Analyzer, 1966).

5. *Determination of protein.* The determination of protein concentration was carried out as earlier described (*Mäkinen*, 1969).

6. *Kinetics of the enzyme reaction.* The effect of substrate concentration on the velocity of the »collagenase»-reaction was studied as earlier described (*Mäkinen, Euranto & Kankare*, 1969). It may be mentioned, however, that the calculations were based on Hanes' equation (*Dixon & Webb*, 1964) and its extension and were carried out by giving each point a weight proportional to $v^4/[S]^2$, where v is the velocity and [S] is substrate concentration. For more details, see the paper cited.

RESULTS

The preliminary experiments showed that enzyme preparations, obtained from human carious dentine by extraction with buffers, were capable of hydrolyzing the substrate used in the study (Fig. 1). After prolonged incubation the amount of measured chromogene had decreased. This was explained in

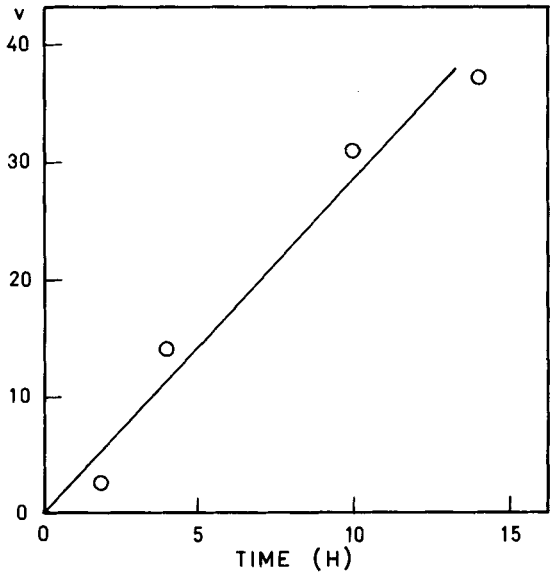

Fig. 1. Rate of hydrolysis, v (in $10^{-6} \times$ M), of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine catalyzed by an enzyme preparation obtained from carious dentine. Substrate concentration: 0.4 mM. Tested in 0.075 M tris-HCl buffer, pH 7.0.

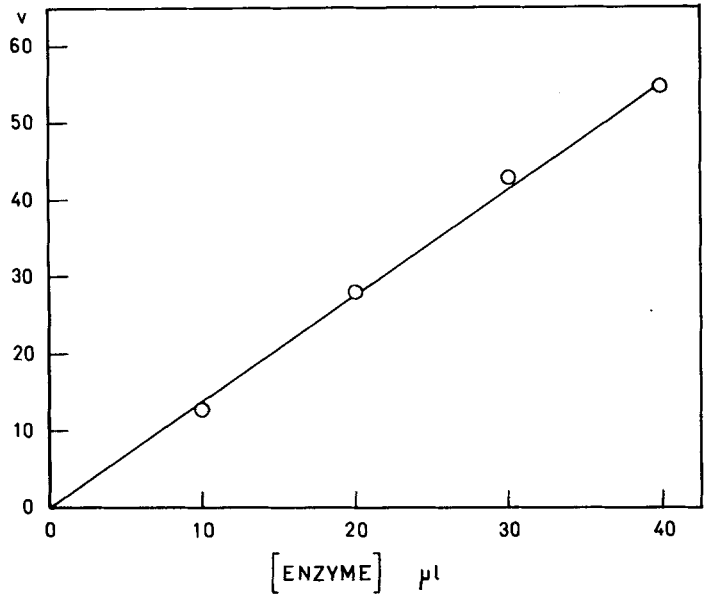

Fig. 2. Effect of enzyme concentration on the rate v (in $10^{-10} \times$ M min^{-1}) of the hydrolysis of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine catalyzed by an enzyme preparation obtained from carious dentine. Substrate concentration: 0.4 mM. Tested in 0.075 M tris-HCl buffer, pH 7.0.

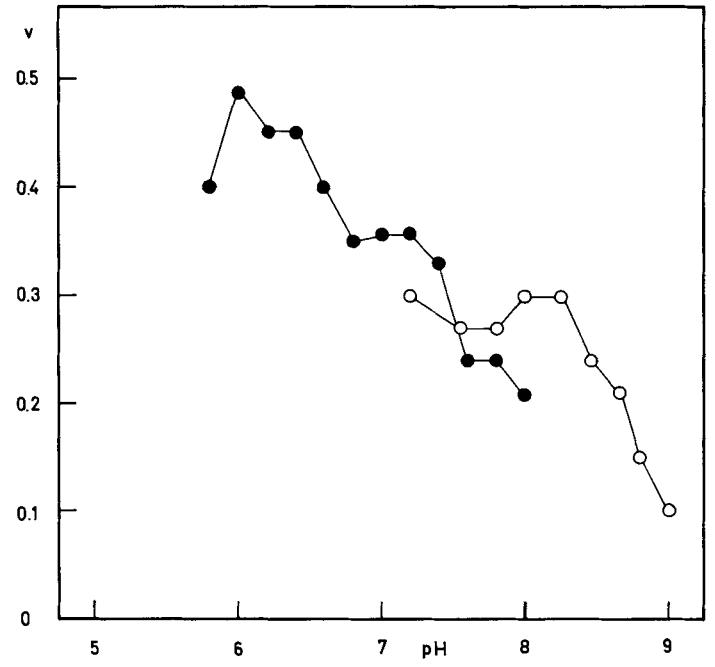

Fig. 3. Effect pH on the rate v (in $10^{-8} \times$ M min^{-1}) hydrolysis of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine. Substrate concentration: 1.7 mM. ●—●, 0.015 M phosphate buffer; O—O, 0.003 M boric acid-borax buffer.

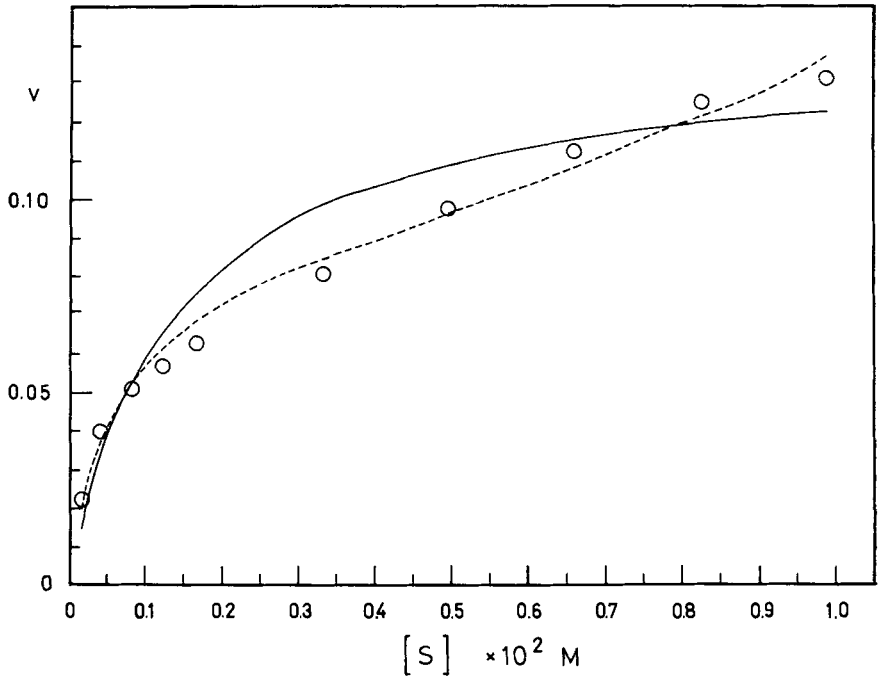

Fig. 4. The Michaelis-Menten plot of the velocity v (in $10^{-8} \times$ M min^{-1}) of the hydrolysis of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine catalyzed by an enzyme preparation (obtained from carious dentine) versus the substrate concentration [S]. The points are experimental and the curves were calculated by the aid of the simple Michaelis-Menten equation (full curve) or an extended form of the equation assuming substrate inhibition (dotted curve). The performance of the calculations is elsewhere described in more detail (*Mäkinen, Euranto & Kankare,* 1969). See text for the explanation of the results.

terms of the occurrence of secondary reactions in which the chromogene participated. In subsequent experiments all enzyme activity determinations were conducted within the linear part of the reaction. It was found that in the conditions used the rate versus time curves were linear up to appr. 10 h. It was evident, however, that initial velocities were not determinable. Consequently, in many experiments presented in this paper (in those concerning the kinetics of the reaction) the results obtained may not describe the accurate nature of the reactions.

The effect of enzyme concentration is shown in Fig. 2. According to the results constantly obtained, the reaction proceeded linearly with increasing enzyme concentration. The effect of pH on the reaction is shown in Fig. 3.

Fig. 4 shows the effect of substrate concentration on the reaction. The resultant Michaelis-Menten curve was not of a typical parabolic form, an indication of some deviation from the classical Michaelis-Menten behaviour

(it is to be noted, however, that initial velocities could not be measured). The two calculated curves in Fig. 4 were obtained either ny the simple form of the Michaelis-Menten equation or its extended form (*Mäkinen, Euranto & Kankare,* 1969). The use of the extended form involves substrate inhibition. No one of the two curves in Fig. 4 seemed to satisfy the experimental points. Substrate activation could perhaps be the reason for the pattern of the experimental data. The maximum velocity, V, was calculated to be $0.14 \pm 0.09 \times 10^{-8}$ M min^{-1}, and K_s, the substrate constant, was calculated to be $1.40 \pm 0.29 \times 10^{-3}$ M, when the simple Michaelis-Menten equation was used. With the extended form the corresponding values were $0.85 \pm 0.05 \times 10^{-9}$ M min^{-1} and $0.56 \pm 0.11 \times 10^{-3}$ M, respectively. It is evident that no one of the values given represents true V or K_s, if substrate activation occurs and when a modified form of the Michaelis-Menten equation is used to obtain the kinetic parameters.

The effect of temperature on the stability of the enzyme(s) responsible for the hydrolysis is shown in Fig. 5. The enzyme was seen to be rather heat-stable.

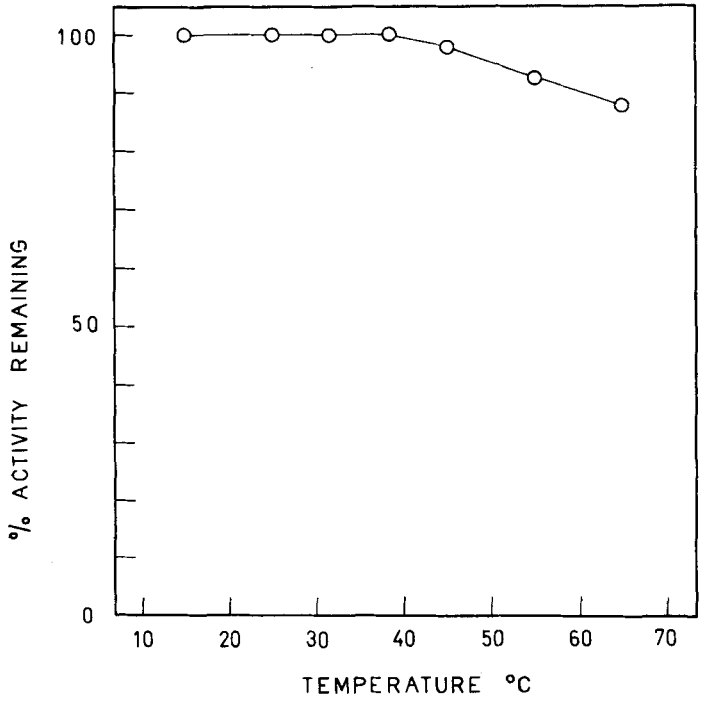

Fig. 5. Effect of temperature on the stability of the enzyme(s) of human carious dentine hydrolyzing 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine. 0.1 ml amounts of an aqueous enzyme solution of carious dentine were preincubated for 30 min. at the temperatures given and the enzyme activity was thereafter tested at 37 C. Substrate concentration: 0.4 mM. Tested in 0.075 M tris-HCl buffer, pH 7.0.

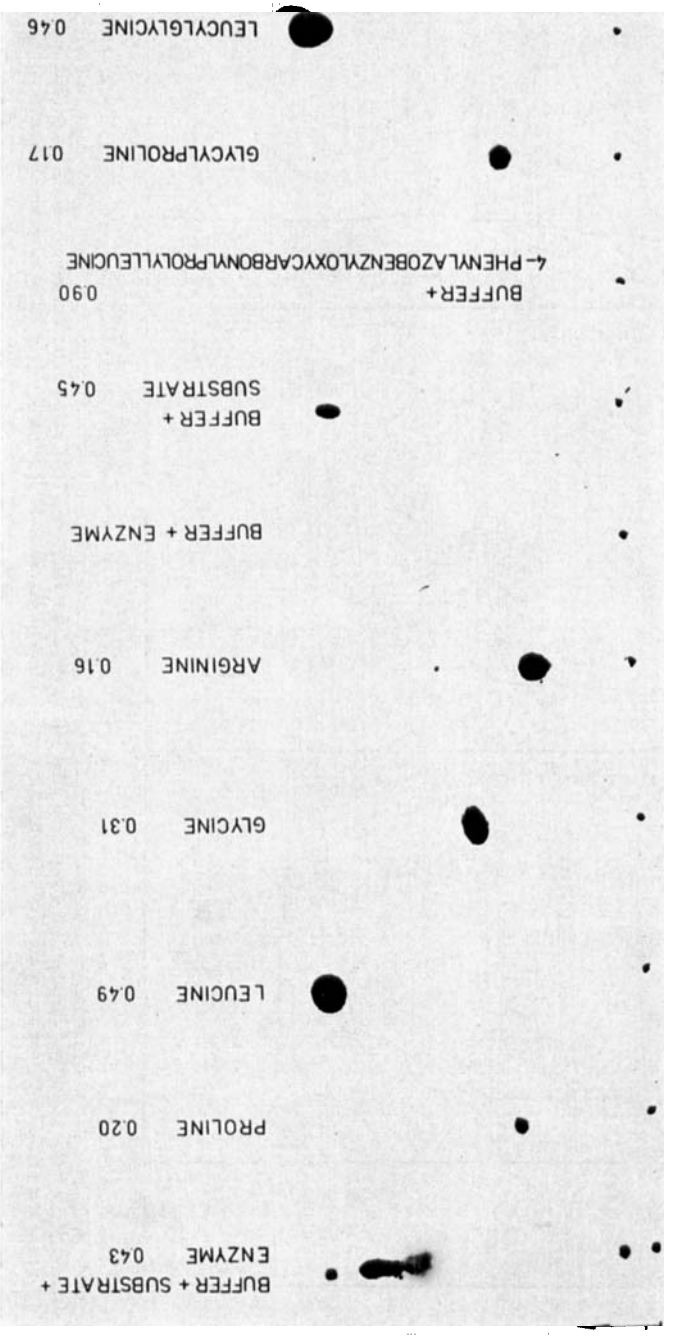

Fig. 6. Thin layer chromatography of the reaction mixtures (100 μl) composed of 0.1 M tris-HCl buffer, pH 7.0 (40 μl), 5 mM collagenase substrate solution (40 μl), and carious dentine enzyme solution (20 μl). The same volume of amino acids or other compounds were applied as references (50 μl, consisting of 40 μl of the above buffer and of 10 μl of aqueous 0.03 M amino acid solution). Development time: 200 min. The figures given represent R_f values of the compounds involved. In general, the R_f value of the chromogene (third row from the right) and substrate (fourth row from the right) varied from 0.87 to 0.90 and from 0.44 to 0.45, respectively. Both yielded yellow spots which were visible also without staining. The small spot in the first row ($R_f = 0.50$) represents leucine. $R_f = 0.43$ in the first row refers to the darkest spot.

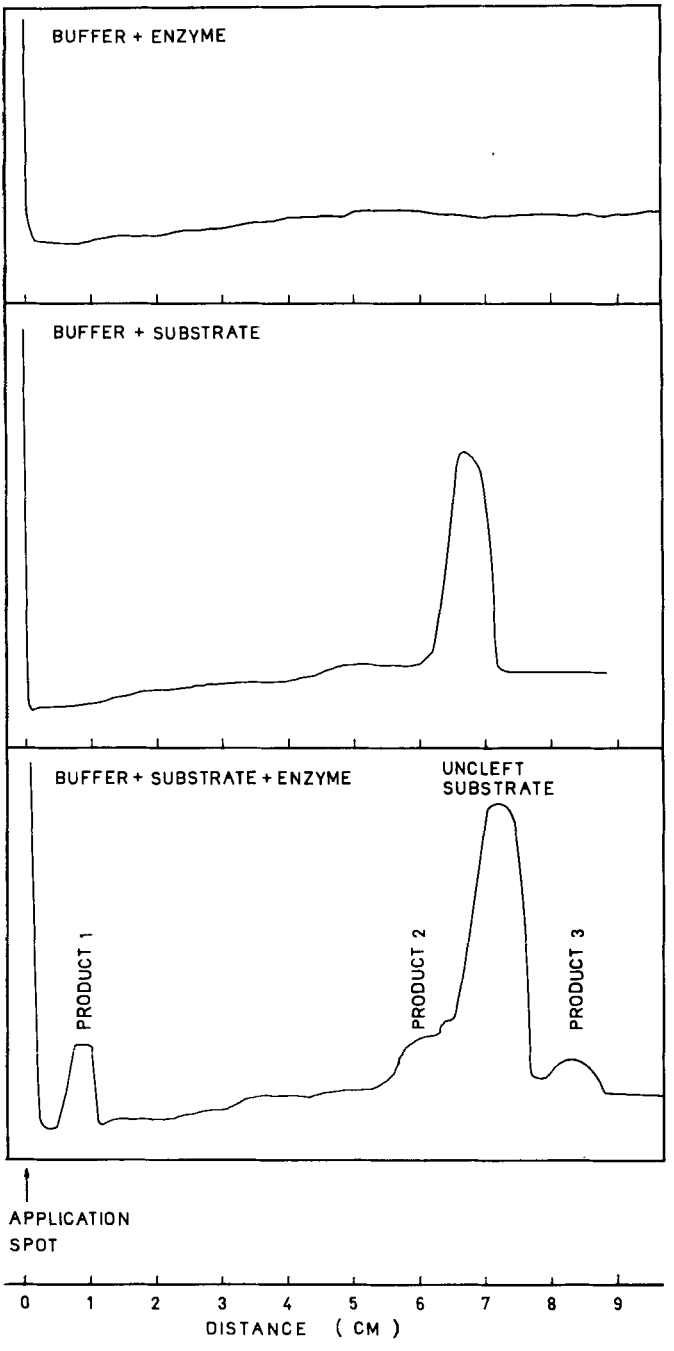

Fig. 7. Densitogram of the thin layer plates from an experiment to determine the products of the hydrolysis of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine catalyzed by an enzyme preparation derived from carious dentine (similar to that shown in Fig. 6). The direction of the ascending chromatography is from the left to the right. Product 3 is assumed to be leucine or leucylglycine. The other products are unidentified and their relative amount varied in different experiments depending on the lot of enzyme preparation.

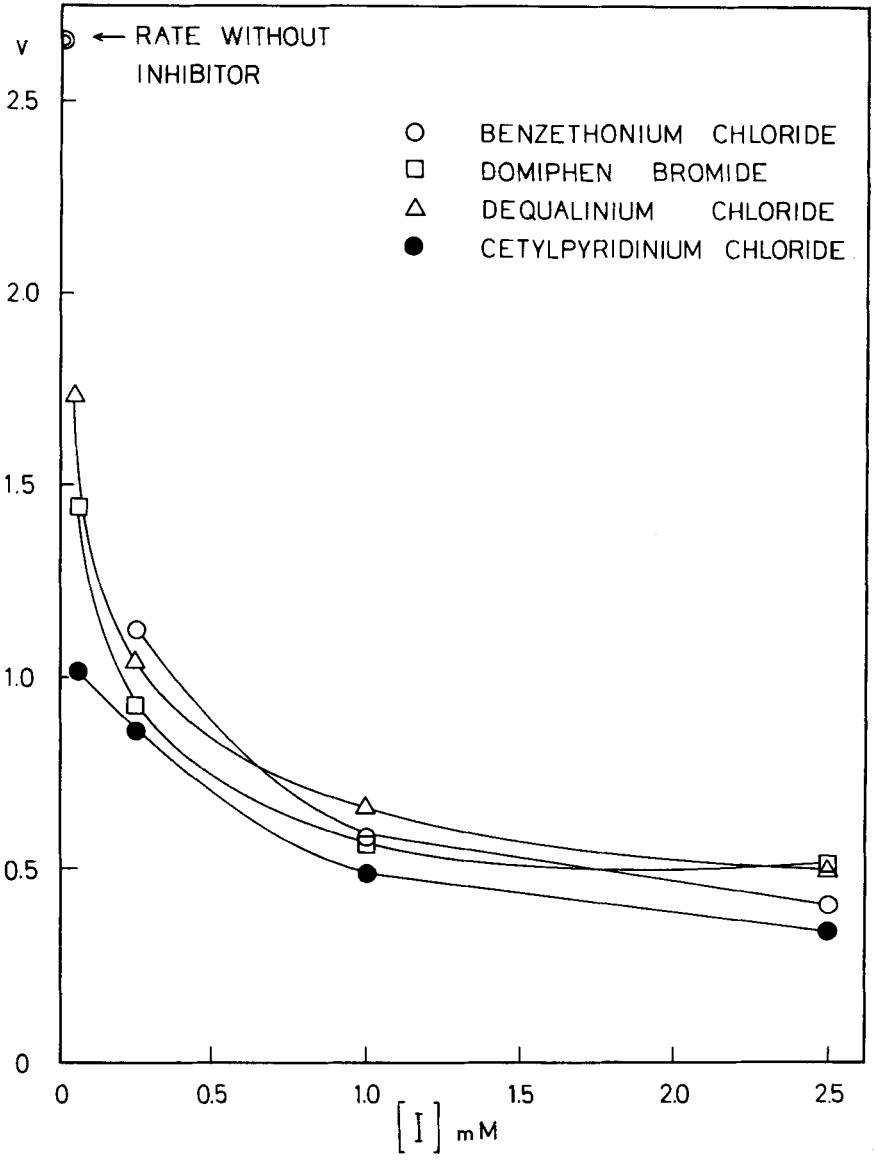

Fig. 8. Effect of some quaternary ammonium salts on the rate v (in $10^{-9} \times M\,min^{-1}$) in the hydrolysis of 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine.

Amino acid analysis did not reveal any free amino acids other than leucine in the reaction mixtures after appr. 10 h. incubation. This must result from secondary reactions (deaminations, cleavage of peptide bonds by proteases

other than collagenase, etc.) in which the chromogene or the substrate participated, due to the use of crude enzyme preparations. The secondary reactions may have converted the products of the »collagenase-reaction» to ninhydrin-negative compounds. Thin layer chromatography on Kieselgel G layers (Fig. 6) showed that the reaction mixtures did not contain any of the following compounds after 10 h. incubation: proline, arginine, glycine, glycyl-proline, or perhaps leucylglycine, although these may have appeared in the course of the reaction. On the other hand, leucine (or a compound with the same R_f value as leucine) was constantly seen on the plates. *Clostridium histolyticum* collagenase (Sigma Chemical Company, St. Louis, Mo., USA) and the carious dentine enzyme preparation, tested in identical conditions (Wunsch & Heidrich, 1963), both yielded the yellow chromophore as the result of enzymic catalysis. For the chromophore (first and eight row from the left) the spot is not shown. Densitograms of the thin layer plates are shown in Fig. 7. Product 3 most likely represents leucine, while products 1 and 2 could not be identified.

The effect of several enzyme inhibitors was also tested (for list of the compounds and other details concerning their dissolution and use, see also *Mäkinen*, 1969). Due to the fact that during the longer reaction time other reactions also take place (protein denaturation, etc.) only some results are concisely mentioned here. Hg^{2+}, Cu^{2+} and Ni^{2+} ions (added as chlorides) inhibited the reaction by 100% at 2.5 mM, by 70% at 1.0 mM and by 30% at 0.1 mM. The effect of some quaternary ammonium compounds on the reaction the reaction is shown in Fig. 8. The most potent inhibitor seemed to be cetylpyridinium chloride.

DISCUSSION

The results showed that the compound used as substrate, and which is claimed to be a specific collagenase substrate (*Wunsch & Heidrich*, 1963), was slowly decomposed in the presence of enzyme preparations obtained from human carious dentine. Due to the use of crude enzyme preparations it is not known whether the reaction was caused by one or several enzymes. The following results may support the involvement of a true collagenase: 1) Cu^{2+} ions inhibited the reaction. This is similar to results obtained by *Tytell* and *Hewson* (1955), although they observed that organic mercurials also inhibit the enzyme they investigated (a *Clostridium* collagenase), while p-chloromercuribenzoate in the present study failed to inhibit at the same concentrations. 2) SH-Groups were not seen to be necessary for the reaction. This is similar to the finding of *MacLennan, Mandl* and *Howes* (1953), who indicate that SH-

groups are not required for activity. 3) It was possible to follow the reaction by determining the amount of the chromogene, which should be one end product of a true collagenase-reaction.

Results were also obtained in this study which do not support the involvement of a classical collagenase (similar to that of *Clostridium*). No one of the metal-sequestering agents used (EDTA and cysteine) had any affect. The collagenase of *Clostridium histolyticum* is inhibited by these compounds (*Hagihara*, 1960). The most probable explanation is that the reaction described in in this paper is due to several enzymes, including a collagenase-like enzyme.

SUMMARY

The enzymic hydrolysis of a collagenase substrate (4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine) was studied with enzyme preparations derived from human carious dentine. The substrate was slowly decomposed to a chromogene (evidently 4-phenylazobenzyloxycaronyl-L-prolyl-L-leucine), indicating that collagenase-like enzymes could be involved. The secondary reactions taking place led to the appearance of, probably, free leucine. No free arginine, proline, glycine, glycyl-proline, or leucylglycine were found in the reaction mixture. Hg^{2+}, Ni^{2+} and Cu^{2+} ions (added as chlorides) at 2.5 mM inhibited by 100%, at 1.0 mM by 70% and at 0.1 mM by appr. 30%. Certain quaternary ammonium salts also inhibited the reaction. The enzyme was seen to be rather heat stable. It is possible that enzymes other than true collagenase were also involved.

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RÉSUMÉ

CARACTÉRISTIQUES DE L'HYDROLYSE DE LA 4-PHÉNYLAZOBENZYLOXYCARBONYL-L-PROLYL-L-LEUCYL-L-GLYCYLPROLYL-D-ARGININE (SUBSTRAT POUR COLLAGÉNASE) PAR DES PRÉPARATIONS ENZYMATIQUES PROVENANT DE LA DENTINE CARIÉE

L'hydrolyse enzymatique d'un substrat pour collagénase (4-phénylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine) a été étudiée au moyen de préparations enzymatiques provenant de dentine humaine cariée. Ce substrat a été lentement décomposé, donnant un chromogène (manifestement la 4-phénylazobenzyloxycarbonyl-L-prolyl-L-leucine), ce qui indique

l'intervention d'enzymes du type collagénase. Les réactions secondaires qui se produisaient provoquaient l'apparition de leucine, probablement à l'état libre. On n'a trouvé à l'état libre dans le mélange de la réaction ni arginine, ni proline, ni glycine, ni glycyl-proline, ni leucyl-glycine. Les ions Hg^{2+}, Ni^{2+} et Cu^{2+} (ajoutés sous forme de chlorures) à 2,5 mM donnaient une inhibition de 100 %, à 1,0 mM une inhibition de 70 % et à 0,1 mM, d'environ 30 %. Certains sels d'ammonium quaternaire inhibaient aussi la réaction. L'enzyme semblait relativement stable à la chaleur. Il est possible que d'autres enzymes que la vraie collagenase aient aussi joué un rôle.

ZUSAMMENFASSUNG

CHARAKTERISTIKA DER HYDROLYSE VON 4-PHENYLAZOBENZYLOXYCARBONYL-L-PROLYL-L-LEUCYLGLYCYL-PROLYL-D-ARGININ (EIN KOLLAGENASE-SUBSTRAT) MITTELS AUS KARIÖSEM DENTIN ERHALTENEN ENZYMPRÄPARATEN

Es wurde die enzymatische Hydrolyse eines Kollagenasepräparates (4-Phenylazobenzyloxycarbonyl-L-Prolyl-L-Leucylglycyl-L-Prolyl-D-Arginin) mit Enzympräparaten untersucht, die aus menschlichem kariösem Dentin stammten. Das Substrat wurde allmählich zu einem Chromogen (offensichtlich 4-Phenylazobenzyloxycarbonyl-L-Prolyl-L-Leucin) abgebaut, ein Hinweis darauf, dass Kollagenase-ähnliche Enzyme eingeschlossen sein könnten. Die weiteren Reaktionen führten zum Auftreten von wahrscheinlich freiem Leucin. Es wurde kein freies Arginin, Prolin, Glycin, Glycylprolin oder Leucylglycin im Reaktionsprodukt gefunden. Hg^{2+}-, Ni^{2+}- und Cu^{2+}-Ionen (als Chloride zugesetzt) wirkten als Inhibitoren: bei 2,5 mM 100%ig, bei 1,0 mM 70%ig und bei 0,1 mM annähernd 30%ig. Bestimmte quaternäre Ammoniumsalze inhibierten die Reaktion auch. Das Enzym erwies sich als ziemlich hitzebeständig. Es ist möglich, dass auch andere Enzyme als eigentliche Kollagenase eingeschlossen waren.

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