Collagenase in mineralized tissues of human teeth

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A collagenase cleaving native type I [^{14}C]collagen but inactive against the synthetic substrate Pz-Pro-Leu-Gly-Pro-D-Arg was extracted from mineralized human dental tissue. The enzyme specifically degrades native collagen into characteristic products (3/4) and (1/4). Its apparent molecular mass of 68 kDa is relatively high in comparison with collagenases from other oral tissues. The enzyme is a metalloproteinase inhibited by low concentrations of the chelating agents EDTA, 1,10-phenanthroline, $\alpha\alpha'$-dipyridyl, and not affected by diisopropylfluorophosphate, soybean trypsin inhibitor, and *p*-chloromercuribenzoate. It is stable to lyophilization and can be stored at -20°C for at least 6 months.

Human collagenase *Collagen* *Periodontitis*

1. INTRODUCTION

Some years ago [1–5] it was pointed out that the destruction of collagen fibers was responsible for periodontal disease. Interest focused on collagenolytic enzymes, the only proteolytic enzymes able to degrade native collagen. Collagenolytic activity was shown in tissue cultures of human gingiva [4–7], and in 1972 Fullmer et al. [8] isolated and characterized a collagenase from this source. More recently, a high collagenolytic activity correlated with the inflammatory process of gingiva tissues from patients under treatment was demonstrated [9].

This collagenase is inhibited by a substance extracted from human teeth [10]. The presence of a specific inhibitor in the tooth suggests a possible occurrence in the same tissue of a collagenase involved in the breakdown of collagen from the mineralized tissues. Dayan et al. [11] reported on the existence of collagenolytic activity in both carious and non-carious dentine. Nevertheless the corresponding enzyme was neither purified nor characterized.

The aim of this work was to purify and characterize the collagenase from mineralized tissues of human teeth.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Bacterial collagenase from *Vibrio alginolyticus* chemovar *iophagus* (EC 3.4.24.8) was prepared in the laboratory. [^{14}C]Acetic anhydride (6 mCi/mM) was purchased from CEA (France). Aqueous counting scintillant ACS II was from Amersham.

2.2. *Preparation of the crude extract*

Whole healthy human teeth were stored after extraction at -20°C. All the following experiments were run at 4°C.

The associated periodontal ligament, soft tissues, and pulp tissue were removed. The teeth were washed with 70% ethanol and demineralized water. The enamel, cementum and dentine were crushed under 30 tons pressure (Press C-30), ground and sieved (60 mesh) to powder.

The powder (1 g) was suspended in 10 ml of NaCl (0.01, 0.1, and 1 M) at 4°C for 24 h with gentle stirring. After centrifugation at 12000 × *g* for 30 min (RC-2B Sorvall), the supernatants were dialyzed against 3 changes of distilled water in a Spectrapor 3 membrane (M_r cut-off 3500) and lyophilized.

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2.3. *[^{14}C]Collagen preparation*

Collagen was prepared from rat tails by the method of Berman et al. [12]; its acetylation by [^{14}C]acetic anhydride was done according to Cawston and Barrett [13]. Purity of [^{14}C]collagen was controlled by slab gel electrophoresis, its helicity by ORD measurements of the value of the negative Cotton effect at 253 nm (polarimeter Perkin-Elmer 241).

2.4. *Collagenase assays*

2.4.1. Diffuse fibril assay

This assay was done according to Cawston and Barrett [13]. Control tubes contained either buffer alone or 2.5 μg trypsin. Total lysis of collagen was estimated from its digest by using 5 or 10 μg *V. alginolyticus* collagenase. One unit of activity was defined as the amount of enzyme required to digest 1 μg collagen per min at 35°C.

2.4.2. Synthetic peptide assay

The assay was performed according to Wunsch and Heidrich [14] using the peptide Pz-Pro-Leu-Pro-Gly-D-Arg in veronal buffer at pH 8.5.

2.4.3. Native collagen

80 μg of type I collagen was incubated at 20°C for up to 72 h with 1 μg of partially purified collagenase, in 200 μl of 50 mM Tris-HCl buffer, pH 7.4, containing 0.2 M NaCl and 10 mM $CaCl_2$ and subjected to electrophoresis.

2.4.4. Inhibition assays

Inhibition was determined by preincubating an enzyme sample with EDTA, $\alpha\alpha'$-dipyridyl, 1,10-phenanthroline, diisopropylfluorophosphate, *p*-chloromercuribenzoate, or soybean trypsin inhibitor, for 1 h at room temperature and then assaying for activity.

2.5. *Protein determination*

All protein concentrations were determined according to Lowry et al. [15]. Crystalline bovine serum albumin (Sigma) was used as a standard.

2.6. *Polyacrylamide gel electrophoresis*

Gel electrophoresis was performed on slabs either in 10% polyacrylamide gels by the method of Laemmli [16] or in a 5–15% gradient according to Ames [17]. The analyses of degradation products were performed in 7.5% polyacrylamide gels according to Furthmayr and Timpl [18]. Coomassie blue was used to stain the protein bands.

2.7. *Chromatography*

Ion exchange chromatography on DE-32 cellulose (Pharmacia) was carried out at 4°C. The column was equilibrated with 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.4. The sample was dialysed against the same buffer. A linear gradient of 0–0.5 M NaCl in the same buffer was applied. High-pressure liquid chromatography (Dupont 8800) was performed on SW G 2000 and SW G 3000 (7.5–300 mm) TSK (IBF-LKB) col-

Fig.1. SDS-polyacrylamide gel electrophoresis of the crude extract from human dentine. All samples were denatured with SDS at 100°C for 3 min. 50 μg and 150 μg of extract pretreated with β-mercaptoethanol (a,b) and without pretreatment (c,d); M_r standards: β-galactosidase (130000), phosphorylase *b* (94000), bovine serum albumin (68000), ovalbumin (43000), cytochrome *c* (11700). 5–15% gradient slab gel, staining with Coomassie blue.

umns. Tris-HCl buffer (70 mM, pH 7.2) containing 50 mM NaCl and 2.5 mM $CaCl_2$ was used for equilibration and elution of proteins.

3. RESULTS

Extraction experiments have shown that the best solubilization of proteins was obtained in 1 M NaCl: 2 mg proteins/g of teeth powder. The pattern of SDS gel electrophoresis of the crude extract is shown in fig.1. By chromatography of crude extract on a DE-32 cellulose column, a fraction devoid of activity was excluded with the first buffer, whereas the collagenolytic activity was eluted with the gradient of NaCl at a low ionic strength (fig.2). The concentration of salt being greater than 0.15 M made the activity assay not applicable; in these fractions, however, no activity was found after dialysis. The pooled active fractions, after concentration with an Amicon UM 10 membrane and dialysis, were lyophilized and analyzed by SDS gel electrophoresis (fig.3). In comparison with the crude extract, specific activity increased 219-times. The purified enzyme gave, on high pressure chromatography under native conditions, a single peak of apparent M_r 62000. It is quite stable to lyophilization and storage at $-20°C$.

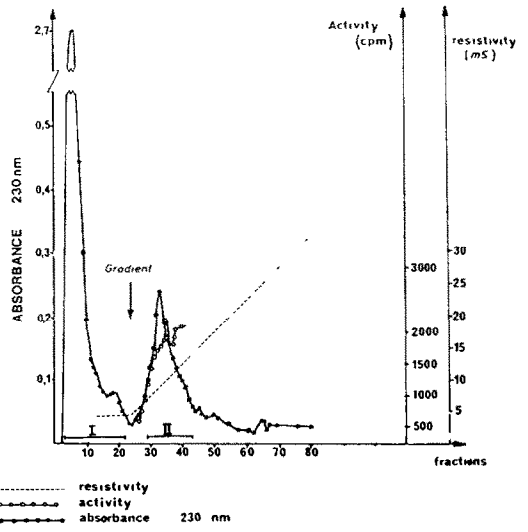

Fig.2. Elution profile of dental crude extract on a DE-32 cellulose column. The column (1.8 × 5 cm) was equilibrated and eluted at a flow rate of 18 ml/h. 3 ml fractions were collected.

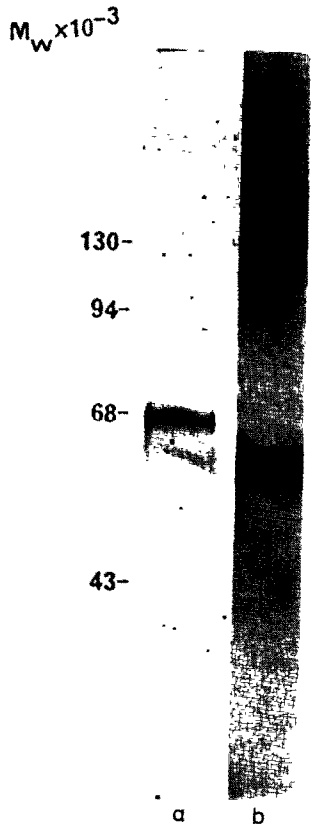

Fig.3. SDS-polyacrylamide gel electrophoresis of the purified collagenase (10% gel). (a) Pool II DE-32; (b) the same sample without reduction. Markers as in fig.1.

Under non-reducing conditions the apparent M_r of the collagenase was found to be 62000 in contrast to the value 68000 obtained in the presence of β-mercaptoethanol (fig.3).

DE-32 cellulose columns gave for the active fraction (apparent M_r 68000) a purification factor of 219.

The purified enzyme cleaves native collagen type I at pH 7.4 into characteristic products 3/4 and 1/4 the length of the chain (fig.4). It has no activity towards the synthetic peptide Pz-Pro-Leu-Gly-Pro-D-Arg.

The inhibition assays showed (table 1) that collagenolytic activity was fully inhibited in the presence of 2 mM 1,10-phenanthroline, 10 mM $\alpha\alpha'$-dipyridyl and 10 mM EDTA. No inhibition was observed in the presence of diisopropylfluorophosphate, *p*-chloromercuribenzoate and soybean trypsin inhibitor.

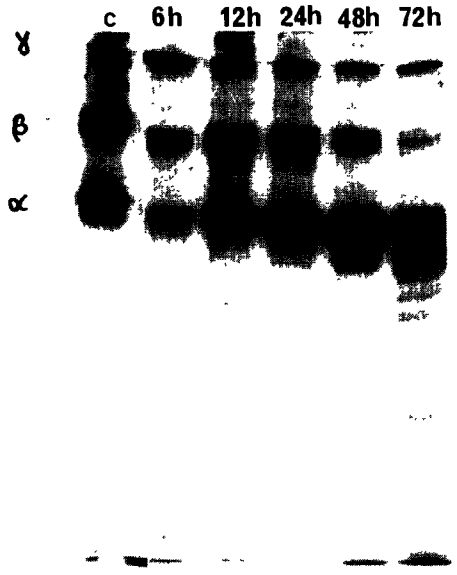

Fig.4. Gel electrophoresis of collagen type I degradation products. Collagen was incubated with purified collagenase at 20°C. (c) Control sample without enzyme after 72 h incubation.

Table 1

Effect of various inhibitors on human teeth collagenase activity

Reagent added	Concentration in assay (mM)	Activity (% of control)
None	–	100
EDTA	0.1	23
	5	7
	10	0
1,10-Phenanthroline	2	0
$\alpha\alpha'$-Dipyridyl	10	0
Diisopropylfluorophosphate[a]	20	0
Soybean trypsin inhibitor	10	87
p-Chloromercuribenzoate	10	100

[a] Diluted from isopropanol solution

Each reagent was dissolved in assay buffer and added to the enzyme for a preincubation at room temperature for 1 h

4. DISCUSSION

From the mineralized dental tissues, we have isolated a collagenase which was homogeneous on SDS electrophoresis and gave a single symmetric peak with high-pressure liquid chromatography.

The apparent M_r of the enzyme is 68000 (fig.3), a value comparable to that of polymorphonuclear leucocyte collagenase [19]. It is higher than that of other collagenases isolated from oral tissues; the collagenases from gingiva, dental pulp, and saliva have apparent M_r values of 40000, 45000 and 50000, respectively [8,20,21]. The increase in apparent molecular mass upon reduction could be explained by an increasing dissymmetry of the molecule after opening of one or more intramolecular disulfide bridges.

The enzyme acts at neutral pH. The effect of various inhibitors or collagenase activity (table 1) revealed that the divalent cation chelating agents 1,10-phenanthroline, $\alpha\alpha'$-dipyridyl and EDTA produced a complete inhibition of the enzyme. On the other hand inhibitors of serine proteinases and SH-proteinases had no effect on enzyme activity. This enables one to classify the collagenase as a metalloproteinase.

Both the ability of the enzyme to split native collagen type I in the 3/4,1/4 pattern and the negative result with the synthetic peptide Pz-Pro-Leu-Gly-Pro-D-Arg, routinely used to characterize the bacterial collagenases [14,22,23], as well as its inhibition by the chelating agents are consistent with the typical characteristics of a true vertebrate collagenase.

Vertebrate collagenases are usually obtained from time-consuming tissue culture techniques in low yields; only collagenases from human skin fibroblast, rat uterus, and polymorphonuclear leucocytes were isolated in quantities sufficient for detailed studies [19,24]. Human teeth collagenase represents a human collagenolytic enzyme available in sufficient amounts for physical, biochemical and immunological studies. It does not require the continuous presence of a non-ionic detergent for the preservation of its activity like uterine and synovial collagenases [25,26].

A detailed study on the enzyme and on its inhibition by natural inhibitors is under way in the laboratory.

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REFERENCES

[1] Narayanan, A.S. and Page, R.C. (1976) J. Biol. Chem. 251, 5464–5471.
[2] Robertson, P.M. and Fullmer, H.M. (1973) J. Med. Sci. 10, 115–118.
[3] Gibson, W. and Fullmer, H.M. (1966) J. Dent. Res. 45, 1225.
[4] Bennick, A. and Hunt, A.M. (1967) Arch. Oral Biol. 12, 1–9.
[5] Beutner, E.H., Triftshauser, C. and Hazen, S.P. (1966) Proc. Soc. Exp. Biol. Med. 51, 349–355.
[6] Fullmer, H.M. (1966) J. Dent. Res. 45, 469–477.
[7] Fullmer, H.M. and Gibson, W.A. (1966) Nature 209, 728–729.
[8] Fullmer, H.M., Taylor, R. and Guthrie, R.W. (1972) J. Dent. Res. 51, 349–355.
[9] Geiger, S. and Harper, E. (1980) J. Dent. Res. 59, 11–16.
[10] Geiger, S.B. and Harper, E. (1981) J. Periodont. Res. 16, 8–12.
[11] Dayan, D., Binderman, I. and Mechanic, G.L. (1983) Arch. Oral Biol. 28, 185–187.
[12] Berman, M.B., Manabe, R. and Davison, P.F. (1973) Anal. Biochem. 54, 522–534.
[13] Cawston, T.E. and Barrett, A.J. (1979) Anal. Biochem. 99, 340–345.
[14] Wunsch, E. and Heidrich, H.G. (1963) Z. Physiol. Chem. 332, 300–304.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Ames, J.F.L. (1974) J. Biol. Chem. 24, 634–644.
[18] Furthmayr and Timpl, R. (1971) Anal. Biochem. 41, 510.
[19] Macartney, H.W. and Tschesche, H. (1983) Eur. J. Biochem. 130, 71–78.
[20] Kishi, J.I., Iijima, K.I. and Hayakawa, T. (1979) Biochem. Biophys. Res. Commun. 86, 27–31.
[21] Sakamoto, S., Sakamoto, M., Goldhaber, P. and Glimcher, M. (1978) Arch. Biochem. Biophys. 188, 438–449.
[22] Lecroisey, A., Keil-Dlouha, V., Woods, D.R., Perrin, D. and Keil, B. (1975) FEBS Lett. 59, 167–172.
[23] Keil, B., Gilles, A.M., Lecroisey, A., Hurion, N. and Tong, N.T. (1975) FEBS Lett. 56, 292–296.
[24] Stricklin, G.P., Bauer, E.A., Jeffrey, J.J. and Eisen, A.Z. (1977) Biochemistry 16, 1607–1614.
[25] Roswit, W.T., Halme, J. and Jeffrey, J.J. (1983) Arch. Biochem. Biophys. 225, 285–295.
[26] Cawston, T.E. and Tyler, J.E. (1979) Biochem. J. 183, 647–686.