INFECTION AND IMMUNITY, Sept. 1995, p. 3567–3575 Vol. 63, No. 9
0019-9567/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Role of the Chymotrypsin-like Membrane-Associated Proteinase from *Treponema denticola* ATCC 35405 in Inactivation of Bioactive Peptides

PIRKKO-LIISA MÄKINEN, KAUKO K. MÄKINEN,* AND SALAM A. SYED

Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan 48109

Received 27 February 1995/Returned for modification 25 April 1995/Accepted 30 May 1995

The ability of washed whole cells of *Treponema denticola* ATCC 35405 to hydrolyze (inactivate) substance P, bradykinin, and angiotensin I was studied. Substance P was attacked primarily at the Phe-8–Gly-9 bond by a chymotrypsin-like proteinase (CTLP), at Pro-4–Gln-5 by an endo-acting prolyl oligopeptidase (POPase), and at Gln-5–Gln-6 by an endopeptidase (FALGPA-peptidase). Bradykinin was cleaved at Phe-5–Ser-6 by the FALGPA-peptidase and at Pro-7–Phe-8 by the POPase. Angiotensin I was rapidly converted to angiotensin II by the CTLP, and both angiotensin I and angiotensin II were further hydrolyzed at Pro-7–Phe-8 by the POPase. All these enzymes were assumed to be cell associated and were easily extracted with a mild (0.05 to 0.1%) Triton X-100 treatment. Because it was conceivable that the hydrolysis of substance P at the Phe-8–Gly-9 bond was catalyzed by a CTLP described earlier (V.-J. Uitto, D. Grenier, E. C. S. Chan, and B. C. McBride, Infect. Immun. 56:2717–2722, 1988), the enzyme was purified to homogeneity by means of conventional fast protein liquid chromatography procedures. For kinetic studies, Phe-8(4-nitro)-substance P (NSP) (absorption maximum at 309.2 nm, $\varepsilon = 545\ M^{-1}\ cm^{-1}$) was synthesized to replace substance P as a substrate in kinetic studies. In reversed-phase chromatography, both NSP and substance P gave identical results with both whole cells and the purified enzyme. The CTLP has a mass of 95 kDa, and its activity is suggested to be based on an active seryl residue, on an active imidazole group, and on an active carboxyl group but not on metal cations. The enzyme hydrolyzes *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroaniline (SAAPFNA, a typical chymotrypsin substrate) at a high rate and several proteins, such as calf thymus histone, human plasma fibrinogen, milk caseins, and gelatin. Among the substrates tested, substance P showed the highest affinity (K_m = 0.22 mM) for the purified enzyme. Depending on conditions, clinically applicable chlorhexidine levels (3.2 mmol/liter, or 0.2%) strongly activated (up to fourfold) the hydrolysis of SAAPFNA by whole cells and the purified CTLP. The hydrolysis of NSP by whole cells and purified CTLP was slightly inhibited by chlorhexidine. The results demonstrated the versatility and the effectiveness of the outer membrane of *T. denticola* in occasioning a rapid breakdown and inactivation of human bioactive peptides and other peptidolytic catalyses. The tests with whole cells resulted in the accumulation of short peptides derived from substance P, bradykinin, and the angiotensins, the resistance of which to further hydrolysis by whole cells deserves additional studies.

Recent studies suggested that the cells of *Treponema denticola* ATCC 35405 can initially exploit short peptides as a source of energy, avoiding complete hydrolysis of the peptides to amino acids before transportation through the cell membrane occurs (35, 38). Because *T. denticola* has been shown to be associated with periodontal disease (with a close cognate, *Treponema pallidum*, being involved in syphilis), and because *T. denticola* has been shown to occasion effective inactivation of several human bioactive peptides (23, 24), such transport of intact peptides or peptide fragments may be linked to the pathogenicity of these organisms. Preliminary screening for potential natural oligopeptide substrates at this laboratory showed that the most rapidly hydrolyzed (inactivated) bioactive peptides included substance P, angiotensin I, and bradykinin, all of which were shown to hydrolyze at peptide bonds involving an imino acid (proline) residue at or near the scissile bond (23, 24). The sizes of the resulting peptide fragments correspond to those (35, 38) of peptides shown to undergo transportation through the cell wall without first undergoing complete hydrolysis.

* Corresponding author. Mailing address: Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI 48109. Phone: (313) 763-6166. Fax: (313) 747-3896.

The primary objective of this study was to characterize the chemical reactions catalyzed by microscopically intact, washed cells of *T. denticola* ATCC 35405 incubated in the presence of substance P, angiotensin I, and bradykinin. When accomplishing this goal, it appeared that substance P was hydrolyzed by whole cells primarily at the Phe-8–Gly-9 bond. The nature of the amino acid residues involved in this peptide bond suggested that the hydrolysis was probably catalyzed by a chymotrypsin-like proteinase (CTLP) (15, 37). For the elucidation of this question, it was found to be necessary to purify and characterize the enzyme responsible for the above catalysis. The purification was accomplished by means of conventional fast protein liquid chromatography (FPLC) procedures. Because the CTLP preferred a proline residue in position P_2 in synthetic peptide derivatives of *p*-nitroaniline (*p*NA) (such as *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-*p*-NA [SAAPFNA] [see Table 3]), this enzyme therefore may be included in the group of proline-specific peptidases of infectious microorganisms. Such enzymes have constituted a primary research object at this laboratory (23, 24). It appeared that washed, whole cells of *T. denticola* ATCC 35405 catalyzed a remarkably fast breakdown of all of the above human bioactive peptides. These reactions, when studied with purified enzymes, are characterized by quite low affinity constants. Consequently, in ad-

dition to elucidating the whole-cell-associated peptidolysis of human bioactive peptides, this paper provides both confirming and new information on the substrate specificity of the recently discovered CTLP of *T. denticola* ATCC 35405.

MATERIALS AND METHODS

Source and cultivation of the organism and treatment of the cells. Cells of *T. denticola* ATCC 35405 were grown anaerobically for 48 h in a tryptone-yeast extract-heart infusion broth containing 10% heat-inactivated rabbit serum (23, 29). For the purpose of enzyme purification, 1.2-liter aliquots of the growth medium in 1.5-liter screw-cap flasks were inoculated with 100-ml aliquots of cultures (23, 29) and incubated anaerobically for 4 days at 37°C. The optical density at 660 nm (OD_{660}) was used to determine the number of cells; an OD_{660} of 0.2 corresponded to 5×10^8 cells per ml. After these procedures, the treatment of the enzyme took place at 0 to 4°C except for FPLC, which was carried out at 22°C. Checking of the disintegration of cells was performed turbidimetrically at 660 nm (14).

Chemicals. Unless specifically indicated, the chemicals used were obtained from Sigma. The water used in this study was prepared with a Millipore Milli-Q system and had a resistance of 18 megaohms cm^{-1}.

Purification of CTLP. The purification of the enzyme was carried out with SAAPFNA as the substrate. The procedure was typically started with a total of ca. 2.5×10^{12} cells. The cells, harvested after 4 days of growth, were washed with 50 mmol of Tris buffer (pH 7.5) per liter and centrifuged for 15 min at 14,500 × *g*. The final cell pellet was suspended in 50 ml of the above buffer, after which small volumes of 20% Triton X-100 (Pierce) were added to a final concentration of 0.1%. The mixture was kept for 150 min in an iced water bath and then centrifuged as above. The supernatant fluid (Triton extract) was subjected to FPLC as detailed below. The detergent-treated cell pellet was examined microscopically for the integrity of the cells; the cells were found to be morphologically intact. The purification of the enzyme was accomplished as described below.

(i) First ion-exchange FPLC and polymyxin treatment. The above supernatant fluid (44 ml) was subjected to ion-exchange FPLC on a Fractogel EMD TMAE-650 column (2 by 60 cm). The column was eluted first with 200 ml of 50-mmol/liter Tris (pH 7.5), containing 0.1% Triton X-100, followed by a shallow salt gradient (increase in NaCl concentration was 2 mmol/liter/min). Fractions (normally 170 through 181; only one enzyme active on SAAPFNA was discovered; Fig. 1A) containing the SAAPFNA-hydrolyzing enzyme were combined, and the resulting enzyme was treated for 80 min with polymyxin B sulfate (2 mg/ml of enzyme) at 37°C for the removal of possible lipopolysaccharide structures associated with the enzyme. The activity of the proteinase was not affected by this treatment. The enzyme solution was then brought to 80% saturation with ammonium sulfate. After 2.5 h of standing at 4°C, the mixture was centrifuged for 15 min at 27,000 × *g*. The resulting protein pellet was dissolved in 50 mmol of Tris (pH 7.5) per liter, containing 0.1% Triton X-100, to a final volume of 2.7 ml. The enzyme was thereafter dialyzed successively two times against 400 volumes of 10 mmol of Tris (pH 7.5) per liter with 0.1% Triton X-100.

(ii) Second ion-exchange FPLC. The enzyme (4.2 ml) resulting from the above step was chromatographed on a Mono Q HR 5/10 column. The column was eluted with an NaCl gradient made in 50 mmol of Tris (pH 7.5) per liter with 0.1% Triton X-100, and the salt concentration was increased by 3.33 mmol/liter/min. The active fractions (normally 34 through 36; Fig. 1B) were combined, and the resulting enzyme was concentrated with Centricon-30 filters (Amicon). The concentrate was treated for 60 min with 8 mmol of sodium dodecyl sulfate (SDS) per liter at 45°C. The SDS-treated enzyme was subjected to the following purification step.

(iii) Gel permeation chromatographic separations. The enzyme (normally 4.0 ml) from the previous step was subjected to two successive separations on a Superose 12 HR 10/30 column, followed by FPLC separation on a Superose 6 HR 10/30 column as for the final step of purification (Fig. 1C). The elution buffer was 20 mmol of phosphate buffer, pH 6.8, per liter containing 0.1% SDS. The purified enzyme was stored in the final elution buffer at 4°C.

Kinetic measurements. The activity of CTLP was routinely tested with SAAPFNA. Other related *p*NA derivatives were also used. The *p*NA substrates were dissolved in 0.1 mol of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5) per liter containing 0.5 mol of NaCl per liter and 10% dimethyl sulfoxide (DMSO). The enzyme reactions were performed in thermostated (+30°C) spectrophotometer cuvettes in 1.0-ml reaction mixtures. The reactions were initiated by the addition of 1 to 10 μl of enzyme. The velocity measurements were carried out at 410 nm, and a value of 8,800 M^{-1} cm^{-1} was used for the extinction coefficient (ε). Phe-8(4-nitro)-substance P (NSP) was synthesized (vide infra) to study the hydrolysis of substance P by the purified enzyme and by washed whole cells of *T. denticola*. The kinetics of the hydrolysis of NSP was studied in the above buffer at 310 nm with a value of 545 M^{-1} cm^{-1} for ε (no value for ε could be found in literature, and the coefficient was calculated for this study with 0.2 mmol of NSP per liter and an excess of enzyme).

The calculation of enzyme kinetic constants (Michaelis constant [K_m], catalytic constant [k_{cat}], and specificity constant [k_{cat}/K_m]) was carried out with Enzpack 3 software (Biosoft, Ferguson, Mo.). The enzyme reactions were carried out in the above buffer at 30°C. The effect of pH on the enzyme reaction was studied in Tris (50 mmol/liter) with SAAPFNA (0.2 mmol/liter) for whole cells (10^9 cells per ml) and for the purified enzyme at 30°C.

Other enzyme measurements. The hydrolysis of proteins by the purified CTLP was studied in mixtures consisting of Tris (0.1 mol/liter, pH 8.35, at 30°C), the protein to be studied (2 mg/ml), and purified CTLP (37 nmol/liter). Reaction times of up to 18 h were used. The reaction mixtures (including blank mixtures from which either the enzyme or the substrate was omitted) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with the Pharmacia Phast System and PhastGel Gradient 8-25, PhastGel SDS buffer strips, and silver staining. The reaction mixtures were also subjected to FPLC on a Superose 12 column.

The enzymatic breakdown of the insulin B chain by several proteinases has been well documented. Therefore, this peptide was used as a model; its hydrolysis by the present CTLP was compared with that by previously studied proteinases, such as elastase and cathepsin G (4, 18). The hydrolysis of insulin B chain and other oligopeptides was studied by dissolving the peptide in phosphate buffer (50 mmol/liter, pH 8.0) (1 mg/ml) and adding 0.1 μg of purified enzyme to the mixture (0.5 ml). After 1 to 60 min of reaction at 30°C, the mixtures were immediately subjected to reversed-phase FPLC on a PepRPC 5/5 column (Pharmacia) with 0.1% trifluoroacetic acid (TFA) in water as eluent A and 0.05% TFA in 2-propanol as eluent B (the increase in eluent B concentration was 0.33%/min) to separate the peptides. The fractions containing the peptides (observed by their absorption at 214 nm) were recovered. After evaporation to dryness, hydrolysis (4 h in HCl [6 mol/liter], 145°C) and further evaporation to dryness, the final dried residues of the fractions were subjected to amino acid analysis and, if necessary, to further reversed-phase high-pressure liquid chromatography (HPLC) on an analytical C_{18} column (type 218TP52; 0.21 by 220 mm; Vydec, Hesperia, Calif.). Amino acid analyses were performed on a Beckman System 6300 High Performance Amino Acid Analyzer.

Chemical modification of CTLP. Modification of seryl residues was performed with diisopropyl fluorophosphate (DFP). Modification of imidazole groups by diethyl pyrocarbonate (DEP) was studied as described by Miles (27). Treatment of the proteinase with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to investigate the involvement of carboxyl groups in enzyme activity was performed at 25°C (30, 34). All other modification reactions were carried out in an iced water bath. All modifications were performed with protection from light.

Experiments with whole cells. The hydrolysis of peptides was studied with whole, washed cells of *T. denticola* ATCC 35405. The reactions were normally performed in phosphate buffer (20 mmol/liter, pH 6.8) at substrate concentrations of 0.2 mmol/liter at 30°C. The cells for these experiments were obtained from 4-day-old cultures, washed once with a large excess of cold Milli-Q-pure water, and checked microscopically for integrity. For velocity measurements, about 5×10^8 washed cells were added to each 0.5-ml reaction mixture in 1.5-ml plastic centrifuge tubes. The reactions were arrested after 1 to 5 min by the addition of 12.5 μl of 10% TFA (final concentration, ca. 0.25%), and the mixtures were spun down for 2 min at 10,000 × *g* at 2°C. The supernatant fluids were passed through 0.2-μm-pore-size Gelman membrane filters and subjected to reversed-phase FPLC on a PepRPC 5/5 column for the separation of peptide fragments. Eluent A was 0.1% TFA in water, and eluent B was 0.05% TFA in 2-propanol. The increase in eluent B concentration was 0.5%/min. The peptides that were separated were subjected to amino acid analysis (vide supra) for identification. The hydrolysis of SAAPFNA by washed whole cells was studied as above, but the reactions were arrested by filtering the reaction mixtures directly into spectrophotometer cuvettes, with Gelman 0.2-μm-pore-size membrane filters. The A_{410} was read as shown above.

Synthesis of NSP. A 56-mg quantity of the nitrated derivative of substance P (NSP) was synthesized at the University of Michigan Medical School Protein and Carbohydrate Structure Core Facility. The purity and the molecular mass of the end product were confirmed by HPLC on a Vydac reversed-phase C_{18} column (4.6 by 250 mm), at a wavelength of 214 nm, followed by compositional amino acid analysis, and by mass spectrometry, respectively. NSP was used especially in kinetic studies of CTLP. The difference spectrum of enzymatic product absorbance minus unhydrolyzed substrate absorbance was determined (not shown).

Protein determination. Protein was determined by means of the bicinchoninic protein assay reagent procedure (Pierce), with bovine serum albumin (Pierce) as a standard protein. In FPLC, the fractionation of proteins was monitored at 280 nm.

RESULTS

Purification of CTLP. The purification of the enzyme is summarized in Fig. 1 and in Table 1. The recovery of enzyme activity after the last purification step was 19.4%, and a purification factor of about 101 was achieved. The magnitude of this factor should be viewed against the relatively small number of proteins present in the mild (0.1%) detergent extract. The purity of the enzyme after step 6 (Table 1) was studied by means of SDS-PAGE (with PhastGel Gradient 8-25 and PhastGel SDS buffer strips) and by means of FPLC on a Superose 6 column. The enzyme was homogeneous in SDS-

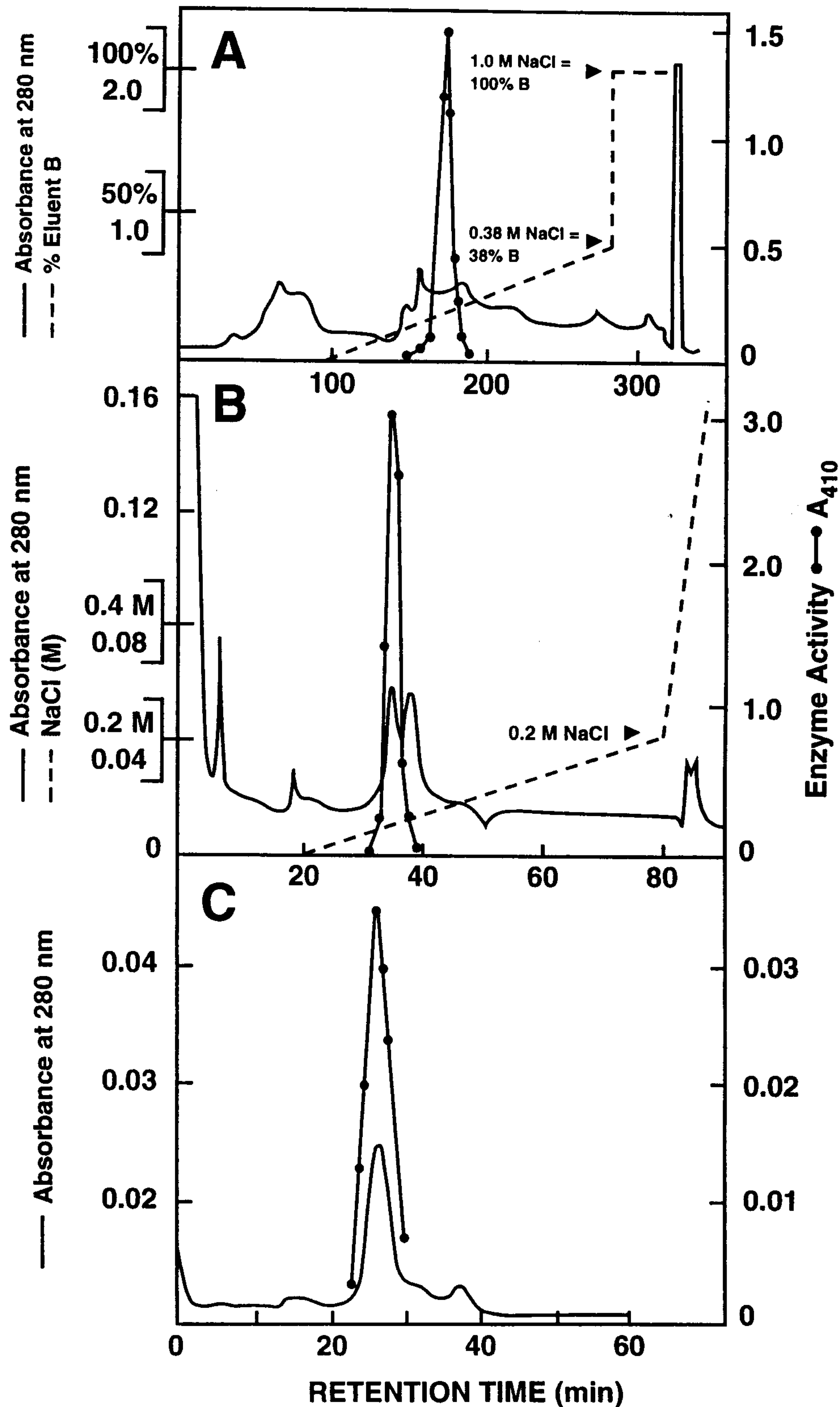

FIG. 1. Purification of the CTLP by FPLC. (A) Separation of the enzyme on a Fractogel anion-exchange column. The elution was performed with Tris (50 mmol/liter, pH 7.5) for 100 min and thereafter with buffer containing the NaCl gradient shown. The fraction volume was 2.0 ml (flow rate, 2.0 ml/min). (B) Separation of the enzyme after the previous step and polymyxin treatment (step 3, Table 1) on a Mono Q anion-exchange column. The column was eluted with the buffer, applying the NaCl gradient shown after 20 min. The fraction volume was 1.0 ml (flow rate, 1.0 ml/min). (C) Final gel permeation separation of the enzyme (step 6, Table 1) on a Superose 6 column. The elution was performed with phosphate buffer (20 mmol/liter, pH 6.8). The fraction volume was 0.5 ml (flow rate, 1.0 ml/min). All elution buffers contained 0.1% Triton X-100. The enzyme activity is in A_{410} units per milliliter per minute.

TABLE 1. Purification of the CTLP from *T. denticola* ATCC 35405

Step	Prepn or procedure	Vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Total activity (nmol/min)	Sp act[a] (nmol/min/mg)
1	Triton X-100 extract	44.0	4.0	176.0	3,498	19.9
2	Fractogel FPLC	24.0	0.36	8.64	2,726	315.5
3	Treatment with polymyxin B, ammonium sulfate precipitation (80%), and dialysis	4.2	0.77	3.23	1,050	324.7
4	Mono Q FPLC	8.8	0.065	0.55	1,145	2,098
5	SDS treatment, concentration, and two consecutive Superose 12 FPLC separations	8.4	0.045	0.38	764	2,007
6	Superose 6 FPLC	3.5	0.097	0.34	680	2,003

[a] Determined with SAAPFNA under the conditions described in Materials and Methods with HEPES (0.1 mol/liter, pH 7.5) containing NaCl (0.5 mol/liter). The substrate concentration was 0.2 mmol/liter.

PAGE and in FPLC (Fig. 1 and 2). The SDS gels of the enzyme which was not heat treated were also sectioned into 1-mm fractions, which were incubated in the presence of SAAPFNA.

Molecular mass and amino acid composition of CTLP. As shown in Fig. 2, the purification procedure resulted in an SDS-PAGE-pure protein with a molecular mass of about 95 kDa. This value was obtained with an enzyme that was not subjected to the customary heat treatment in the presence of SDS. The activity and the sole protein peak coincided. Treatment of the enzyme at 100°C under standard SDS-PAGE conditions resulted in the formation of molecular forms with average molecular masses of 76, 39, and 36 kDa (Fig. 2). This behavior of the enzyme was in principle similar to that observed by Uitto et al. (37), except that the reported values for the molecular masses differed to a certain extent from those observed here. The amino acid composition of the CTLP is shown in Table 2. The average molecular mass range of the SDS-PAGE-pure CTLP, based on amino acid analysis, was 95.009 to 95.185 kDa, the estimated minimum length of the peptide being 950 amino acid residues.

Substrate specificity. The CTLP could be conveniently assayed with SAAPFNA as the substrate. This peptide derivative was, however, not the best substrate discovered, since NSP was hydrolyzed far more rapidly, displaying higher affinity for the enzyme (as evidenced by a low K_m) and a high specificity constant (k_{cat}/K_m) (Table 3). In scissile peptides, Phe in the P_1 position was preferred over Ala, Val, and Leu. A prolyl residue at P_2 in the *p*NA derivatives accelerated the rate of hydrolysis. From these studies, it appeared that the purified CTLP could be active on natural (unmodified) oligopeptides as well. This was ascertained by incubating substance P and angiotensin I in the presence of the enzyme under the conditions shown in Table 3. Substance P was hydrolyzed at a high rate at Phe-8–Gly-9, indicating that nitration of Phe-8 did not alter this peptide's function as a substrate, while angiotensin I was converted (upon hydrolysis of Phe-8–His-9) to angiotensin II, also at a high rate. Fragments of angiotensin II and substance P were not further hydrolyzed by the purified enzyme but were broken down by washed whole cells (vide infra). The CTLP had no effect on bradykinin.

The following proteins were rapidly broken down by the purified enzyme into fragments that could be visualized by means of SDS-PAGE and FPLC on Superose 12: calf thymus histone (type II-AS), human plasma fibrinogen (fraction I), and bovine milk α-, β-, and κ-casein. Human immunoglobulin A (IgA) and IgG were also hydrolyzed, but at a lower rate. No enzymatic breakdown was observed by means of the above procedures with soybean trypsin inhibitor (type 1-S) and bovine insulin. Figure 3 shows an SDS-PAGE proof of the hydrolysis of fibrinogen, κ-casein, and histone, all of which produced several low-molecular-weight products during the 18-h reaction period. When κ-casein was used as the substrate, most of the products were too small to be revealed by the silver-staining procedure used, although this pattern was also ob-

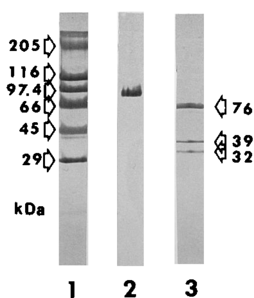

FIG. 2. SDS-PAGE of purified CTLP. Lanes: 1, molecular size markers (Sigma); 2, untreated purified enzyme (after step 6; Table 1); 3, purified enzyme after conventional heat treatment in the presence of SDS. Silver staining was used.

TABLE 2. Amino acid composition of the CTLP from *T. denticola* ATCC 35405[a]

Amino acid	No. of residues (nearest integer)
Asparagine/aspartic acid	90
Threonine	33
Serine	49
Glutamine/glutamic acid	62
Proline	289
Glycine	129
Alanine	88
Valine	32
Methionine	9
Leucine	33
Isoleucine	27
Tyrosine	21
Phenylalanine	27
Histidine	8
Lysine	25
Tryptophan	12
Arginine	16
Unknown	0
Total	950

[a] The hydrolysis of the enzyme (1.2 pmol) was carried out for 60 min at 200°C. Values were not corrected for possible loss of amino acids during hydrolysis. Values for half-cystine were not determined. Values for tryptophan were determined after hydrolyzing the enzyme for 22.5 h at 110°C in mercaptoethanesulfonic acid (3 mol/liter).

TABLE 3. Kinetic constants of the hydrolysis of *p*NA derivatives, NSP, and angiotensin I by the CTLP from *T. denticola* ATCC 35405

↓ Substrate[a] (P_5-P_4-P_3-P_2-P_1-P_1')	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Suc-Ala-Ala-Ala-*p*NA	1.95	0.013	6.7
Suc-Ala-Ala-Phe-*p*NA	2.42	0.21	86.8
Suc-Ala-Ala-Val-*p*NA			NR[b]
Suc-Ala-Ala-Pro-Ala-*p*NA	5.47	4.81	879.3
Suc-Ala-Ala-Pro-Leu-*p*NA	8.47	10.04	1,185.4
SAAPFNA (Suc-Ala-Ala-Pro-Phe-*p*NA)	3.35	34.88	10,411.9
Suc-Ala-Ala-Pro-Val-*p*NA			NR
Gly-Phe-*p*NA			NR
NSP (-Gln-Gln-Phe-Phe(NO_2)-Gly-)	0.22	10.57	48,054.4
Angiotensin I (-Pro-Phe-His-Leu)	0.51	63.3	123,633

[a] Each scissile substrate was hydrolyzed at only one bond (arrow). The enzyme reactions were performed in HEPES (0.1 mol/liter, pH 7.5) containing NaCl (0.5 mol/liter) and 10% DMSO at 30°C. The enzyme concentration was 4.3 to 43 nmol/liter. For NSP, only the sequence for Gln-5 through Gly-9 is shown. For angiotensin I, the sequence from Pro-7 to Leu-10 is shown.

[b] NR, no reaction.

served with other hydrolyzable protein substrates. The pattern of proteolysis is further illustrated in Fig. 4, with fibrinogen, κ-casein, and gelatin, whose hydrolysis products were subjected to FPLC. Most of the end products from these reactions had a molecular mass of <12 kDa. These results showed that the CTLP is a relatively powerful and versatile proteinase. Although the insulin molecule was not hydrolyzed at a detectable rate, insulin B chain was rapidly broken down primarily at Leu-15–Tyr-16, while Tyr-16–Leu-17 and Phe-24–Phe-25 were good secondary sites of hydrolysis during the 18-h reaction period.

Other properties of CTLP. The purified enzyme hydrolyzed SAAPFNA optimally at pH 7.8 (tested in Tris [50 mmol/liter] and phosphate [20 mmol/liter] buffers). Addition of Ca^{2+} (1.0 mmol/liter) had no effect on the rate of hydrolysis. Metal-chelating agents [EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA)] had no effect on the catalysis when purified CTLP was used (with whole cells, EGTA inhibited the reaction in phosphate buffer in the absence of Ca^{2+}; also, crude CTLP preparations were inhibited by EGTA). 2-Mercaptoethanol was also without effect. The enzyme was strongly inhibited by the Bowman-Birk soybean trypsin-chymotrypsin inhibitor (Sigma): a 50-μg/ml concentration of the inhibitor caused 71% inactivation of the enzyme

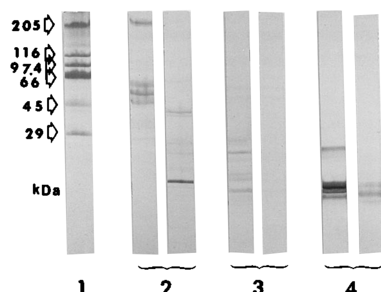

FIG. 3. Hydrolysis of proteins by the CTLP demonstrated by SDS-PAGE. Lanes: 1, molecular size markers (Sigma); 2, human plasma fibrinogen (before and after 18 h of incubation at 30°C); 3, bovine milk κ-casein (before and after incubation); 4, calf thymus histone (before and after incubation). Silver staining was used.

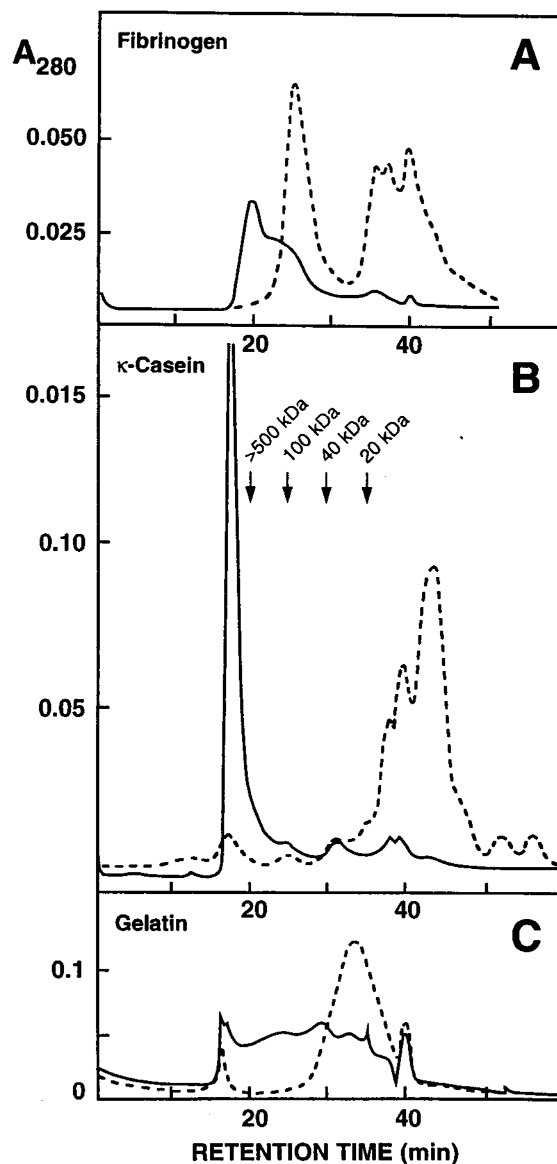

FIG. 4. Hydrolysis of proteins by the CTLP. FPLC was done on a gel filtration column (Superose 12) of human plasma fibrinogen (A), bovine milk κ-casein (B), and gelatin (C) before (——) and after (– – –) 18 h of incubation with the enzyme at 30°C. The elution was performed with phosphate buffer (20 mmol/liter, pH 8.0) containing EDTA (0.1 mmol/liter) and NaCl (0.25 mol/liter). The proteins were monitored at 280 nm. The flow rate was 0.5 ml/min. Panel B shows the approximate retention times of molecular size markers (Sigma).

(with 0.8 μg of enzyme and SAAPFNA as the substrate under standard assay conditions). This inhibition was also observed with whole cells. *p*-Hydroxymercuribenzoic acid was only slightly inhibitory. This effect was considered to be unspecific and not indicating the involvement of an active sulfhydryl group in enzyme activity.

For the classification of the proteinase, extensive chemical modification experiments were carried out and are summarized as follows. The experiments focused on the study of the catalytic triad Ser-His-COOH, known to be present in CTLPs. The enzyme was potently (99%) inhibited by DFP (0.1 mmol/liter) in Tris (50 mmol/liter) with EDTA (0.1 mmol/liter) and NaCl (0.25 mol/liter) (pH 7.5). Because chymotrypsin activity depends on an active imidazole residue, the effect of DEP on

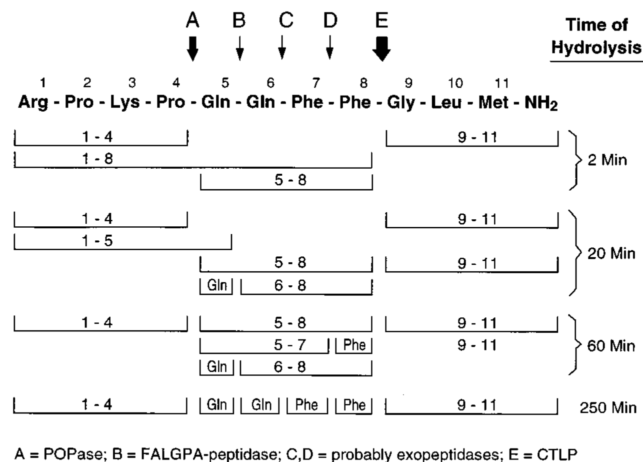

FIG. 5. Time course of the hydrolysis (inactivation) of substance P by 5×10^8 whole cells of *T. denticola* ATCC 35405 in phosphate buffer (20 mmol/liter, pH 6.8), with an initial peptide concentration of 0.2 mmol/liter (30°C). The sizes of the arrows reflect the preference for the site of hydrolysis.

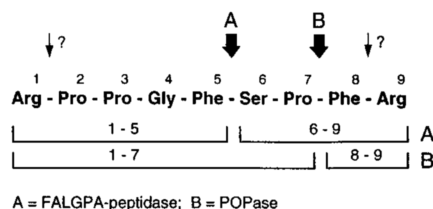

FIG. 6. Hydrolysis (inactivation) of bradykinin by 5×10^8 whole cells of *T. denticola* ATCC 35405 in MES (40 mmol/liter, pH 6.5), with an initial peptide concentration of 0.1 mmol/liter (30°C). The cell-associated enzymes responsible for the catalyses are indicated. In addition to the bradykinin fragments shown, free arginine (liberated from either terminus or both termini) and fragments 5 to 7 and/or 6 to 8 were also present after the 3-h reaction time. The reactions were confirmed with purified enzymes.

enzyme activity was studied in phosphate buffer (0.1 mol/liter). The enzyme was inhibited in a time-, dose-, and pH-dependent manner by DEP. Thus, the activity of the enzyme was effectively lost at pH 6.0 and was potently restored by 80% by a relatively mild treatment with NH_2OH (20 mmol/liter). Both the inactivation at pH 6.0 and the low concentration of NH_2OH needed for a high degree of reactivation are considered specific for an active imidazole group (a seryl residue can also be modified in chymotrypsin by DEP at low pH, but the reaction cannot be reversed with NH_2OH).

The enzyme was also potently inactivated by EEDQ in a dose-, time-, and pH-dependent manner. When the modifications were carried out at pHs 6.0, 5.5, and 5.0 in phosphate buffer (0.1 mol/liter), it appeared that the lower the pH of the modification mixture, the stronger the inactivation of the enzyme. For example, EEDQ at 2.0 mmol/liter caused a 70% inactivation in 60 min at pH 6.0. Because EEDQ reacts with the protonated form of a carboxyl group and because these experiments were carried out under conditions that are especially sensitive to COOH groups, it is conceivable that the activity of CTLP depends on such an active group. The chemical modification results collectively suggest that the activity of the *T. denticola* ATCC 35405 CTLP depends on an active seryl residue, on an active imidazole group, and on an active carboxyl group.

Experiments with whole cells. It was first established that 300 min of incubation of washed cells at 37°C did not release any peptides. The washed whole cells caused rapid hydrolysis of SAAPFNA, liberating the nitrophenolate ion. The pH optimum of this hydrolysis was studied in Tris (50 mmol/liter) and phosphate (20 mmol/liter) buffers and found to be 7.8. NSP was, however, hydrolyzed four times faster than SAAPFNA (both substrates were tested in HEPES [0.1 mol/liter, pH 7.5] containing NaCl [0.5 mol/liter] and with a substrate concentration of 0.2 mmol/liter). This suggests that the whole cells exhibited high affinity for NSP, prompting a more detailed study of the hydrolysis of substance P and other human bioactive peptides by washed whole cells. Figure 5 shows that substance P was primarily attacked at the Phe-8–Gly-9 bond by an enzyme that was subsequently determined to be the CTLP described above. The cleavage of this bond terminates the biological activity of substance P. Substance P was also hydrolyzed, at a somewhat lower rate, at Pro-4–Gln-5 by the endo-acting POPase described earlier (24). During the course of the reaction, other peptide bonds were also hydrolyzed. After 250 min, the only detectable end products of hydrolysis were substance P fragments 1 to 4 and 9 to 11 and free glutamine and phenylalanine. The accumulation of substance P fragments 1 to 4 and 9 to 11 in the reaction mixtures was striking and somewhat unexpected. Overall, the hydrolysis of substance P took place in two major differentiable steps. The first involved the present CTLP, the POPase, and the FALGPA peptidase (23), which cleaved the Phe-8–Gly-9, the Pro-4–Gln-5, and the Gln-5–Gln-6 bonds, respectively. At a later stage, the Gln-6–Phe-7 bond was hydrolyzed. The exact timing of the hydrolysis of Phe-7–Phe-8 and the nature of the catalysis could not be determined. It is possible that Phe-8 was liberated as a result of a carboxypeptidase reaction from substance P fragments 5 to 8 or by another exopeptidase.

Washed whole cells were also active on bradykinin, the primary sites of hydrolysis being Pro-7–Phe-8, occasioned by the POPase (Fig. 6), and Phe-5–Ser-6, catalyzed by the FALGPA peptidase. After very long incubation at 30°C, only fragments 1 to 5 and 6 to 8 (or 5 to 7) were present as intact peptides, in addition to free arginine. At this stage, it was not determined from which end the arginine was derived. Also, since bradykinin fragments 5 to 7 and 6 to 8 both contain the same amino acids (Phe, Ser, and Pro), future research should elucidate the question of which of the two fragments is the dominant end product or if both are. In summary, whole cells of the organism rapidly inactivated bradykinin under the conditions described in the legend to Fig. 6.

The conversion of angiotensin I to angiotensin II and the latter's further breakdown by whole cells are shown in Fig. 7. The most prominent reaction was the hydrolysis of the Phe-His bond, leading to the formation of angiotensin II. This hydrolysis was catalyzed by CTLP. Other peptide bonds were subsequently hydrolyzed. CTLP also hydrolyzed the Tyr-Ile bond at a lower rate. These experiments were repeated with purified CTLP and established the breakdown of Phe-His and Tyr-Ile by CTLP.

Effect of CH on CTLP. Chlorhexidine (CH) is a cationic detergent with marked antibacterial and antiplaque activity, also exerting bacteriostatic effects. Although it has been assumed that CH exerts its effect through disruption of cell membranes, other, more specific mechanisms may be involved. The following experiments with CTLP may shed light on this question. Both CH acetate and CH gluconate activated the hydrolysis of SAAPFNA in a similar way; the results shown below were obtained with CH acetate. The hydrolysis of SAAPFNA was affected by CH in the same way regardless of

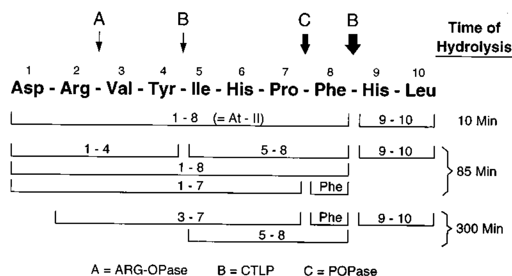

FIG. 7. Conversion of angiotensin I to angiotensin II (At-II) and further degradation of angiotensin II by whole cells of *T. denticola* ATCC 35405 in MES (0.1 mol/liter, pH 6.5), at an initial peptide concentration of 0.1 mmol/liter (30°C) and with 5×10^8 cells. The cell-associated enzymes responsible for the catalyses are indicated. The reaction times were 10 min (by which time virtually all angiotensin I was converted into angiotensin II), 85 min, and 300 min (by which time the other reactions indicated were dominant). After 300 min, 15% of the cells had been broken down (tested as described by Grenier [14]). The sizes of the arrows reflect the preference and velocity of hydrolysis. It is possible that some compositional amino acids (Asp, Arg, Val, and Tyr) may have been metabolized by the cells during the 300-min reaction, avoiding detection. Reactions A, B, and C were confirmed with purified enzymes. Arg-OPase refers to the trypsin-like oligopeptidase (most likely oligopeptidase B; EC 3.4.21.83) described previously (22, 29). POPase was described recently (24).

whether purified CTLP or washed whole cells were used. The degree of activation depended on the buffer used: the activation was more pronounced in Tris and MES (morpholineethanesulfonic acid) than in HEPES. The activation versus the CH concentration curve was not linear at higher CH levels (Fig. 8). Control experiments showed that it was the enzyme activity that was accelerated by CH in the whole-cell system and that the effect did not result from CH's effect on the absorption of *p*NA or from other nonenzymatic reactions. Tests with purified CTLP showed that CH had no effect on the values of K_m in the hydrolysis of SAAPFNA but did have a strong effect on V_{max}. CH also reversed the chelation-based enzyme inhibition caused by EGTA in the presence of whole cells.

The hydrolysis of NSP was, instead, slightly inhibited by CH, tested with both purified CTLP and washed whole cells (not shown). Consequently, the effect of CH clearly depended on the structure of the substrate used. CH also inhibited the

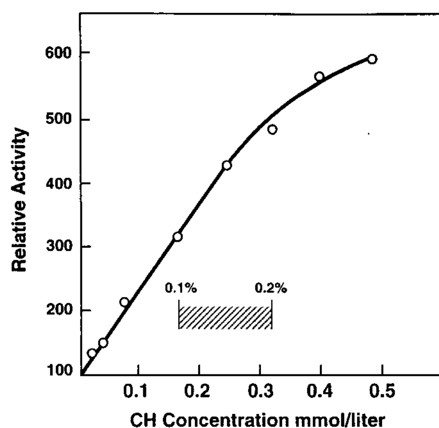

FIG. 8. Time course of CH-caused activation of the hydrolysis of SAAPFNA by whole cells (2.5×10^8) in MES (0.08 mol/liter), with an initial substrate concentration of 0.16 mmol/liter. The reaction time was 15 min at 30°C. The hatched bar shows the concentration range of CH frequently used in clinical practice. A value of 100 was given to the velocity obtained without CH addition.

conversion of angiotensin I to angiotensin II by purified CTLP (not shown).

DISCUSSION

Based on enzyme studies of the past several years, the outer cell surface of the organism *T. denticola* is now emerging as a structure that figuratively can be compared with a workbench equipped with a powerful and versatile proteolytic and peptidolytic armamentarium. The organism can be both highly motile and adherent, and its outer surface thus serves as a type of matrix for immobilized hydrolases that have the ability to process host proteins and peptides for the advantage of the pathogen as it moves and propagates within its biological niche.

The present paper and previous research (23, 24) indicate that whole cells of *T. denticola* as well as individual cell surface peptidases and proteinases can accomplish the breakdown and inactivation of not only several human bioactive peptides but also different types of protein molecules present in human tissues. Considering the overall large number of peptidases obviously involved, the ability of the cells to inactivate angiotensin I, angiotensin II, bradykinin, substance P, etc., as well is not surprising. Future research will perhaps elucidate whether the inactivation of such peptides is a coincidental phenomenon resulting from the mutual similarity between these peptides and the true in vivo substrates or whether these reactions indeed directly contribute to pathogenicity. Several of the peptide hydrolyses observed are characterized, however, by affinity constants that are too low (indicating high affinity) and by specificity constants that are too high (indicating high specificity) (Table 3) (24) to be categorically ignored. At minimum, the bioactive peptides studied in this and previous reports (23, 24) should display close similarity to the true in vivo substrates (23, 24). Uitto et al. (37) and Grenier et al. (15) showed that the present CTLP hydrolyzes fibrinogen, transferrin, α_1-antitrypsin, IgA, IgG, gelatin, serum albumin, laminin, and certain other proteins but not collagen. The present study confirmed this in the case of fibrinogen and gelatin and additionally showed that thymus histone and milk caseins are suitable substrates. No information on the suitability of salivary proteins as substrates is yet available.

Two research groups initially reported on CTLP activity in cells of *T. denticola*. One enzyme was partially purified from strain ATCC 35405 (37) and was suggested to be attached to the outside of the cell envelope (15). Another CTLP from the same strain was isolated after cloning of the enzyme gene in *Escherichia coli* (1, 31). The molecular mass of the latter enzyme was reported to be ca. 76 kDa, while the former CTLP had a mass of about 95 kDa (37). From the reported enzyme characteristics, it appears that the present CTLP, purified by conventional FPLC techniques, is identical to the enzyme described previously (15, 37) but differs from the enzymes studied by Kuramitsu's group (1, 31). The present and previous studies (15, 37) thus progressed to the same result by different methods. All CTLPs can be conveniently assayed with SAAPFNA as the substrate. A cell-bound CTLP activity was also described in the cells of *T. denticola* ATCC 33520, but the characteristics so far reported for this enzyme do not allow it to be identified as one of the above CTLPs (26).

It appears that the CTLP of *T. denticola* ATCC 35405 is a true proteinase, with specificity and inhibition characteristics similar to those of chymotrypsin. The practice of naming this enzyme chymotrypsin-like should, however, be reconsidered if the molecule turns out to display insignificant homology with the true chymotrypsin molecule, which has a mass of ca. 23.8

kDa. The customary reference to a trypsin-like enzyme when the proteinase hydrolyzes some synthetic trypsin substrates was recently discussed (22); the case described exemplifies a situation for which reservation should be used until complete homology data become available. The high content of proline observed in CTLP also draws attention. Because the enzyme was found to be homogeneous in SDS-PAGE and FPLC, it is possible that a proline-rich domain is an integral part of the SDS-PAGE-pure CTLP molecule. Proline-rich regions in proteins are relatively common.

The catalytic similarity of the present CTLP and bovine pancreatic chymotrypsin was, however, evidenced by the chemical modification studies and by experiments on insulin B chain. The activity of CTLP is most likely based on active seryl, carboxyl, and histidyl groups, which are also involved in the activity of chymotrypsin. The functional similarity between the present CTLP and chymotrypsin was indeed evidenced by several substrate specificity features: both enzymes fail to cleave bradykinin, most likely because Pro in positions P_1 and P_3 causes a discrimination of the amidolytic power of chymotrypsin (11). The CTLP showed a preference for Phe over Ala, Val, or Leu at position P_1 when a series of succinyl-peptide-*p*NAs were tested. With Val in position P_1, the CTLP showed no hydrolysis; the values of the specificity constants (Table 3) decreased in the order of Phe > Leu > Ala > Val. Pro in position P_2 greatly enhanced the hydrolysis of those substrates. Elastase prefers Val in position P_1, while cathepsin G, a homolog of chymotrypsin, prefers Phe in this position, suggesting that the present CTLP differs from elastase but resembles cathepsin G catalytically (25, 28, 36). The CTLP also resembles another chymotrypsin-like enzyme, the neutrophil CTLP, since both enzymes (as well as cathepsin G) catalyze the hydrolysis in insulin B chain primarily at Leu-15–Tyr-16, other common cleavage sites being Tyr-16–Leu-17 and Phe-24–Phe-25 (4, 18). It is thus likely that the present peptidase and chymotrypsin have several common structural and catalytic features.

Although the effect of CH against the subgingival spirochetes has been reported to be only moderate or weak and often of short duration (discussed by Fiehn [10]), CH has still been claimed to be the most effective antiplaque and antigingivitis agent known today (17) and also potently affects mutans streptococci and human caries (8). CH inhibits certain proteolytic enzymes (2, 13, 22, 32), but CH is not a common, specific enzyme inhibitor that categorically reacts with selected active-site groups. Several enzyme reactions, such as the CTLP-catalyzed hydrolysis of SAAPFNA by whole cells, can indeed be strongly activated by CH. The reason for such remarkably diverse effects on enzymes that hydrolyze chemically similar peptide bonds may not be found solely in the structure of the enzymes' active sites but rather in the overall protein structure and the protein's electric charge distribution. Consequently, some studies may have ignored the action of CH as a typical membrane-active, detergent-like antibacterial agent. The effect of CH on proteolytic enzymes may physicochemically be explained in terms of CH's specific interaction with the enzyme protein, depending on the isoelectric point of the latter, as reported 25 years ago for certain related membrane-active agents (20, 21). The membrane-destabilizing property of CH was recently reemphasized (3). It is also remarkable that Ca^{2+} and CH have similar effects on several enzyme reactions. This was shown with whole cells, so that when the CTLP-catalyzed hydrolysis of SAAPFNA was inhibited by EGTA followed by addition of Ca^{2+} (which reactivated the enzyme), further addition of CH had no additional activating effect. However, if Ca^{2+} was not added, CH activated the enzyme as described above. The effect of CH on enzyme activities is, however, a complex reaction that depends on the concentration of divalent cations present, the buffer used, and the state of purity of the enzyme.

In spite of the versatile overall peptidolytic capacity of the outer cell envelope, it is remarkable that certain peptide fragments derived from substance P, angiotensin, and bradykinin were not hydrolyzed further to form the compositional amino acids. It is possible that such peptides may get transported, however, and utilized by the pathogen's other peptidases located intracellularly or perhaps by those located in the periplasmic space. Two recent studies suggest that the cells of *T. denticola* can initially exploit short peptides as a source of energy, thus avoiding complete hydrolysis before transport (35, 38).

It is possible that the inactivation of bioactive peptides by *T. denticola* has significant pathophysiologic consequences. Peptide-based regulatory systems have increasingly been implicated in inflammatory processes via their actions on vascular functions (38). For example, peptides released by sensory nerves (substance P) or generated by the endothelium (angiotensin II) or by damaged tissue (bradykinin) regulate several biological phenomena, such as vascular tone, permeability, and proliferation (38). In a healthy tissue, the activities of regulatory peptides are modulated by membrane peptidases, i.e., the activities of these peptides are under tight control in vivo (38). Interference with this control by bacterial peptidases and proteinases that are reminiscent of the host's own enzymes could contribute to the development of an inflammatory process.

In conclusion, whole cells of *T. denticola* were capable of inactivating substance P, bradykinin, and angiotensin II (and of catalyzing the latter's formation from angiotensin I), based on the reported potency of fragments of these peptides (5–7, 9, 12, 16, 19, 33). The purified CTLP alone had no effect on bradykinin but inactivated substance P rapidly, converted angiotensin I to angiotensin II, and finally inactivated angiotensin II by slowly breaking it down into two tetrapeptides. Future research may reveal the significance of these peptidolytic reactions in the etiology of periodontal disease. At minimum, the described ready hydrolysis of human bioactive peptides by *T. denticola* exemplifies the potent peptidolytic capacity of the spirochete.

REFERENCES

1. **Arakawa, S., and H. K. Kuramitsu.** 1994. Cloning and sequence analysis of a chymotrypsinlike protease from *Treponema denticola*. Infect. Immun. **62:** 3424–3433.
2. **Beighton, D., J. Decker, and K. A. Homer.** 1991. Effect of chlorhexidine on proteolytic and glycosidic enzyme activities of dental plaque bacteria. J. Clin. Periodontol. **18:**85–89.
3. **Berrettbee, K., L. Newboult, and S. Edwards.** 1994. The membrane destabilising action of the antibacterial agent chlorhexidine. FEMS Microbiol. Lett. **119:**249–253.
4. **Blow, A. M. J., and A. J. Barrett.** 1977. Action of human cathepsin G on the oxidized B chain of insulin. Biochem. J. **161:**17–19.
5. **Bury, R., and M. Mashford.** 1976. Biological activity of C-terminal partial sequences of substance P. J. Med. Chem. **19:**854–856.
6. **Bury, R. W., and M. L. Mashford.** 1975. Substance P: its pharmacology and physiologic roles. Aust. J. Exp. Biol. Med. Sci. **55:**671–735.
7. **Cascieri, M. A., and T. Liang.** 1983. Characterization of substance P receptor in rat brain cortex membranes and the inhibition of radioligand binding by guanine nucleotides. J. Biol. Chem. **258:**5158–5164.
8. **Emilson, C. G.** 1994. Potential efficacy of chlorhexidine against mutans streptococci and human caries. J. Dent. Res. **73:**682–691.
9. **Erdös, E. G.** 1979. Kininases, p. 427–487. *In* E. G. Erdös (ed.), Handbook of experimental pharmacology, vol. 25 (suppl.). Springer, Heidelberg.
10. **Fiehn, N.-E.** 1987. Susceptibility of small-sized oral spirochetes to eight antibiotics and chlorhexidine. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **95:**325–329.
11. **Fischer, G., H. Bang, E. Berger, and A. Schellenberger.** 1984. Conformational specificity of chymotrypsin toward proline-containing substrates. Biochim. Biophys. Acta **791:**87–97.
12. **Giannis, A., and T. Kilter.** 1993. Peptidomimetics for receptor ligands—discovery, development, and medical perspectives. Angew. Chem. Int. Ed. Engl. **32:**1244–1267.

13. **Grenier, D.** 1993. Reduction of proteolytic degradation by chlorhexidine. J. Dent. Res. **72:**630–633.
14. **Grenier, D.** 1994. Effect of proteolytic enzymes on the lysis and growth of oral bacteria. Oral Microbiol. Immunol. **9:**224–228.
15. **Grenier, D., V.-J. Uitto, and B. C. McBride.** 1990. Cellular location of a *Treponema denticola* chymotrypsinlike protease and importance of the protease in migration through the basement membrane. Infect. Immun. **58:**347–351.
16. **Hall, M. E., F. Miley, and J. M. Stewart.** 1989. The role of enzymatic processing in the biological actions of substance P. Peptides **10:**895–901.
17. **Lang, N., and M. C. Brecx.** 1986. Chlorhexidine digluconate—an agent for chemical plaque control and prevention of gingival inflammation. J. Periodont. Res. **21**(Suppl. 16)**:**74–89.
18. **Levy, H., and G. Feinstein.** 1979. The digestion of the oxidized B chain of insulin by human neutrophile protease: elastase and chymotrypsin-like protease. Biochim. Biophys. Acta **567:**35–42.
19. **Liang, T., and M. A. Cascieri.** 1981. Substance P receptor on parotid cell membranes. J. Neurosci. **1:**1133–1141.
20. **Mäkinen, K. K., R. Brummer, and A. Scheinin.** 1970. Selective effect of quaternary ammonium salts on the activity of arylaminopeptidases of the dental pulp, and on trypsin, chymotrypsin and leucine aminopeptidase. Acta Odontol. Scand. **28:**389–398.
21. **Mäkinen, K. K., and P.-L. Mäkinen.** 1970. Selective effect of domiphen bromide [dodecyldimethyl (2-phenoxyethyl) ammonium bromide] on some hydrolytic enzymes. Biochim. Biophys. Acta **206:**143–151.
22. **Mäkinen, K. K., P.-L. Mäkinen, W. J. Loesche, and S. A. Syed.** 1995. Purification and general properties of an oligopeptidase from *Treponema denticola* ATCC 35405—a human oral spirochete. Arch. Biochem. Biophys. **316:** 689–698.
23. **Mäkinen, K. K., P.-L. Mäkinen, and S. A. Syed.** 1992. Purification and substrate specificity of an endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35405, active on furylacryloyl-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267:**14285–14293.
24. **Mäkinen, P.-L., K. K. Mäkinen, and S. A. Syed.** 1994. An endo-acting proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. Infect. Immun. **62:**4938–4947.
25. **McRae, B., K. Nakajima, J. Travis, and C. Powers.** 1980. Studies on reactivity of human leukocyte elastase, cathepsin G, and porcine pancreatic elastase toward peptides including sequences related to the reactive site of α_1-protease inhibitor (α_1-antitrypsin). Biochemistry **19:**3973–3978.
26. **Mikx, F. H. M., F. Jacobs, and C. Satumalay.** 1992. Cell-bound peptidase activities of *Treponema denticola* ATCC 33520 in continuous culture. J. Gen. Microbiol. **138:**1837–1842.
27. **Miles, E. W.** 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol. **47:**431–442.
28. **Nakajima, K., J. C. Powers, B. M. Ashe, and M. Zimmerman.** 1979. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. J. Biol. Chem. **254:**4027–4032.
29. **Ohta, K., K. K. Mäkinen, and W. J. Loesche.** 1986. Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. **53:**213–220.
30. **Pougeois, R., M. Satre, and P. V. Vignais.** 1978. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a new inhibitor of the mitochondrial F_1-ATPase. Biochemistry **17:**3018–3023.
31. **Que, X.-C., and H. K. Kuramitsu.** 1990. Isolation and characterization of the *Treponema denticola prtA* gene coding for chymotrypsinlike protease activity and detection of a closely linked gene encoding PZ-PLGPA-hydrolyzing activity. Infect. Immun. **58:**4099–4105.
32. **Radford, J. R., K. A. Homer, M. N. Naylor, and D. Beighton.** 1992. Inhibition of human subgingival plaque protease activity by chlorhexidine. Arch. Oral Biol. **37:**245–248.
33. **Regoli, D., N.-E. Rhaleb, S. Dion, and G. Drapeau.** 1990. New selective bradykinin receptor antagonists and bradykinin B_2 receptor characterization. Trends Pharmacol. Sci. **11:**156–161.
34. **Saccomani, G., M. L. Bercellona, and G. Sachs.** 1981. Reactivity of gastric ($H^+ + K^+$)-ATPase to *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. J. Biol. Chem. **256:**12405–12410.
35. **Shah, H. N., S. E. Gharbia, and M. I. N. Zhang.** 1993. Measurement of electrical bioimpedance for studying utilization of amino acids and peptides by *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Treponema denticola*. Clin. Infect. Dis. **16**(Suppl. 4)**:**S404–S407.
36. **Tanaka, T., Y. Minematsu, C. Reilly, J. Travis, and J. C. Powers.** 1985. Human leukocyte cathepsin G: subsite mapping with 4-nitroanilides, chemical modification, and effect of possible cofactors. Biochemistry **24:**2040–2047.
37. **Uitto, V.-J., D. Grenier, E. C. S. Chan, and B. C. McBride.** 1988. Isolation of a chymotrypsinlike enzyme from *Treponema denticola*. Infect. Immun. **56:** 2717–2722.
38. **Walsh, D. A., J. Wharton, D. R. Blake, and J. M. Polak.** 1993. Species and tissue specificity of vasoactive regulatory peptides. Int. J. Tissue React. **15:**109–124.
39. **Wyss, C.** 1993. Aspartame as a source of essential phenylalanine for the growth of oral anaerobes. FEMS Microbiol. Lett. **108:**255–258.