Hydrolysis of the Leu-Gly Bond of Phenylazobenzyl-oxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (a substrate of microbial collagenases) by Treponemes Isolated from the Subgingival Plaque of Periodontitis Patients

Kauko K. Mäkinen, Salam A. Syed, Sergio L. Salvador, and Pirkko-Liisa Mäkinen

Department of Biologic and Materials Sciences, School of Dentistry, The University of Michigan, Ann Arbor, Michigan, USA

Abstract. Cell extracts prepared from several oral treponemes isolated from the subgingival plaque of periodontitis patients showed high enzyme activity toward phenylazobenzyl-oxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (a compound used as a substrate for microbial collagenases). One major enzyme hydrolyzing this substrate at the Leu-Gly bond only was partially purified from an unspeciated treponeme (strain US), *Treponema denticola* ATCC 35405, and 29 different clinical isolates of *T. denticola*. The *Treponema* US enzyme also hydrolyzed furylacryloyl-L-leucylglycyl-L-prolyl-L-alanine (another substrate of bacterial collagenases) at the Leu-Gly bond. This enzyme also hydrolyzed various collagens and collagen-derived peptides. These treponemal proteases were sensitive to metal chelators and *p*-chloromercury compounds. The results indicate that human oral treponemes contain enzymes that readily hydrolyze in chromogenic protease substrates the Leu-Gly bond only that is the cleavage site of these substrates also by "true" microbial collagenases.

Several studies have demonstrated proteolytic activity in subgingival plaque [1, 3] and in specific microorganisms that are considered to be associated with periodontal disease [5–13, 15]. Incubation of gingival fibroblasts in the presence of extracts from dental plaque caused drastic changes in the cells' appearance [7]. Such changes can be expected to result from bacterial hydrolytic activity. It is thus obvious that chronic exposure of the periodontal tissues to bacterial enzymes leads to chemical reactions that are harmful to the tissue. Of special interest are various endopeptidases, since they may attack tissue proteinaceous substances, releasing chemotactic or chemokinetic products that contribute to the maintenance of inflammatory conditions. We have previously employed a synthetic and soluble pentapeptide derivative as a substrate of endopeptides present in oral microorganisms [10, 11, 13, 14]. This compound, phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (PZ-PLGPA), is hydrolyzed by "true" microbial collagenases and collagenolytic enzymes at the Leu-Gly bond, and it has been suggested to be a suitable substrate for such enzymes [2, 10, 18].

The purpose of this paper is to provide information about the presence of PZ-PLGPA-hydrolyzing enzymes in several organisms that have been claimed to be associated with periodontal disease. An enzyme that hydrolyzed the above pentapeptide derivative at the Leu-Gly bond only was demonstrated in 33 treponemes and in 5 other human oral microorganisms.

Materials and Methods

A medium-sized *Treponema* sp. (strain US) was isolated from the subgingival plaque of a periodontitis patient. The cells were maintained and cultivated in a tryptone–heart infusion–yeast extract broth containing 5% rabbit serum, as described earlier for *T. denticola* [9, 11]. The harvesting and pretreatment of the cells, including their disintegration with ultrasound, were also described previously [9]. The source and cultivation of other treponemes [9, 11], the fusobacteria [9], *Capnocytophaga* [9], *Bacillus cereus* [10], *B. gingivalis* [9], and *Streptococcus faecalis* [14] were described in cited references. *S. faecalis* (human oral strain 0G1-10) was included in this study as a PZ-PLGPA-nega-

Table 1. Ability of various oral microorganisms to hydrolyze PZ-PLGPA[a]

Organisms	Rate of hydrolysis[b]
Treponema denticola ATCC 35405	1.70
Myr	1.25
Sim	1.30
Corha	1.70
H$_1$	1.05
Spira	1.80
Al$_2$	1.15
H$_{22}$	1.15
T. vincentii ATCC 35580	1.53
T. (US) species	0.89
T. socranskii	0.20
T. denticola isolates	2.3 ± 0.6 (n = 22)
Fusobacterium nucleatum (FN2)	0.4
F. nucleatum (FN3)	0.4
Capnocytophaga sp.	0.5
Bacillus cereus	2.5
B. gingivalis	1.8
Streptococcus faecalis (OGI-10)	Nil

[a] The reactions were carried out in 50 mM Tris-HCl, pH 8.0 (containing 0.1 mM CaCl$_2$), at 30°C and 0.6 mM substrate concentration. The reactions were carried out with the supernatant fluids (10 min; 23,500 g) of cell extracts made by ultrasonic treatment of washed cells.

[b] In μmol min^{-1} mg^{-1} (× 10^3).

tive reference organism (although its gelatinase readily hydrolyzes various soluble and insoluble collagens).

The endopeptidase activity was routinely determined with PZ-PLGPA as substrate [18]. The hydrolysis of the Leu-Gly bond of this substrate was ascertained as reported earlier [13]. with reversed-phase chromatography and amino acid analysis. The Cd–ninhydrin method of Doi et al. [4] was used to compare the rates of the hydrolysis of several synthetic and natural peptidase substrates. In these cases the assay mixtures consisted of 100 μl of substrate solution (or homogeneous suspension when the substance was insoluble) made in 50 mM Tris-HCl, pH 7.5 (2 mg/ml), and 5–10 μl of enzyme. After incubation, 0.4 ml of ninhydrin reagent and 1.0 ml of ethanol were added, followed by reading of the color intensities at 570 nm. L-Leucine was used as a standard. The hydrolysis of N-aminoacyl-2-naphthylamines (2NAs), N-α-benzoyl-L-arginyl-2NA (BANA), $N\alpha$-benzoyl-L-arginyl-p-niroaniline (BAPNA) and furylacryloyl (FA) derivatives was determined as described earlier [11, 12]. Proteinase activity was in some cases also tested with azocoll, azocasein and elastin–orcein [11]. The kinetics of the enzyme from certain treponemes was studied as reported previously [13, 16, 17].

The ability of the *Treponema* US enzyme to hydrolyze collagen was studied in a reaction mixture consisting of 5 mg of Sigma #4387 insoluble collagen (Classical Type I, from bovine achilles tendon) suspended in 1 ml of 20 mM Tris-HCl (pH 7.5), and of about 0.1 μg of enzyme. Mixtures in which either the enzyme or the collagen was omitted were included. The mixtures were incubated for 24 h at 30°C, followed by filtration through 0.2 μm Acro LC 13 membranes (Gelman, Ann Arbor, Michigan). The filtrates were evaporated to dryness at room temperature, and each of the dry residues was dissolved in 20 μl of water, 10-μl aliquots of these soutions were mixed with 10 μl of denaturing

buffer, followed by treatment for 12 min in a boiling water bath. Of the resulting preparations, 1.0-μl aliquots were subjected to PhastGel electrophoresis with the Pharmacia PhastSystem (8%–25% gel; silver staining; Pharmacia, Uppsala, Sweden). Protein concentrations were determined by the Bradford method with Sigma bovine serum albumin as the standard protein [6]. The Pharmacia PhastSystem, including the Phastgels and SDS Buffer Strips, was also used to study the ability of the *Treponema* US enzyme to degrade other collagens (Table 3). The method was optimized for 8%–25% PhastGel gradient media and silver staining. SDS–PAGE was also used to study the hydrolysis of [^{3}H]-rat tail tendon collagen (New England Nuclear, Boston, Massachusetts) catalyzed by this enzyme.

The complete FPLC unit was purchased from Pharmacia (Uppsala, Sweden). For this study the unit was equipped with Superose 6 and Superose 12 columns, a Mono Q anion exchange column, an arginyl-sepharose column, and a phenyl-superose column. The proteins were monitored at 280 nm or 214 nm. Because of the rapidity of the FPLC separations, the unit was operated at 22°C. The FPLC programs used were described below. Unless otherwise indicated, all chemicals were from Sigma (St. Louis, Missouri). The water used in the study was Millipore-treated and had a resistance of at least 18 MΩ cm^{-1}. The culture media were prepared from Difco Products (Detroit, Michigan).

Results and Interpretations

Ability of cell extracts to hydrolyze PZ-PLGPA. Cell extracts prepared by ultrasonic treatment of the cells were tested for proteinase activity with PZ-PLGPA as substrate (Table 1). All treponemes, the fusobacteria, *Capnocytophaga*, and the *Bacillus* species exhibited high PZ-PLGPA-hydrolyzing activity. The cell-free growth media obtained after removal of the cells showed high (extracellular) activity on PZ-PLGPA, especially in the case of *B. cereus*. The reference organism *Streptococcus faecalis* did not hydrolyze this substrate. A major PZ-PLGPA-hydrolyzing enzyme was thereafter partially purified from the medium-sized unspeciated treponeme (strain US), from *Treponema denticola* ATCC 35405, *T.* vincentii ATCC 35580, *T.* socranskii, from all 29 *T. denticola* strains or clinical isolates listed in Table 1, and from *Fusobacterium nucleatum* (strain FN2). The purification procedure and results from partial characterization studies of the treponeme enzymes are summarized below. Unless separately mentioned, all procedures were performed in the cold (+4°C). It was also established that the enzymes studied in this paper cleaved PZ-PLGPA at the Leu-Gly bond only [13].

PZ-PLGPA-hydrolyzing proteinase from *Treponema* US. The supernatant fluid of the cell extract (70 ml; resulting from a 1-L culture) was dialyzed for 72 h against 200 volumes of 10 mM Tris-HCl (pH 7.0, containing 1.0 mM CaCl$_2$). The dialysate was

centrifuged for 10 min at 23,500 g before being filtered through a 0.45-μm Millipore filter for FPLC. The filtrate was injected into a Mono Q anion exchange column with a Superloop. The column was eluted with the above buffer at a flow rate of 1.0 ml min^{-1}, applying a three-phase NaCl gradient shown in Fig. 1A. This FPLC program resulted in the appearance of one enzyme peak (21–25 min) with high activity toward PZ-PLGPA. The active fractions were pooled, and the pool was concentrated to 2 ml with an Amicon Microconcentrator (cut-off 30,000 mol. wt.). Concentrated enzymes from three similar separations were combined for the next step.

The enzyme from the previous step was injected into a Superose 6 Prep column. One enzyme peak that showed activity toward PZ-PLGPA appeared (not shown). The active fractions were pooled (15 ml), and the pool was dialyzed for 24 h against 1000 volumes of 3 mM Tris-HCl (pH 7.5, containing 0.3 mM CaCl$_2$). The dialysate was applied on an arginyl-sepharose affinity column (1.0 cm × 25 cm) and eluted with the above buffer, which contained a linear NaCl gradient from zero to 1.0 M (Fig. 1B). This separation resulted in the appearance of one PZ-PLGPA-hydrolyzing enzyme. The active fractions were pooled (30 ml) and concentrated on an Amicon YM10 membrane to 2.0 ml. The development of the specific activity of the enzyme is shown in Table 2. The molecular weight of the enzyme was estimated to be near 100,000.

The PZ-PLGPA-hydrolyzing proteinase did not hydrolyze BANA, BAPNA, or N-aminoacyl-2NAs. The most rapidly hydrolyzed substrates were PZ-PLGPA and FA-Leu-Gly-Pro-Ala (FALGPA; Table 3), both of which have been used as substrates of microbial collagenases. Both were hydrolyzed at the Leu-Gly bond only. The following FA derivatives were not hyrolyzed: FA-Phe, FA-Phe-Phe, FA-Phe-Gly-Gly, FA-Ala-Phe-NH$_2$, FA-Trp, FA-Gly-Leu-Ala-NH$_2$), FA-Leu methyl ester, and FA-Trp methyl ester. Other substrates that were hydrolyzed included the soluble collagen digests (Sigma Polypep preparations), Type VI soluble collagen, and fibrinogen. Sigma #4387 insoluble collagen (classical Type I; from bovine achilles tendon) was readily hydrolyzed in 20 mM Tris-HCl (pH 7.5), producing, in 20 h incubations, soluble fragments whose molecular weights ranged from 10,000 to 60,000 (not shown). ^{3}H-labeled collagen was hydrolyzed as tested with the New England Nuclear collagenase assay kit. Figure 2 shows that the enzyme hydrolyzed PZ-PLGPA most rapidly at pH 7.5. The enzyme was sensitive to all metal chelators tested
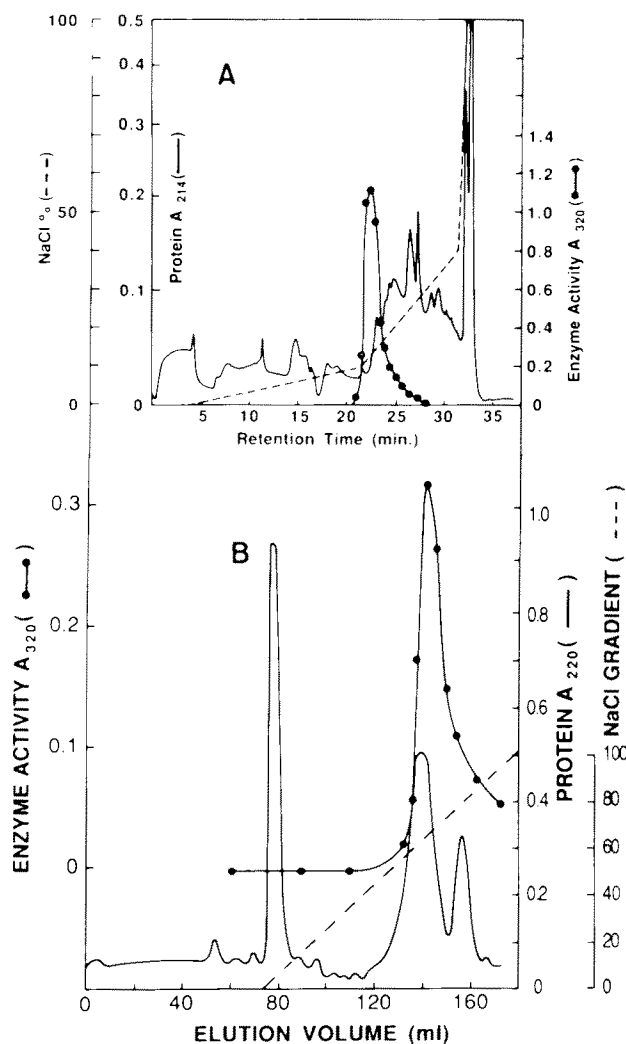

Fig. 1. Panel A: Separation by FPLC of a PZ-PLGPA-hydrolyzing proteinase from the cell extract of the unspeciated treponeme (strain US). Thoroughly dialyzed enzyme (supernatant fluid of cell sonicate) was injected into a Mono Q anion exchange column that was equilibrated and eluted with 10 mM Tris-HCl (pH 7.0, containing 1.0 mM CaCl$_2$). A three-step NaCl gradient was applied after all sample had entered the column (with a Superloop; at 4 min). The active fractions (21.5–23.5 min) were pooled, and the pool was dialyzed for the subsequent gel permeation chromatography (see text). Panel B: Further purification of the PZ-PLGPA-hydrolyzing proteinase from the unspeciated treponeme (strain US) on an arginyl-sepharose column (1 cm × 25 cm). A 6-ml sample from the previous purification step (see text) was applied (a typical separation is shown), eluting the column with 3 mM Tris-HCl (pH 7.5, containing 0.3 mM CaCl$_2$ and a NaCl gradient from zero to 1.0 M). The active fractions were pooled and concentrated.

(Table 4). Because PCMPS (Table 4) was a strong inhibitor of the reaction, it is possible that the enzyme activity depended on an essential sulfhydryl residue.

Table 2. Purification of the major PZ-PLGPA-hydrolyzing enzyme from medium-size *Treponema* US

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (nmol mg^{-1}min^{-1})
Cell sonicate (after centrifugation)	70	0.75	52.5	0.95
After Mono Q separations and concentrations	6	1.0	6.0	14.0
After Superose 6 gel	15	0.15	2.25	21.0
After affinity chromatography on arginyl-sepharose	2	0.096	0.19	252.0

Table 3. Ability[a] of the *Treponema* strain US proteinase to hydrolyze various peptidase substrates in the presence of Ca^{2-} and without added Ca^{2-}

Substrate	1 mM Ca^{2-}	No No added Ca^{2-}
PZ-PLGPA (Sigma)	0.975	0.160
Furylacryloyl-L-Leu-Gly-L-Pro-L-Ala (Sigma) (FALGPA)	0.822	0.816
Furylacryloyl-Gly-Leu-Amide (Sigma)	–	–
BAPNA (Sigma)	–	–
Polypep, low-viscosity (Sigma)[b]	0.0125	0.031
Polypep, high-viscosity (Sigma)[b]	0.0262	0.0187
Collagen (Sigma #7521; Type VI, soluble, from human placenta)	0.0162	–
Gelatin (Baker & Adamson)	–	–
Fibrinogen (Mann; bovine, plasminogen-free)	0.022	0.015
Serum albumin (Sigma; bovine)	–	–
Casein (Calbiochem)	0.005	0.004
Hemoglobin (Pierce)	–	–

[a] In nmol min^{-1}mg^{-1}. The reactions were performed at 37°C in 50 mM Tris-HCl, pH 7.8, with a ninhydrin method [4] to determine the liberation of free amino groups.
[b] Soluble collagen polypeptides (made by digestion). –, No activity detected.

PZ-PLGPA-hydrolyzing activity in *Treponema denticola*. An essentially similar purification procedure was used for the enzyme from *T. denticola* ATCC 35405. The partially purified enzyme showed no activity toward azocasein, serum albumin, and the N-aminoacyl-2NAs previously listed [13]. The enzyme was inhibited by 90% and 70% in the presence of 0.0167 mM EDTA and 1,10-phenantroline, respectively. *p*-Chloromercuribenzoic acid (PCMB) (1.67 µM) inhibited the enzyme totally, while 0.0167 mM dithiothreitol had no effect. The enzyme hydrolyzed PZ-PLGPA most rapidly at pH 8.0 in 0.1 M phosphate buffer. The optimum pH values in 50 mM Tris-HCl and 50 mM borate buffers were 8.0

and 8.6, respectively. The hydrolysis of PZ-PLGPA displayed sigmoidal Michaelis curves and substrate inhibition. The latter started to appear above 0.46 mM substrate level. The enzyme was stable at pH 5.1 (0.1 M phosphate buffer). The presence of a similar PZ-PLGPA-hydrolyzing enzyme was subsequently discovered and partially purified from all *T. denticola* strains or isolates mentioned in Table 1. The enzymes obtained from 17 of these isolates were subjected to characterization studies. The pH optimum of the hydrolysis of PZ-PLGPA by these enzymes in 50 mM Tris-HCl was near pH 7.5–8.0; the enzyme from the isolate designated as #62, however, showed the highest rate of hydrolysis at pH 7.0. The kinetics of the hydrolysis of PZ-PLGPA by these enzymes was not exactly identical, as evidenced by the distinctly different K_m values obtained (Fig. 3). The values shown were determined by the Hanes' plot, but similar values were obtained from the double reciprocal plot and the Hofstee plot (velocity versus v/[S]). Typical Michaelis kinetics was observed at [PZ-PLGPA] up to 0.62 mM. The PZ-PLGPA-hydrolyzing enzymes from all 17 clinical isolates of *T. denticola* were sensitive to PCMB; 1.67 µM inhibitor reduced the rate of the hydrolysis by 90% to 95%, and even at 0.0167 µM inhibitor level the inhibition was 10%–20%. The enzymes were inhibited by 35%–75% in the presence of 0.0167 mM 1,10-phenantroline, while EDTA at the same concentration inhibited by 80%–98%. The thiols (Table 4) were tested at a high concentration; these results do not exclude the possible importance of an SH group for enzyme activity.

All treponemes studies in this paper contained at least one major enzyme capable of hydrolyzing PZ-PLGPA, a substrate used in the study of microbial collagenases and collagenolytic enzymes. The *Treponema* US enzyme hydrolyzed also FALGPA, various collagens, and collagen-derived peptides. A "true" bacterial collagenase degrades in the helical regions of the native collagen, preferentially the X-
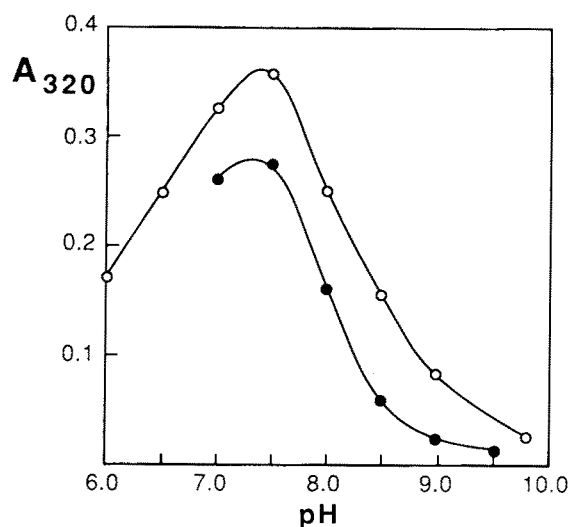
Fig. 2. Effect of pH on the rate of the hydrolysis of PZ-PLGPA catalyzed by the proteinase present in the unspeciated treponeme (strain US). The reactions were carried out in 0.1 M Tris-HCl (open circles) and 0.05 M phosphate buffers (closed circles), at 37°C, with 0.5 mM substrate concentration.

Fig. 3. Hanes' plot of substrate concentration divided by the initial velocity ([S]/v) versus the substrate concentration in the hydrolysis of PZ-PLGPA catalyzed by proteinases separated by FPLC from eight clinical isolates of *Treponema denticola*. The reactions were performed at 30°C in 50 mM Tris-HCl, pH 8.0 (containing 0.01 mM CaCl$_2$). The values obtained for K$_m$ were 0.2 mM, 0.5 mM, 0.5 mM, and 0.6 mM for enzymes 1, 36, 39, and 40, respectively (Panel A); and 0.1 mM, 0.1 mM, 0.25 mM and 0.35 mM for enzymes 9, 48, 81, and 43, respectively (Panel B). The enzyme numbers given are those of the clinical isolates studied.

Gly bond. It is necessary to emphasize that the hydrolysis of the peptide of a composition similar to sequences existing in collagen, or the fact that the enzyme degrades collagen, is not sufficient evidence that a true collagenase is involved. However, the clinical significance of bacterial collagenolysis should not be belittled even if a "true" collagenase is not involved. Therefore, all bacterial enzyme activity that is directed toward insoluble and soluble collagens, synthetic model substrates, or stained collagen preparations (Azocoll) should be regarded as interesting from the pathological point of view. Furthermore, the criteria set for bacterial collagenases will undergo changes when all current information on substrate specificity is considered.

In spite of the obviously constitutive presence of the PZ-PLGPA-hydrolyzing enzymes in *T. denticola*, *T. vincentii*, and *T. socranskii*, the respective enzymes seem to differ as proteins. For example, the *T. denticola* enzyme has a higher molecular weight than the *T. vincentii* enzyme [13]. The fact that all enzymes were sensitive to metal chelators suggests that they may be metalloendopeptidases.

Conclusions

1. All of the 33 treponemes isolated from the subgingival plaque of periodontis patients displayed enzyme activity toward PZ-PLGPA, a substrate that has been used in the study of microbial col-
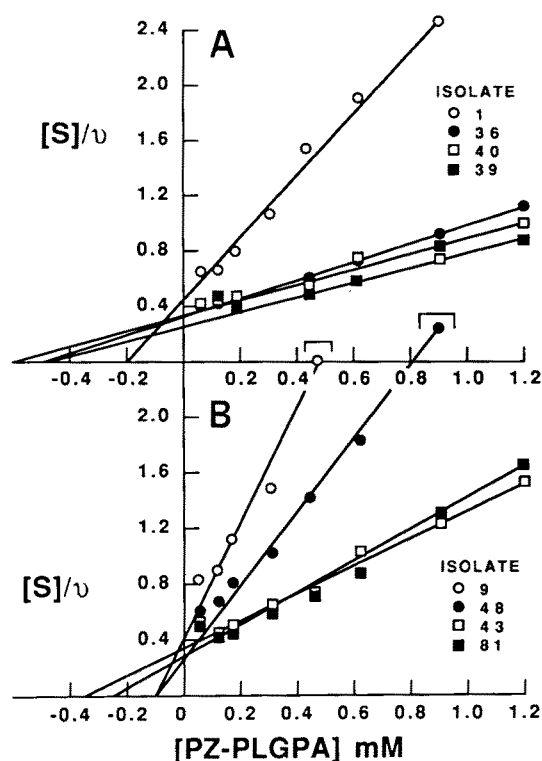
Table 4. Effect of various chemical reagents on the hydrolysis of PZ-PLGPA by the collagenolytic proteinase of the medium-size *Treponema* (strain US)[a]

Reagent	Inhibition %
Chelators	
EDTA (1.0 mM)	97.6
1,10-Phenantroline (1.0 mM)	94.3
8-Hydroxyquinoline 5-sulfonic acid (1.0 mM)	69.8
EGTA (1.0 mM)[b]	62.5
Thiols:	
Dithiothreitol (1.0 mM)	51.8
L-Cysteine (1.0 mM)	7.8
PCMPS[c] 5 μM	86.3
10 μM	89.2
50 μM	90.9

[a] The reactions were carried out in 0.1 M Tris-HCl, pH 7.5, with 0.5 mM PZ-PLGPA as substrate at 37°C.
[b] Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.
[c] p-Chloromercuriphenylsulfonic acid (tested in 50 mM phosphate buffer, pH 7.5).

lagenases and collagenolytic enzymes. Fusobacteria, *Capnocytophaga,* and *Bacillus* species also exhibited this type of activity.

2. A major enzyme hydrolyzing PZ-PLGPA at the Leu-Gly bond only was partially purified from all the treponemes studied. The enzyme was inhibited by metal chelators and *p*-chloromercury compounds.

3. The major PZ-PLGPA-hydrolyzing enzyme from a medium-sized treponeme (US) cleaved various collagens and collagen-derived peptides, and also a furylacryloyl derivative (another substrate of collagenase-like bacterial enzymes) at the Leu-Gly bond only.

4. The preferential hydrolysis of the Leu-Gly bond in the above substrates and the hydrolysis of collagen by *Treponema* US suggests that a more detailed chemical study should be conducted on the relationship between the degradation of periodontal collagen and the human oral treponemes.

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