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Proteolytic profile of *Treponema vincentii* ATCC 35580 with special reference to collagenolytic and arginine aminopeptidase activity

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The proteolytic profile of *Treponema vincentii* ATCC 35580 was studied using PZ-PLGPA (phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine; a substrate of bacterial collagenases) and amino acid 2-naphthylamides (2NA) as substrates. The cell extracts showed high activity toward PZ-PLGPA, *Nα*-L-arginyl-2NA and *Nγ*-L-glutamyl-2NA. Gel permeation chromatography revealed 2 major endopeptidases (I and II) hydrolyzing PZ-PLGPA, the molecular weights of which were 75,000 and 23,000, respectively. Enzyme I was stable enough for subsequent fast protein liquid chromatography on an anion exchange column. The enzyme had a broad pH optimum of 6.5 to 7.5 with PZ-PLGPA as substrate, hydrolyzed gelatin and was moderately inhibited by metal chelators, but was very sensitive to *p*-chloromercuribenzoic acid (PCMB). Enzyme II with a pH optimum of 7 to 8 was more labile, quite sensitive to PCMB and moderately inhibited by chelators. A high-molecular weight arginine aminopeptidase (mol. wt. >200,000) was sensitive to PCMB and showed a value of 0.55 mM for K_m in the hydrolysis of *Nα*-L-arginyl-2NA. The hydrolysis of PZ-PLGPA and gelatin suggests that this organism may contain collagenolytic proteinases. Because the insoluble proteinase substrate Azocoll was not hydrolyzed, these enzymes may be active on soluble collagenous substances only. *T. vincentii* ATCC 35580 typifies an organism rich in PZ-PLGPA-endopeptidase, arginine aminopeptidase and γ-glutamylpeptidase activity.

Key words: spirochetes; proteolytic enzymes; periodontal disease

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Spirochetes are helically shaped, heterotrophic bacteria which are particularly prominent in the subgingival plaque when there is evidence of clinical disease, such as gingival bleeding, periodontal inflammation and suppuration (16). The chemical aggressiveness of oral spicochetes may partly be associated with the presence of proteoytic enzymes which are active toward host tissue proteins, such as collagen and other connective tissue components. The physiology, morphology and evolution of spirochetes have been actively studied (8, 13), but the chemistry of proteolytic enzymes produced by spirochetes has received attention only recently (11, 20, 21, 28, 40, 43).

Many of the oral spirochetes are not readily mass-cultured because they are nutritionally fastidious (39). The cultivable spirochetes are, therefore, generally grown in complex media supplemented with serum or other biological fluids. Such strict nutritional requirements of many spirochetes should be reflected in the number and types of proteolytic enzymes formed by these organisms. Previous studies have indeed demonstrated several proteolytic enzymes produced by *Treponema denticola,* a host-associated spirochete present in the human periodontal pocket (11, 20, 21, 28, 43). The enzymes demonstrated in *T. denticola* represent endopeptidases, iminopeptidases, aminopeptidases, collagenolytic proteinases and peptidases showing so called BANA-activity (connoting that the enzymes hydrolyze *N*-benzoyl-DL-arginine-2-naphthylamide) (20, 28). Only one proteolytic enzyme from a human oral spirochete (the BANA-hydrolyzing peptidase of *T. denticola* strain ASLM) has been subjected to chemical studies (Mäkinen KK, Chen C-Y, et al. 1988). Chemical modification of the benzoyl-arginine peptidase from *T. denticola* (strain ASLM), a human oral spirochete: evidence for active site carboxyl groups. Submitted). Virtually nothing is known about the proteolytic enzymes produced by another human oral spirochete, *Treponema vincentii* ATCC 35580. The purpose of this study was to characterize this organism with regard to its overall proteolytic activity and to report results on collagenolytic endopeptidases and arginine aminopeptidases present in this spirochete. The collagenolytic activity of *T. vincentii* ATCC 35580 was studied using as substrate a chromogenic pentapeptide derivative, i.e. phenyl-

azobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (PZ-PLGPA) which is typically hydrolyzed at the Leu-Gly bond by "true" collagenases (3, 22, 46) and several other collagenolytic enzymes. In fact, microbial extracellular proteinases specifically hydrolyzing PZ-PLGPA or similar synthetic substrates at the Leu-Gly bond, have been regarded as "collagenolytic enzymes" (3, 22, 46). The presence of such activity in *T. vincentii* suggests that the pathogenicity of this organism in periodontal disease may partly depend on the activity of those enzymes.

Material and methods

Source of the organism and treatment of the cells

The cells of *T. vincentii* ATCC 35580 were maintained with bi-weekly transfers in Tryptone-heart infusion-yeast extract broth (39). The cells were grown anaerobically normally in 100–500 ml broth for 72 h. The cells were harvested by centrifugation for 10 min at 16,500 g, washed 3 times with 10 mM Tris-HCl, pH 7.0 (containing 0.1 mM $CaCl_2$), and finally resuspended in 10 ml of buffer. The suspensions were treated for 2 min with a Branson Sonifier model W1850D (Branson Sonic Power Company, Plainview, NY) in an iced water bath. The sonicates were centrifuged for 10 min at 12,000 g and the supernatant fluids were tested with various proteolytic enzyme substrates as indicated below (in the experiment shown in Fig. 1, centrifugation took place at 4,500 g). The sonicates were stored at $-20\,^{\circ}C$ and required recentrifugation after thawing for further studies. *T. denticola* ATCC 35405 was cultivated and the cells were treated as above.

Enzyme and protein assays

Aminopeptidase activity with 2-naphthylamine (2NA) derivatives of amino acids was determined using an azo-coupling metod (19). Benzoylarginine peptidase activity was studied with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) (10, 18). Endopeptidase activity (collagenolytic) was tested using PZ-PLGPA as substrate as described by Wünsch and Heidrich (46). Azocoll (9, 24), Azocasein (33) and elastin-orcein (30) were tested as substrates according to the specific methods given. In kinetic studies initial velocities were measured,

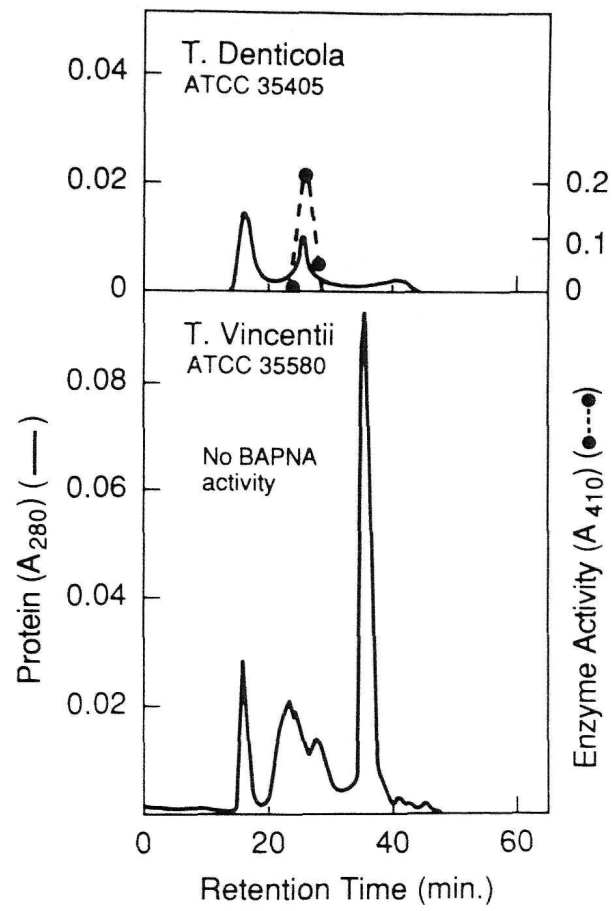

Fig. 1. FPLC on a Superose 12 gel column of the proteins present in the centrifuged (10 min 4,500 g) and ACRO-filtered (0.2μ) sonicates of *T. denticola* ATCC 35405 and *T. vincentii* ATCC 35580. The chromatographic fractions were tested for enzymes hydrolyzing BAPNA. The column was equilibrated and eluted with 0.1 M Tris-HCl, pH 7.5 (containing 50 mM NaCl) at a flow rate of 0.5 ml/min.

whereas in FPLC and Sephadex G-200 chromatography the reaction times were sometimes prolonged (up to 17 h) for the detection of minor enzyme peaks (which were undetectable in short incubation). Protein was determined according to Bradford (6) using Sigma bovine serum albumin as a standard protein, or by using the "absolute" method of Whitaker and Granum (45).

Chemicals and other materials

Unless otherwise indicated, the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The water used in this study was prepared with a Millipore-Milli-Q system and had a resistivity of 18 M Ω cm^{-1}. All samples intended for FPLC were filtered immediately before injection through ACRO LC13 0.2μ filters (Gelman Sciences, Ann Arbor, MI). Concentration of enzymes was performed with Centricon-10 or Centricon-30 membranes (Amicon, Lexington, MA).

Determination of K_m values

The K_m values were determined by plotting the velocity data using the Lineweaver-Burk's, Hanes' and Hofstee's methods with special consideration of substrate inhibition (32).

Amino acid analysis

The nature of the peptide bond (Leu-Gly) hydrolyzed in PZ-PLGPA by the *T. vincentii* enzymes was checked by studying the amino acid conposition of the hydrolysis products. For this purpose a Beckman Model 6300 Automated Amino Acid Analyzer was used. The hydrolysis products of PZ-PLGPA, separated by reversed-phase chromatography on a PepRPC HR5/5 column, were treated for 4 h at $+145\,^{\circ}C$ before analysis. Norleucine was used as an internal standard.

Results

Peptidolytic activity of the sonicates

The supernatant fraction derived from ultrasonic preparations of washed cells of *T. vincentii* displayed considerable activity toward PZ-PLGPA and Azocasein, but hydrolyzed BANA at a very low rate (Tables 1, 2). Table 2 shows that out of the 24 amino acid-2NAs tested only N-L-arginine-2NA (arg-2NA) and γ-L-glutamyl-2NA were hydrolyzed at a high rate. Based on these specificity studies, the fractions in subsequent chromatographic separations were tested for collagenolytic and arginine aminopeptidase activity using PZ-PLGPA and arg-2NA as substrates. The hydrolysis of PZ-PLGPA observed in this study occurred at the Leu-Gly bond only. This was proven by subjecting the reaction mixtures to reversed phase

Table 1. Activity of the cell sonicate of *T. vincentii* (strain ATCC 35580) toward some peptidase substrates*

Substrate	Specific activity
PZ-PLGPA	1.53 μmol $min^{-1} \cdot mg^{-1}$ ($\times 10^{-3}$)
Azocasein	5.1×10^{-3} enzyme units $min^{-1} \cdot mg^{-1}$**
BAPNA	1.5 μmol $\cdot min^{-1} \cdot mg^{-1}$ ($\times 10^{-5}$)
Azocoll	Nil
Elastin-orcein	Nil

* The reactions were performed in 50 mM Tris-HCl buffer (pH 7.0) at 30°C using methods indicated in the Material and methods section, and the supernatant obtained after centrifugation of the cell sonicate for 10 min at 12,000 g.

** In collagenase units as defined in Calbiochem-Boehring Doc. No. 3805–880.

FPLC (only PZ-Pro-Leu and Gly-Pro-Arg were detected) and determining the amino acid composition of the products of enzymatic breakdown of PZ-PLGPA.

In contrast to the sonicates obtained from *T. denticola* (20, 21) the *T. vincentii* sonicates were more difficult to treat on Amicon and Gelman membranes because of the presence of large amounts of high-molecular weight proteinaceous substances. This is shown in Fig. 1 which compares the protein chromatograms of *T. vincentii* ATCC 35580 and *T. denticola* ATCC 35405. *T. vincentii* always produced a more complex protein pattern and higher absorption of fractions at 280 nm. The nature of these proteinaceous substances remains to be studied. These chromatograms were obtained with small (25 ml) volumes of cells and by using several membrane filters to clarify the sonicates.

As expected (20, 21, 28), FPLC separation on a Superose 12 column showed *T. denticola* to contain enzymes hydrolyzing BAPNA at a high rate, whereas *T. vincentii* did not produce measurable BAPNA-hydrolyzing activity; the low activity indicated in Table 1 was, thus, not sufficient for chromatography.

Table 2. Activity of the cell sonicate of *T. vincentii* (strain ATCC 35580) and the high-molecular weight arginine aminopeptidase preparation on various amino acid 2NAs in 50 mM Tris-HCl buffer (pH 7.0; 30°). The sonicated sample was centrifuged for 10 min at 10,000 g before use. This enzyme was obtained by concentrating the void volume fractions of a Sephadex G-200 separation (similar to Fig. 2). BANA = *Na*-benzoyl-DL-arginyl-2NA.

Substrate	Cell sonicate (μmol · min^{-1} · mg^{-1} × 10^{-5})	Arginine aminopeptidase (Relative activity; arg-2NA = 100)
Aspartyl-2NA	1.2	12
Alanyl-2NA	7.8	29
Arginyl-2NA	158.3	100
BANA	0.8	14
α-Glutamyl-2NA	7.8	58
γ-Glutamyl-2NA	49.1	280*
Histidyl-2NA	14.0	12
Isoleucyl-2NA	2.0	0
Leucyl-2NA	8.0	17
Lysyl-2NA	14.7	31
Methionyl-2NA	2.1	13
Prolyl-2NA	2.9	0
Pyrrolidonyl-2NA	3.5	0
Threonyl-2NA	4.0	5
Tryptophyl-2NA	3.0	3
Tyrosyl-2NA	3.2	3
Valyl-2NA	1.6	8
Hydroxyprolyl-2NA	1.0	5
Cysteinyl-2NA	2.5	2
Glycyl-2NA	1.2	4
Seryl-2NA	1.5	8
Glycylprolylleucyl-2NA	1.4	0
Glycylglycyl-2NA	3.6	5
Leucylglycyl-2NA	1.5	13

* Reflects most likely the presence of a γ-glutamyl peptidase.

Preparative gel chromatography

Separation of *T. vincentii* collagenolytic enzymes and arginine aminopeptidases from 100- to 500-ml cultures on a Sephadex G-200 gel column is shown in Fig. 2. Because of the presence of large amounts of substances clogging the filters normally used before FPLC, the supernatant fluids of the sonicates were chromatographed on a larger Sephadex G-200 column. Two major collagenolytic enzymes with a molecule weight of about 75,000 and 23,000, respectively, were revealed. Both displayed some heterogeneity which in repeated separations was found to result mostly from experimental conditions (normal variations in long-term incubation) rather than from true enzyme microheterogeneity. The arginine aminopeptidase activity appeared as several distinctly different peaks, most of which were considered to represent true enzyme heterogeneity.

The chromatographic pattern shown in Fig. 2 was found to be reproducible for the collagenolytic enzyme II, whereas the relative size of collagenolytic enzyme peak I and the fractionation pattern of the arginine aminopeptidases showed greater variability. Depending on conditions that remain to be elucidated more thoroughly, other similar cultivations resulted in a much higher void volume arginine aminopeptidase peak than that shown in Fig. 2. Since the activity of the low-molecular weight arginine aminopeptidases was rapidly lost and since the high-molecular weight enzyme turned out to be more stable, the latter was partly characterized. The active fractions of the 2 collagenolytic enzymes and the major arginine aminopeptidase peaks were pooled, the pools were concentrated on Amicon membranes, and the resulting PZ-PLGPA-hydrolyzing enzymes subjected to FPLC as shown below.

FPLC on a Mono Q column

The concentrates from the previous step were injected into a Mono Q anion exchange column. Applying a three-phase NaCl gradient indicated in Fig. 3, a successful separation of collagenolytic enzyme I was achieved. However, this procedure was destructive to collagenolytic enzyme II. Pretreatment of this enzyme with sulphydryl compounds, metal chelators or metal cations, or the presence of these substances in elution buffers, did not significantly improve the result. Collagenolytic enzyme I was eluted out of the column at the highest NaCl concentration employed, indicating tight binding to the gel. This enzyme did not contain detectable amounts of other proteolytic enzymes (tested on substrates mentioned in Tables 1, 2) and it was considered suitable for various characterization studies. Table 3 shows the specific activity of the enzyme at various stages of the partial purification. Enzyme II showed the highest specific activity before FPLC suggesting that this enzyme may be the dominant collagenolytic proteinase of *T. vincentii* ATCC 35580. However, its lability did not allow further purification and hence this enzyme was characterized following simple Sephadex G-200 chromatography. This enzyme was devoid of other significant proteolytic activity, i.e. out of the substrates shown in Tables 1 and 2, it hydrolyzed only PZ-PLGPA at a high rate.

Properties of collagenolytic enzyme I

The effect of pH on the hydrolysis of PZ-PLGPA by enzyme I is shown in Fig. 4A. The highest rate of hydrolysis

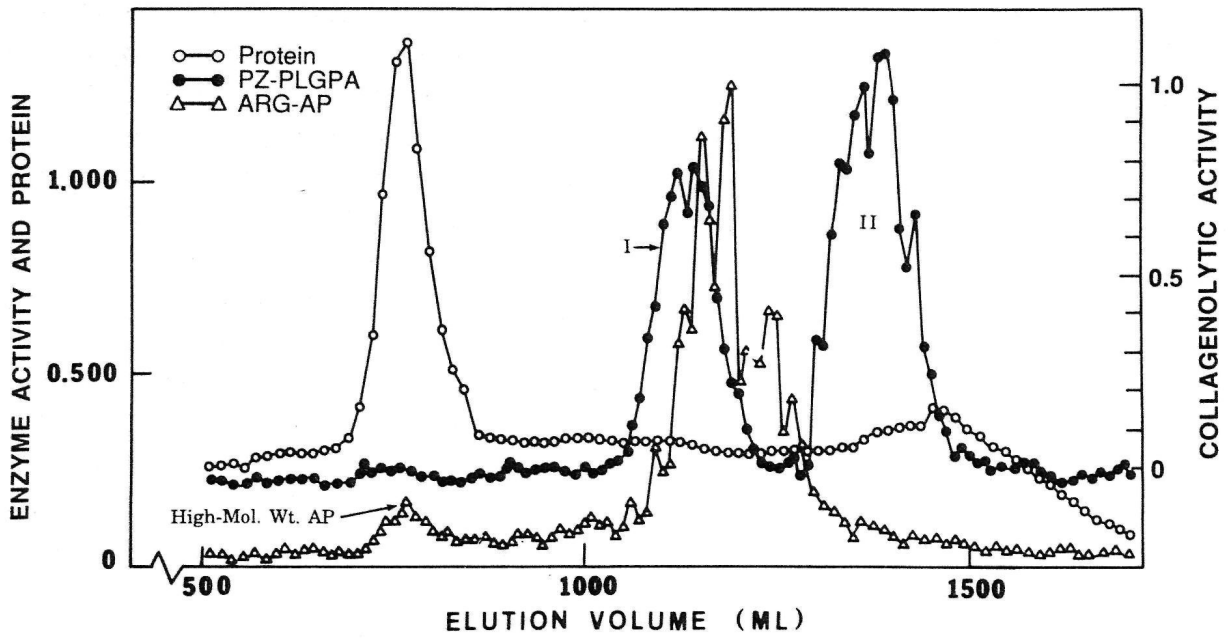

Fig. 2. Separation of collagenolytic enzymes and arginine aminopeptidase-like enzymes of the cell sonicate (centrifuged for 10 min at 12,000 g) of *T. vincentii* ATCC 35580 on a Sephadex G-200 column (5 cm × 88 cm). The column was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.8, containing 1.0 mM $CaCl_2$) at a flow rate of 0.5 ml/min. 40 ml of the centrifuged sonicate was applied on the column. The active fractions were pooled and dialyzed. I and II stand for the major collagenolytic enzymes revealed. The elution of the high-molecular weight arginine aminopeptidase (AP) is indicated. Collagenolytic activity is in A_{320} (PZ-PLGPA as substrate), protein in A_{280} and arginine aminopeptidase activity ("enzyme activity") in A_{525}.

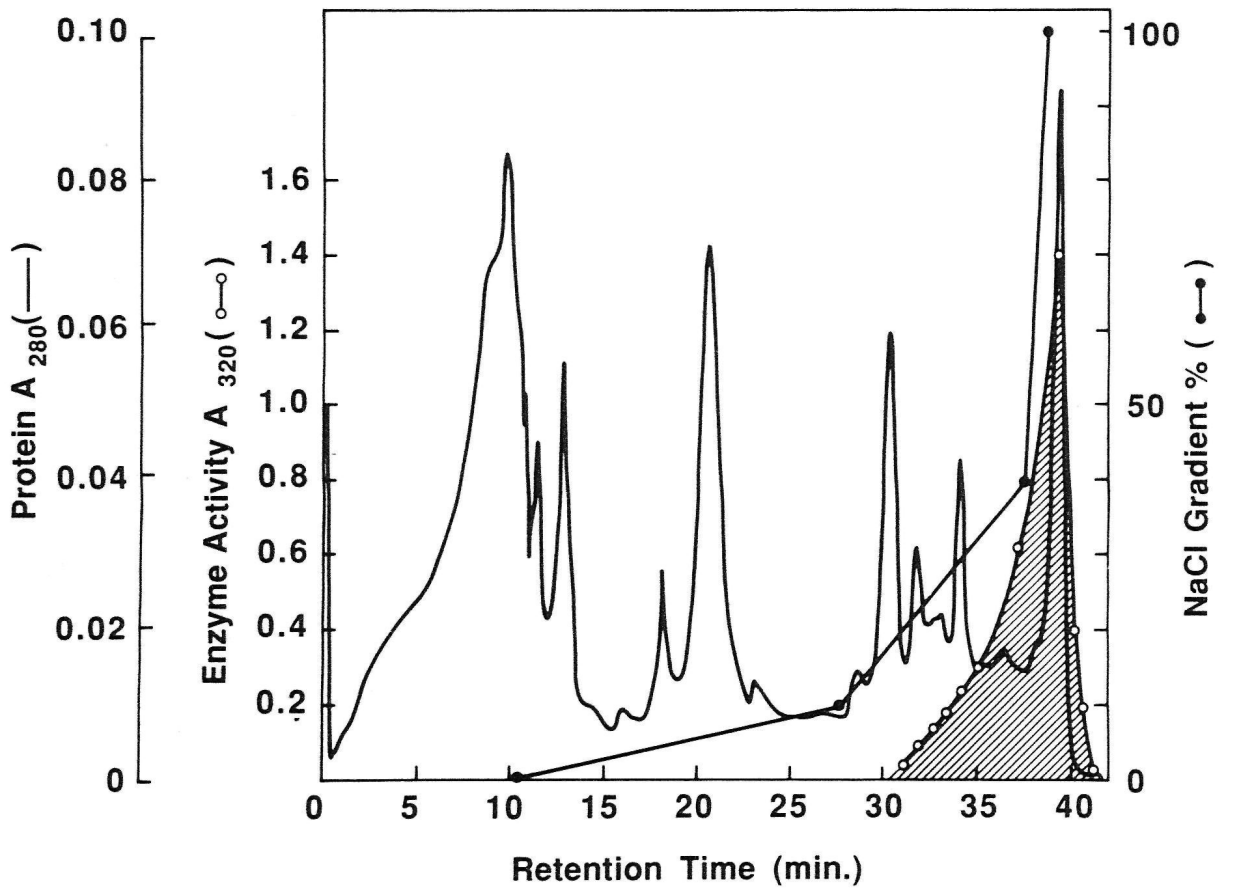

Fig. 3. FPLC on a Mono Q anion exchange column of the collagenolytic enzyme I obtained from Sephadex G-200 chromatography. A thoroughly dialyzed enzyme was injected into the column and eluted at a flow rate of 2.0 ml/min with 50 mM Tris-HCl, pH 7.8 (containing 1.0 mM $CaCl_2$; = buffer A) using, in buffer B, the three-phase NaCl gradient indicated; the proportion of NaCl in buffer B is shown in %. The shaded area shows the elution of the enzyme (tested with PZ-PLGPA). The portion eluted at 38–41 min was dialyzed for removal of NaCl, concentrated and subjected to characterization studies.

was detected between pH 6.5 and 7.5 in the buffer systems shown. The following experiments were performed in 50 mM Tris-HCl, pH 7.0, using an approximate enzyme concentration of 10^{-8} M. The enzyme was inhibited by 0.0167 mM EDTA by 70% and by 8-hydroxyquinoline-5-sulfonic acid and 1,10-phenantroline by 60% and 80%, respectively. These compounds are typical metal chelators. 1.67 μM and 0.167 μM *p*-chloromercuribenzoate (PCMB) inhibited the hydrolysis of PZ-PLGPA by 80% and 54%, respectively, while 0.167 mM dithiothreitol inhibited by 65%. Thus, this enzyme was strongly inhibited by a typical inhibitor of sulphydryl enzymes, although it was not activated by a thiol. Very long-term (17 h) incubation used to test the presence of other enzyme activities with Azocasein, Azocoll, BAPNA, BANA, and the 2NAs of L-arginine, L-histidine, L-lysine, L-leucine, L-alanine and γ-L-glutamic acid as substrates, showed nil or insignificantly low rates of hydrolysis under conditions where PZ-PLGPA was hydrolyzed at a high rate.

The kinetics of the hydrolysis of PZ-PLGPA was studied at different substrate concentrations in 50 mM Tris-HCl (pH 7.8). The kinetics was characterized by substrate inhibition at [PZ-PLGPA] > 0.46 mM (Fig. 5). Thus, typical Michaelis-Menten behavior was not observed. Furthermore, the rate versus [PZ-PLGPA] curves were not parabolic at low substrate concentrations. A value of 0.15 mM was estimated for K_m (or rather K_s; *vide infra*), although this value should be checked after establishing the more exact nature of this kinetic behavior which may appear to be complex. This enzyme hydrolyzed gelatin (22), at a low but clearly measurable rate.

Characterization of collagenolytic enzyme II

The effect of different chemical reagents on the enzyme activity is shown in Table 4. According to these studies, the enzyme was quite sensitive to PCMB, was slightly activated by a low concentration of dithiothreitol and moderately inhibited by metal chelators. The effect of pH on the rate of hydrolysis of PZ-PLGPA catalyzed by the collagenolytic enzyme II is shown in Fig. 4B. The highest rate of hydrolysis was observed between pH 7 and 8. The general pH profile of the hydrolysis of PZ-PLGPA by both collagenolytic enzymes was relatively similar. The effect of [PZ-PLGPA] on the rate of the hydrolysis differed from that observed with enzyme I; the kinetics more closely followed Michaelis-Menten behavior, although substrate inhibition was observed. A K_m value of about 0.65 mM was obtained using the three plotting methods indicated (Fig. 6). This enzyme hydrolyzed arg-2NA as a result of partial overlapping of arginine aminopeptidases with this enzyme (Fig. 2). However, because this preparation was de-

Table 3. Partial purification of the PZ-PLGPA-hydrolyzing collagenolytic enzymes I and II from the cell sonicate of *T. vinentii* (strain ATCC 35580)

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (nmol · min^{-1} · mg^{-1})	Purification
Cell extract (after centrifugation)	500	7.8	3,900	1.38	–
After Sephadex G-200 (before concentration)					
Enzyme I	500	0.110	55	3.10	2.2
Enzyme II	220	0.042	9	29.9	22
Enzyme I after FPLC (Mono Q)*	6	0.012	0.07	33.8	24
Enzyme I after dialysis	6	0.010	0.06	48.3	35

* In gel filtration on Sephadex G-200, two major peaks with activity toward PZ-PLGPA appeared. Enzyme I was further purified as indicated, whereas the lability of enzyme II necessitated its characterization after Sephadex G-200 chromatography.

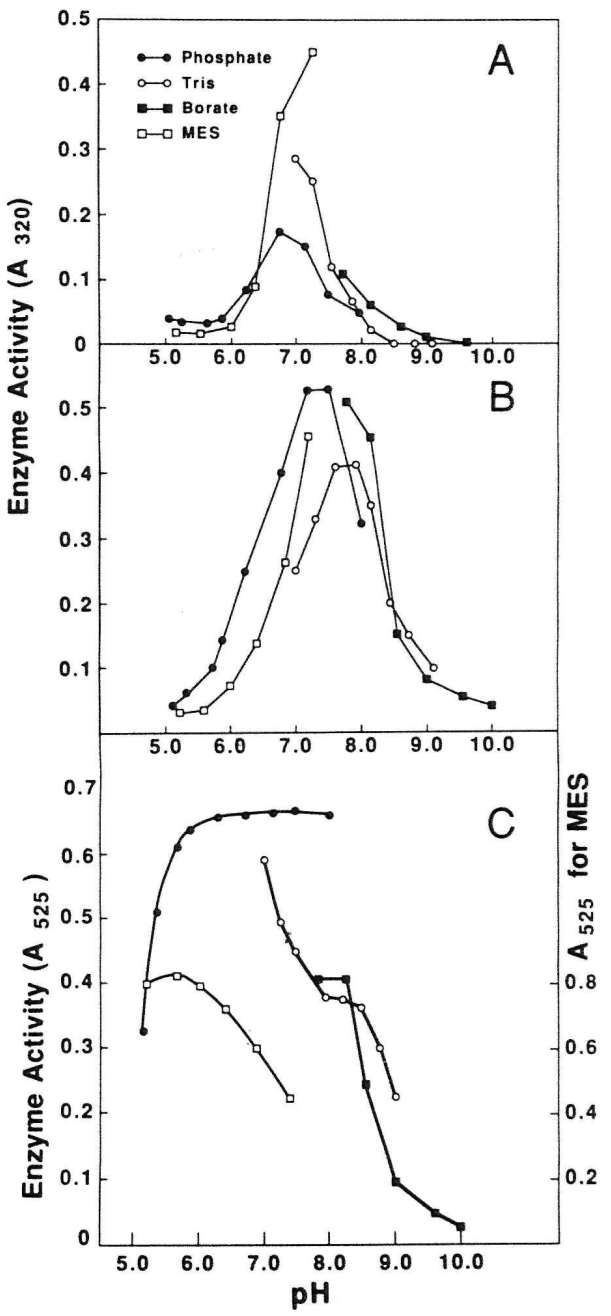

Fig. 4. Dependence on pH of the rate of the hydrolysis of PZ-PLGPA catalyzed by collagenolytic enzymes I (A) and II (B), and that of the hydrolysis of arg-2NA catalyzed by the high-molecular weight arginine aminopeptidase (C), of *T. vincentii* ATCC 35580. The reactions were carried out at 30 °C in 0.1 M phosphate, 50 mM Tris-HCl, 50 mM Mes and 50 mM borate buffers. The reactions in C were performed in the presence of 0.167 mM 1,10-phenantroline.

void of other endopeptidase activity (it hydrolyzed PZ-PLGPA as the endopeptidase substrate; the other substrates shown in Table 1 were not hydrolyzed), the above characterization experiments were considered justified.

Characterization of the high-molecular weight arginine aminopeptidase

The low-molecular weight arginine aminopeptidases of *T. vincentii* ATCC 35580 lost a considerable part of their activity upon standing, dialysis and various chromatographic procedures. The enzyme eluted in the void volume of the Sephadex G-200 column (Fig. 2) was more stable under these conditions. For this study, this enzyme was obtained from a separation yielding about a sixfold activity compared to the argininge aminopeptidase peak shown in Fig. 2. This enzyme had a molecular weight of >200,000 (assuming approximate globular shape and unhindered filtration of the enzyme). The enzyme was inhibited by 80% and 10% in the presence of 1.67×10^{-6} M and 0.167×10^{-6} M PCMB, respectively.

The effect of pH on the rate of the hydrolysis of arg-2NA in different buffer systems is shown in Fig. 4C (tested in the presence of 0.167 mM 1,10-phenantroline which stabilized the enzyme). The pH optimum greatly depended on the buffer used; except for Mes buffer, hibher rates of hydrolysis were measured between pH 7 and 8. The relative rate of hydrolysis of various amino acid 2NAs by this enzyme is shown in Table 2. The void volume peak contained also other proteolytic enzymes, the present arginine aminopeptidase and γ-glutamyl peptidase activities being, however, dominant (judged on the basis of experiments using the substrates shown in Tables 1, 2). The activities shown in Table 2 may not necessarily reflect the true situation with regard to all amino acid 2NAs studied, but these data suitably characterize the overall aminopeptidase (and BANA-hydrolyzing) activity of the void volume fraction.

The effect of [arg-2NA] on the rate of the hydrolysis was studied as above using 3 different methods of plotting (Fig. 7). These methods yielded a mean value of 0.055 mM for K_m, tested in 50 mM Tris-HCl, pH 7.5. No significant deviation from Michaelis-Menten kin-

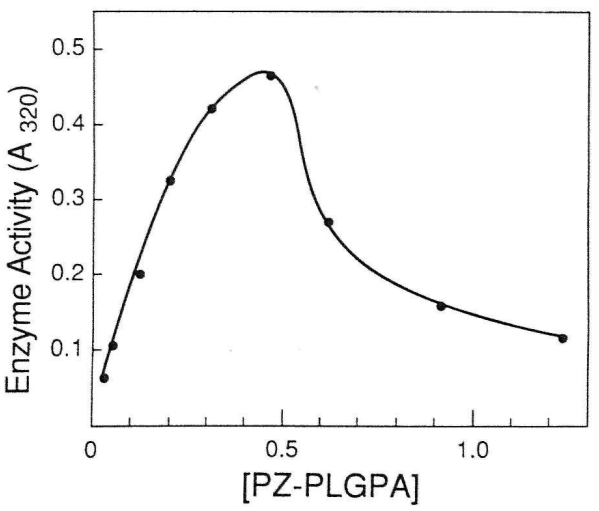

Fig. 5. The Michaelis-Menten plot of the initial velocity (in A_{320}) of the hydrolysis of PZ-PLGPA catalyzed by collagenolytic enzyme I from *T. vincentii* ATCC 35580, showing substrate inhibition and other deviation from "normal" kinetics. The reactions were performed in 0.1 M Tris-HCl, pH 7.8 (containing 0.1 mM $CaCl_2$); at 30 °C.

Table 4. Effect of various chemical reagents on the rate of the hydrolysis of PZ-PLGPA by collagenolytic enzyme II from *T. vincentii* ATCC 35580*

Affector (M)		Percentage of inhibition
EDTA	1.67×10^{-5}	33
	1.67×10^{-6}	23
HQSA**	1.67×10^{-4}	77
	1.67×10^{-5}	13
1,10-PHE**	1.67×10^{-5}	51
	1.67×10^{-6}	0
Dithiothreitol	1.67×10^{-4}	16
	1.67×10^{-5}	(+30)***
PCMB	1.67×10^{-6}	90
	1.67×10^{-7}	61
	1.67×10^{-8}	17

* The reactions were performed in 50 mM Tris-HCl, pH 7.8 at 30 °C using a substrate concentration of 0.5 mM and an enzyme concentration of about 10^{-8} M. The values shown are means of 3 of 4 determinations.
** HQSA = 8-hydroxyquinoline sulfonic acid; 1,10-PHE = 1,10-phenantroline.
*** Activation.

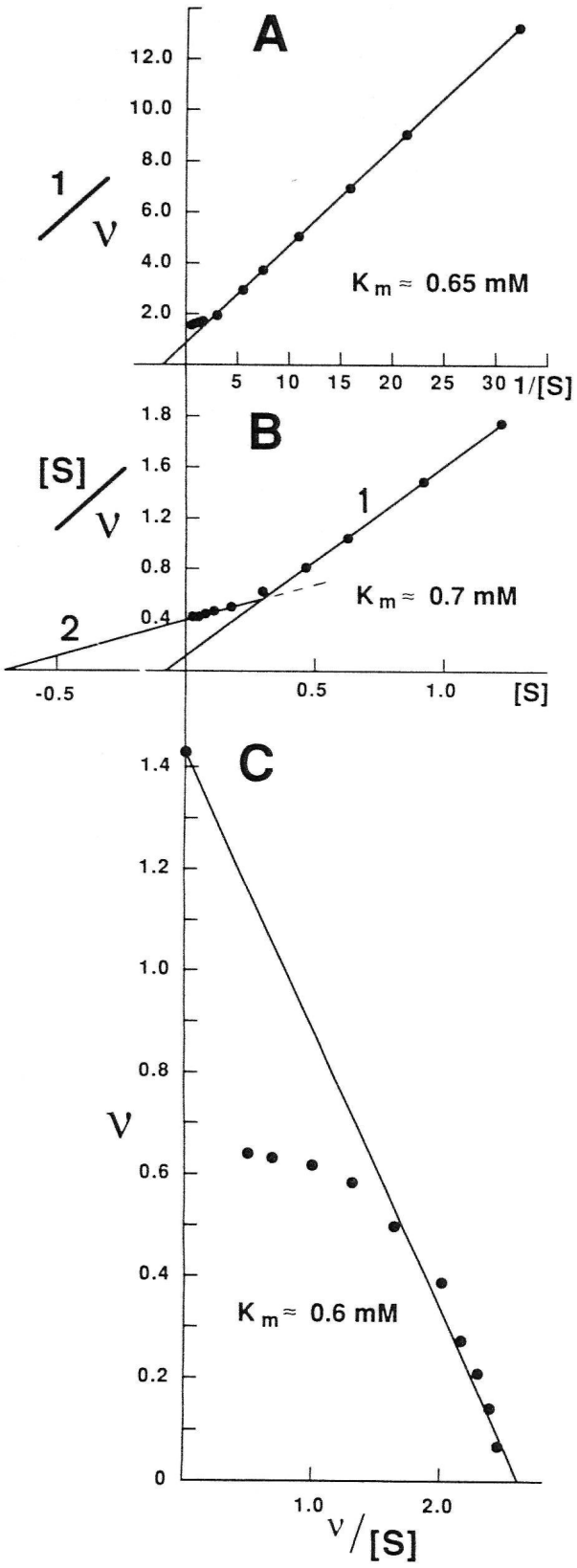

Fig. 6. Lineweaver-Burk's (A), Hanes' (B) and Woolf-Hofstee's (C) plots for the hydrolysis of PZ-PLGPA catalyzed by collagenolytic enzyme II of *T. vincentii* ATCC 35580. The reactions were carried out in 0.1 M Tris-HCl, pH 7.5 (containing 0.1 mM $CaCl_2$), at 30°C. As a result of substrate inhibition, the 4 highest substrate concentrations were disregarded in the determination of K_m (see text). Thus, in the plot of [S]/v versus [S], Curve 1 gave an erroneous value and Curve 2 a more correct estimation of K_m (see Discussion).

etics was observed. The presence of 1,10-phenantroline greatly affected the course of the reaction (Fig. 8). The presence of the chelator induced substrate inhibition and slightly increased the affinity between enzyme and arg-2NA at low substrate concentrations. Therefore, this chelator was used in some characterization studies (Fig. 4C), although its presence seemed to complicate the kinetics of the hydrolysis of arg-2NA.

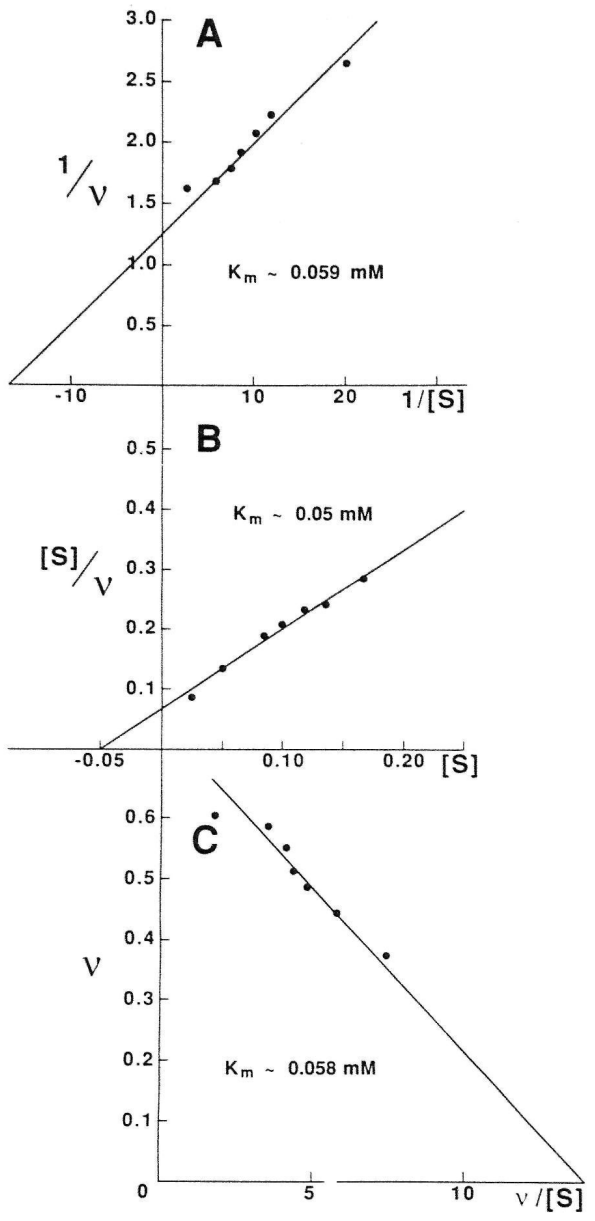

Fig. 7. Lineweaver-Burk's (A), Hanes' (B) and Woolf-Hofstee's (C) plots for the hydrolysis of arg-2NA catalyzed by the high-molecular weight arginine aminopeptidase of *T. vincentii* ATCC 35580. The values shown are those of K_m. The reactions were carried out in 50 mM Tris-HCl, pH 7.0.

Discussion

The current information renders it possible to speculate about the role of the proteolytic activity exhibited by cultivable oral treponemes in the gingival crevice. *T. denticola* was characterized by high proline iminopeptidase, BANA and BAPNA activity (21, 28), whereas an unspeciated treponeme (strain US) displayed considerable activity toward PZ-PLGPA (38). *Treponemo socranskii*, a saccharolytic organism, was active toward hydroxyprolyl-2NA (38). *T. vincentii* studied in this investigation, contained high activity toward PZ-PLGPA, arg-NA and γ-glu-2NA. These specific characteristics suggest that although the human oral treponemes seem to specialize in the hydrolysis of specific substrates, the overall proteolytic specificity of the treponemes is relatively broad. It is interesting from the clinical point of view that the hydrolysis of PZ-PLGPA has been a common denominator in all oral treponemes studied by us (20, 38). The PZ-PLGPA-hydrolyzing enzymes also showed more constant and consistent activity levels than many other peptidases whose activities often differed more significantly between species and strains (20). The PZ-PLGPA-hydrolyzing enzymes may thus be regarded as constitutive proteinases of these treponemes.

This study does not claim that the two major PZ-PLGPA-hydrolyzing enzymes of *T. vincentii* represented "true" collagenases. True microbial collagenases have been more thoroughly studied only in *Clostridium histolyticum* (2–5, 12), *Achromobacter* (42), and *Bacillus cereus* (22). Further studies on radioactively labelled collagen and more detailed comparative enzymology are needed to elucidate this question. However, PZ-PLGPA has been recommended and intensively used as a chromogenic substrate for bacterial collagenases (3, 22, 46). Bond and van Wart, for example, stated that the best way to compare the activities of bacterial collagenases is to employ PZ-PLGPA as substrate (3). The present collagenolytic enzymes I and II showed high activity toward PZ-PLGPA and did not significantly hydrolyze other peptidase substrates tested. Furthermore, enzyme I hydrolyzed gelatin, a modified collagen. However, these enzymes may not be active on insoluble collagen, as evidenced by the inability

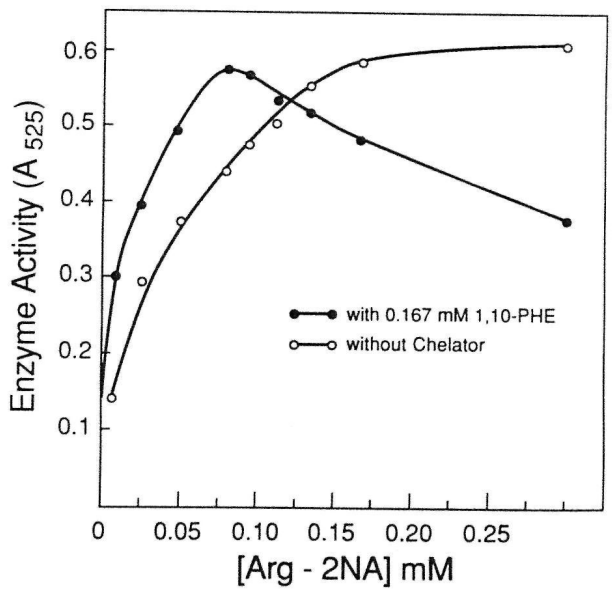

Fig. 8. The Michaelis-Menten plot of the initial velocity (in A_{525}) of the hydrolysis of arg-2NA catalyzed by the high-molecular weight arginine aminopeptidase in the presence of 0.167 mM 1,10-phenantroline and without added chelator. The reactions were carried out at 30°C in 0.1 M Tris-HCl buffer, pH 7.5.

of the crude enzyme preparation and the partially purified enzymes to hydrolyze Azocoll. The *in vivo* substrate(s) of these enzymes should then be liberated by other mechanisms, for example, by tissue collagenases.

The Michaelis law was obeyed at lower substrate concentrations with collagenolytic enzyme II, while the kinetics of enzyme I deviated from this law at most substrate levels tested. The velocity fell off at high concentrations with both enzymes. This effect may have resulted from a number of different causes. In a "simple" peptidolytic reaction, such as the cleavage of the Leu-Gly bond by a monoreactant enzyme, it is likely that the enzyme, with its most likely extended binding domain, may contain 2 or more groups, each combining with a particular part of the molecule. At high [PZ-PLGPA] this may result in the formation of ineffective complexes. This situation corresponded to competitive inhibition by the substrate itself and thus the value obtained from the intercept of the line in the double reciprocal plot, for example (Fig. 6A), does not give K_m, but $K_{s'}$, which is the dissociation constant of an inactive ES_2 complex. Endopeptidases that hydrolyze peptide bonds often contain an extended active site (25) which would render this type of kinetic behavior possible. Because this type of kinetic examinations require more detailed studies, we are here restricting ourselves to the use of K_m rather than $K_{s'}$ (Fig. 6).

The plots of v versus $v/[S]$ can be used to prove if one or more enzymes active on the same substrate are present (32). If more than one enzyme is involved, this plot tends to display pronounced curvature which is quite different from that associated with substrate inhibition (in the former case the experimental points arranged on a concave curve). In none of the cases studied was such kinetic behavior noted. This suggests that the collagenolytic enzymes I and II were essentially free from other enzymes hydrolyzing PZ-PLGPA entitling us to draw conclusions about the data obtained. Nor was such behavior observed in the hydrolysis of arg-2NA.

Although inflammatory processes in the periodontal pocket are frequently characterized by the presence of highly active host tissue enzyme participating in numerous biochemical events, bacterial enzymes are also present and most likely contribute significantly to the entire process. It was stated more than twenty years ago that the close proximity of oral tissues to microbial enzymes almost certainly results in the hydrolysis of the former (17). This direct role of "periodontophatic" hydrolases has received abundant attention recently. A larger number of studies have described the presence of different peptidases in periodontopathic organisms (11, 14, 15, 20–23, 26–29, 31, 35–37, 40, 41, 43) or in subgingival plaque (1, 7), and (bacterial) collagenase activity was demonstrated in the gingival sulcal debris, cervicular fluid, or periodontopathic organisms (1, 22, 23, 29, 34, 41). It is of special interest that a bacterial collagenase was shown to be a chemoattractant for human neutrophils (44). Among these enzymes collagenolytic proteinases elicit special interest, as the evolution of these enzymes, like the spirochete endopeptidases, must have taken place in close association with the evolution of the connective tissue itself. Such periodontopathogens as the treponemes, thus represent an old peptidolytic trait. This view may be further extrapolated to comprise other organisms isolated from subgingival plaque: studies which will be published later suggest that PZ-PLGPA-hydrolyzing enzymes are present not only in treponemes, but also in fusobacteria, and in *Capnocytophaga, Bacillus* and *Streptococcus* species, all of which display pathogenicity in the periodontium or elsewhere in host tissues. Furthermore, all PZ-PLGPA-hydrolyzing enzymes discovered in periodontopathic organisms also hydrolyze various soluble and/or insoluble collagenous substances.

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