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Purification and Characterization of an Enzyme Produced by *Treponema denticola* Capable of Hydrolyzing Synthetic Trypsin Substrates

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An enzyme from *Treponema denticola* that hydrolyzes a synthetic trypsin substrate, *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), was purified to near homogeneity, as judged by gel electrophoresis. The molecular weight of the enzyme was estimated to be ca. 69,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ca. 50,000 by gel filtration on Sephadex G-100. The pH optimum for the hydrolysis of BAPNA was around 8.5. The enzyme was heat labile and irreversibly inactivated at low pH values. Enzyme activity was enhanced by Ca^{2+}, Mg^{2+}, and Ba^{2+} but inhibited by Mn^{2+}, Hg^{2+}, Co^{2+}, and Zn^{2+}. Metal chelators and sulfhydryl reagents had no effect on this activity. The enzyme was inhibited by certain protease inhibitors such as diisopropyl fluorophosphate, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, phenylmethylsulfonyl fluoride, L-1-tosylamide-2-phenylethylchloromethyl ketone, α-1-antitrypsin, and soybean trypsin inhibitor. The K_m values for BAPNA and *N*-α-benzoyl-L-arginine ethyl ester were 0.05 and 0.12 mM, respectively, and the V_{max} values were higher than those observed with trypsin. Although the purified enzyme hydrolyzed some low-molecular-weight synthetic trypsin substrates, it did not hydrolyze casein, hemoglobin, azocasein, azocoll, bovine serum albumin, or gelatin. Thus, this enzyme is probably not a protease but is capable of hydrolyzing ester, amide, and peptide bonds involving the carboxyl group of arginine and lysine.

Observations by microscopy of subgingival plaques have demonstrated elevated numbers and proportions of spirochetes involved with various types of periodontal disease (19, 21, 22, 24, 28, 34). Spirochetes compose about 45% of the microscopic count in plaques removed from sites associated with adult periodontitis (28). In acute necrotizing ulcerative gingivitis, spirochetes compose about 30% of the microscopic count (26) and can be demonstrated to be invading the underlying connective tissue and margin of the lesion well in advance of other plaque bacteria (20). Spirochetal invasion of the gingival tissue has also been observed in periodontitis (7, 37). These observations, as well as clinical trials which show that successful treatments result in significant decreases in spirochetal proportions in plaques (23, 24, 27), implicate the spirochetes as periodontopathic organisms. More-detailed investigation of the role that spirochetes play in periodontal disease has been hampered by either the difficulty of growing many of these organisms or their inability to grow.

Treponema denticola is one of the cultivable spirochetes found in subgingival plaques associated with periodontal disease (24, 31). Laughon et al. (17) found that *T. denticola*, as well as *Bacteroides gingivalis*, has a trypsinlike enzyme which hydrolyzes *N*-α-benzoyl-DL-arginine-2-naphthylamide (BANA). Yoshimura et al. (43) have reported the partial purification and characterization of a trypsinlike enzyme from *B. gingivalis*, but the *T. denticola* enzyme has not been studied. The trypsinlike activity of *T. denticola* is not only useful for the identification of this organism from among the other cultivable oral spirochetes (S. A. Syed, unpublished data), but it may contribute to, or be associated with, periodontal pathology. Thus, Loesche et al. (W. J. Loesche, S. A. Syed, and J. Stoll, J. Dent. Res. vol. 64, A. A. D. R. abstract number 77) were able to significantly correlate the presence of this enzyme with the depth of the periodontal pocket, with clinical impressions of disease severity, and with plaque levels of spirochetes.

These considerations led us to isolate and purify the trypsinlike enzyme from *T. denticola* and to characterize its properties relative to trypsin. We report here that this enzyme is apparently not a protease, although its specificity against synthetic substrates is similar to that of trypsin in that it preferentially catalyzes the hydrolysis of ester, amide, and peptide bonds involving the carboxyl group of arginine or lysine.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *T. denticola* ASLM, which had been isolated from human subgingival plaque, was maintained and grown at 34°C in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in a broth medium which had the following composition (per liter): tryptone (Difco Laboratories, Detroit, Mich.), 10.0 g; veal heart infusion broth, 5.0 g; yeast extract, 10.0 g; gelatin, 10.0 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2HPO_4, 1.13 g; KH_2PO_4, 0.9 g; and NaCl, 1.0 g (TYGVS medium). The pH was adjusted to 7.2 with 4 N KOH before the autoclaving of the medium. The following ingredients were mixed, neutralized with KOH, sterilized by filtration, and added aseptically to the autoclaved medium containing (final concentration, per liter): glucose, 1.0 g; cysteine hydrochloride, 1.0 g; thiamine pyrophosphate, 0.0125 g; sodium pyruvate, 0.25 g; acetic acid, 0.27 ml; propionic acid, 0.10 ml; *n*-butyric acid, 0.064 ml; *n*-valeric acid, 0.016 ml; isobutyric acid, 0.016 ml; isovaleric acid, 0.016 ml; DL-methylbutyric acid, 0.016 ml; and heat-inactivated rabbit serum, 10% (vol/vol).

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Enzyme assay. The enzyme activity was measured during the course of purification by using *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as the substrate. The standard assay mixture contained 50 mM Tris hydrochloride buffer (pH 8.5), 20 mM $CaCl_2$, and 0.2 mM BAPNA in a final volume of 2 ml. The enzyme activity was measured at 37°C by recording the rate of the increase in the A_{410} (6) with a Gilford model 240 spectrophotometer equipped with a cell compartment containing a thermostat. One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline per min by use of the molar extinction coefficient of *p*-nitroaniline at 410 nm of 8,800 M^{-1} cm^{-1}. The hydrolysis of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide was assayed by the same method as BAPNA. The rate of hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester (BAEE) was determined by recording the change of the A_{255} in the same reaction mixture by using a molar absorption difference of 808 M^{-1} cm^{-1} (11). The hydrolysis of *N*-α-*p*-tosyl-L-arginine methyl ester and *N*-α-*p*-tosyl-L-lysine methyl ester was followed at an A_{247} (9). The hydrolysis of BANA and of various L-amino acid 2-naphthylamides was measured by the chromogenic method described by Makinen and Makinen (30), with the exception that 50 mM Tris hydrochloride buffer (pH 8.5) was used in the reaction mixtures.

The ability of the enzyme to degrade heat-denatured casein and urea-denatured hemoglobin was assayed by measuring the release of trichloroacetic acid-soluble substances from the substrates at an A_{280} as described by Laskowski (16). Azocasein hydrolysis was measured by the procedure described by Leighton et al. (18). Activity against azocoll was determined by measuring the A_{520} of the liberated dye in the filtered reaction mixture. The hydrolysis of bovine serum albumin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Yoshimura et al. (43). Gelatin hydrolysis activity was estimated by a plate diffusion procedure in which the enzyme to be assayed was placed into wells (diameter, 4 mm) cut into a 1% gelatin–1% agarose plate. After incubation for 24 to 72 h at 37°C, active enzyme preparations were identified as clear zones that developed after an overlaying of a saturated ammonium sulfate solution on the plates. All chemicals used as enzyme substrates were obtained from Sigma Chemical Co., St. Louis, Mo., except for casein (Nutritional Biochemicals Corp., Cleveland, Ohio) and gelatin (Difco).

Purification of the enzyme. The following experiments were conducted at 4°C, except where noted.

(i) Cell extraction. Approximately 15 g (wet weight) of cells was obtained by the centrifugation of 5 liters of an early-stationary-phase culture at 13,000 × *g* for 15 min. The cells were washed three times with phosphate-buffered saline (pH 7.2; 0.15 M NaCl, 10 mM potassium phosphate) and suspended in 100 ml of 10 mM Tris hydrochloride buffer (pH 7.4) containing 0.1 mM dithiothreitol ([DTT]; Tris-DTT buffer).

The cells were disrupted by sonic oscillation (100 W) for a total of 5 min at 1-min intervals with a Branson sonifier (model W185D, Branson Sonic Power Co., Danbury, Conn.). The resulting suspension was centrifuged at 13,000 × *g* for 15 min, and the supernatant was recentrifuged at 104,000 × *g* for 60 min to remove the cell envelope fractions. The final supernatant was used for the purification of the enzyme.

(ii) Ammonium sulfate precipitation. Solid ammonium sulfate was added slowly to the crude enzyme extract with stirring to yield 60% saturation. After standing for 2 h, this suspension was centrifuged at 13,000 × *g* for 20 min. More ammonium sulfate was added to the supernatant to yield 80% saturation, and this preparation was kept standing overnight. After centrifugation at 13,000 × *g* for 20 min, the resulting precipitate was washed with saturated ammonium sulfate three times, dissolved in a small amount of the Tris-DTT buffer, and dialyzed for 24 h against the same buffer with several changes of buffer.

(iii) Bio-Gel A-5m gel filtration. The dialyzed preparation was applied on a Bio-Gel (Bio-Rad Laboratories, Richmond, Calif.) A-5m column (2.5 by 98 cm) that had been equilibrated with the Tris-DTT buffer. The column was eluted with the same buffer at a flow rate of 10 ml/h, and 4.5-ml fractions were collected.

(iv) DEAE–Bio-Gel A chromatography. The active fractions obtained from the Bio-Gel A-5m gel filtration were pooled and applied to a DEAE (Bio-Rad)–Bio-Gel A column (1.6 by 20 cm) that was previously equilibrated with the Tris-DTT buffer. The column was washed with several volumes of the equilibrating buffer, and the enzyme was eluted with a 200-ml linear gradient of 0 to 0.4 M NaCl in the same buffer at a flow rate of 10 ml/h. The active fractions were concentrated by ultrafiltration in a pressure cell (Amicon Corp., Lexington Mass.) equipped with a Diaflo PM-10 membrane and were dialyzed against the Tris-DTT buffer.

(v) Sephadex G-100 gel filtration. The concentrated preparation was applied on a column (1.6 by 59 cm) of Sephadex G-100 Superfine (Pharmacia, Uppsala, Sweden) equilibrated with the Tris-DTT buffer, and the column was eluted with the same buffer at a flow rate of 4 ml/h.

(vi) Fast-protein liquid chromatography. Fast-protein liquid chromatography (FPLC) was carried out at 22°C by use of the Pharmacia FPLC system. The enzyme from the Sephadex G-100 column was dialyzed against 1,000 volumes of 10 mM Tris hydrochloride buffer (pH 7.0) and concentrated by ultrafiltration. The sample was applied to a Mono Q HR5/5 prepacked anion-exchange column (Pharmacia) and eluted by a linear gradient of NaCl (0 to 0.1 or 0.2 M) in the buffer at a flow rate of 1 ml/min (1-ml fractions were collected). The active fractions were dialyzed against 1,000 volumes of 10 mM Tris hydrochloride buffer (pH 7.0) and rechromatographed on the Mono Q HR5/5 prepacked anion-exchange column. The conditions for the elution were the same as for the first separation.

Protein assay. The protein concentrations were measured by the method of Lowry et al. (29), with bovine serum albumin as the standard. The protein concentration of the final preparation was determined by measuring the A_{214}, with bovine serum albumin as the standard.

PAGE and molecular weight determination. Disc gel polyacrylamide electrophoresis was performed under nondenaturing conditions with the running buffer (pH 9.5) and the 7% gel system described by Davis (5). The enzyme activity on BANA was revealed in the gel by the method described by Muller-Estel and Fritz (32). Immediately after electrophoresis, the gel was incubated with BANA (2.8 mM in 50 mM Tris hydrochloride buffer containing 20 mM $CaCl_2$ [pH 8.5]) for 10 to 20 min at room temperature. The gel was transferred to a freshly prepared 0.1% Fast Garnet GBC salt (Sigma) solution made in the above buffer and kept in this solution until an orange band appeared. The gel was then removed and placed in 7% acetic acid to stop the reaction and to remove the background stain. The enzyme activity on BAPNA was also demonstrated in the gel. After incubation of the gel with BAPNA (2 mM in 50 mM Tris hydrochloride

TABLE 1. Purification of the BAPNA-hydrolyzing enzyme from *T. denticola*

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	96.9	451	0.215	1.0	100
Ammonium sulfate precipitation	59.2	83.2	0.712	3.3	61
Bio-Gel A-5m gel filtration	45.9	10.3	4.46	20.7	47
DEAE–Bio-Gel A chromatography	37.7	4.75	7.94	36.2	39
Sephadex G-100 gel filtration	25.8	1.26	20.5	95.3	26
Mono Q HR5/5 prepacked ion-exchange chromatography[a]	4.7	0.07	67.2	313.0	4.8

[a] FPLC system.

containing 20 mM $CaCl_2$ [pH 8.5]) for 5 to 10 min, the gel was placed in 7% acetic acid and immediately scanned for a band with A_{410}.

SDS-PAGE was carried out by the method of Laemmli (15), with a 12% separation gel and a 4% stacking gel. Samples were dissolved in the sample buffer containing 2% SDS and 5% 2-mercaptoethanol and were heated at 100°C for 3 min. A mixture of seven protein standards (SDS molecular weight marker, Dalton Mark VII-L; Sigma) was electrophoresed in parallel. Gels were stained for protein with Coomassie brilliant blue R-250. Reagents for gel electrophoresis were obtained from Bio-Rad Laboratories.

The molecular weight of the enzyme by gel filtration on a Sephadex G-100 Superfine column was estimated by the method of Andrews (1). The standard proteins used (molecular weight) were: bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400) (Sigma). Blue dextran (Pharmacia) was used to determine the void volume. The molecular weight of the enzyme was also determined by FPLC on a Superose 12 HR10/30 gel column (Pharmacia).

Effect of metal cations and various protease inhibitors. The purified enzyme was preincubated for 15 min at 37°C in 1 ml of a mixture of 100 mM Tris hydrochloride buffer (pH 8.5) and various metal chlorides and protease inhibitors at the indicated concentration. The substrate was then added, and the enzyme activity upon hydrolysis of BAPNA was measured as described above. For these experiments, the purified enzyme was dialyzed against 10 mM Tris hydrochloride buffer (pH 7.4). The various protease inhibitors were purchased from Sigma.

Determination of kinetic parameters. The K_m and V_{max} values of the purified enzyme from *T. denticola* and trypsin (Type III-S; Sigma) for BAPNA and BAEE were determined by double-reciprocal plots under the same experimental conditions as described above. The V_{max} was estimated from the reaction velocity at infinite substrate concentration and was expressed as micromoles of hydrolysis product per minute per milligram of protein. All experiments were repeated at least three times.

RESULTS

A *T. denticola* enzyme that catalyzed the hydrolysis of BAPNA was found in the supernatant obtained after ultracentrifugation of the sonicated cell preparation. The enzyme activity in the crude enzyme extract accounted for about 93% of the total activity of the washed cell suspension. No activity was detected in the spent culture fluid obtained

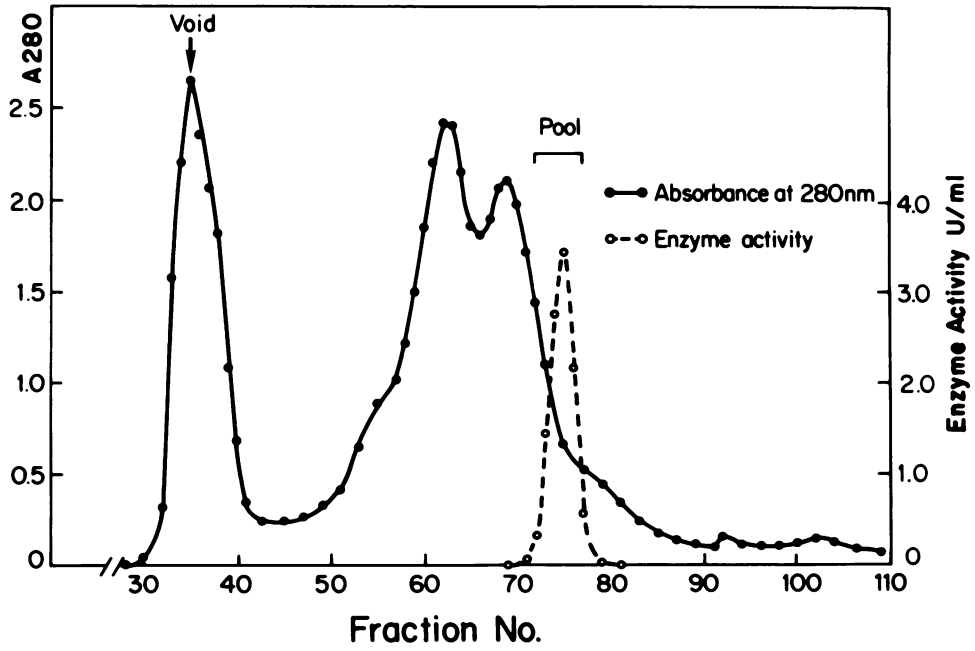

FIG. 1. Bio-Gel A-5m gel filtration chromatography of the BAPNA-hydrolyzing enzyme from *T. denticola* (step iii). The enzyme from the ammonium sulfate precipitation step was applied to the column and eluted as described in the text. The fractions (4.5 ml) were measured for protein at an A_{280}. The enzyme activities were assayed in the standard reaction mixture as described in the text. Fractions 72 to 77 were pooled for further purification.

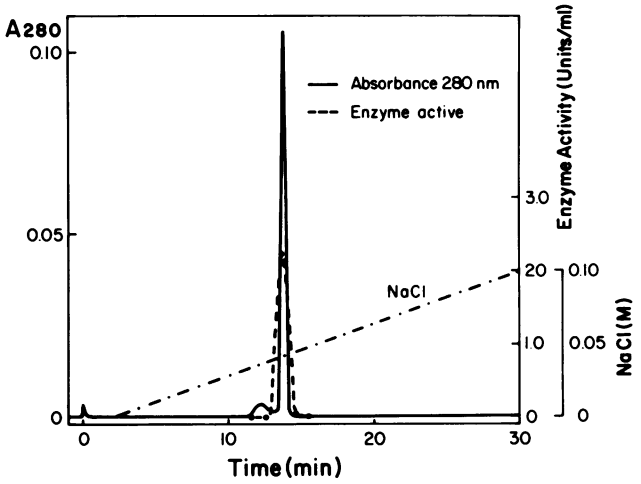

FIG. 2. Elution profile of the *T. denticola* enzyme from the second FPLC separation on the Mono Q HR5/5 prepacked ion-exchange column (step vi). ——, A_{280}; – – –, enzyme activity; – · – · –, NaCl gradient.

from either log- or stationary-phase cultures. These results indicate that the BAPNA-hydrolyzing enzyme was located intracellularly.

A summary of the purification of the enzyme from the crude extract is shown in Table 1. The enzyme was precipitated from the crude extract at between 60 and 80% ammonium sulfate saturation. Gel filtration on Bio-Gel A-5m separated most of the higher-molecular-weight proteins, and the enzyme was eluted as a single symmetrical peak (Fig. 1). When the pooled enzyme fractions were applied to a DEAE–Bio-Gel A column, the enzyme was eluted as a single peak at about 0.1 M NaCl. The enzyme was rechromatographed on Sephadex G-100 and further purified by two consecutive runs on the Mono Q HR5/5 prepacked ion-exchange column with the FPLC system. A typical elution profile from the second run, in which a single protein peak coinciding with the enzyme activity was obtained, is shown in Fig. 2. The enzyme was purified about 310-fold.

Electrophoresis of the purified enzyme on polyacrylamide gels under nondenaturing conditions yielded a single band which could be demonstrated either by protein staining with Coomassie blue or by active-enzyme staining with either BANA (Fig. 3) or BAPNA (data not shown). These bands migrated over the same distance in the gel. The purified enzyme yielded a single band upon SDS-PAGE corresponding to a molecular weight of approximately 69,000, which coincided with the enzyme activity. Several faint bands of lower molecular weight could also be observed; these accounted for about 1 to 2% of the total protein present (Fig. 4). The molecular weight of the purified enzyme was estimated to be about 50,000 by gel filtration on a Sephadex G-100 Superfine column and was estimated to be about 40,000 by FPLC on the Superose 12 HR10/30 gel column.

The purified enzyme (15 mg/ml) could be stored at −20°C for at least 1 month and at 4°C for several days in 10 mM Tris-DTT buffer (pH 7.4) without detectable loss of enzyme activity. Repeated freezing and thawing did not affect the enzyme activity. The enzyme obtained after gel filtration on Sephadex G-100 was diluted with various buffers (pH 4, 5, 6, 7, 8, and 10) and assayed for BAPNA-hydrolyzing activity in the standard assay mixture after storage at 4°C for 18 h. The enzyme was stable in the pH range from 6 to 10 but was

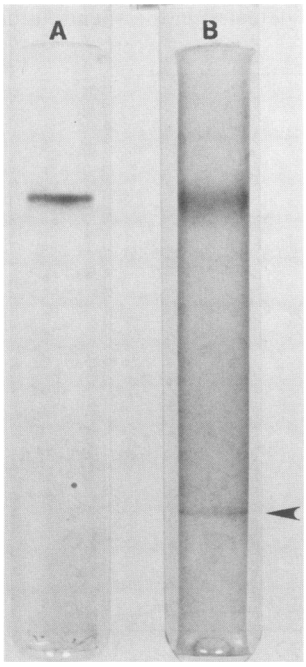

FIG. 3. Disc gel polyacrylamide electrophoresis of the purified enzyme from *T. denticola* under nondenaturing conditions. (A) Staining for protein with Coomassie brilliant blue. (B) Staining for protein with 0.1% Fast Garnet GBC salt solution after incubation of the gel with BANA, as described in the text. The arrow indicates the position of bromophenol blue tracking dye.

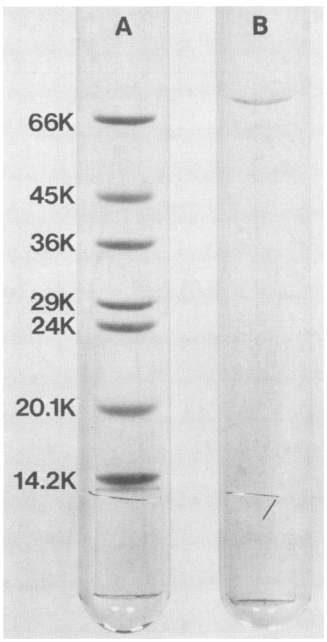

FIG. 4. SDS-PAGE of the purified enzyme from *T. denticola* (B) compared with the following marker proteins of known molecular weight (A): bovine serum albumin (66K), 66,000; ovalbumin (45K), 45,000; glyceraldehyde-3-phosphate dehydrogenase (36K), 36,000; carbonic anhydrase (29K), 29,000; trypsinogen (24K), 24,000; soybean trypsin inhibitor (20.1K), 20,100; and β-lactalbumin (14.2K), 14,200. Experimental details are given in the text.

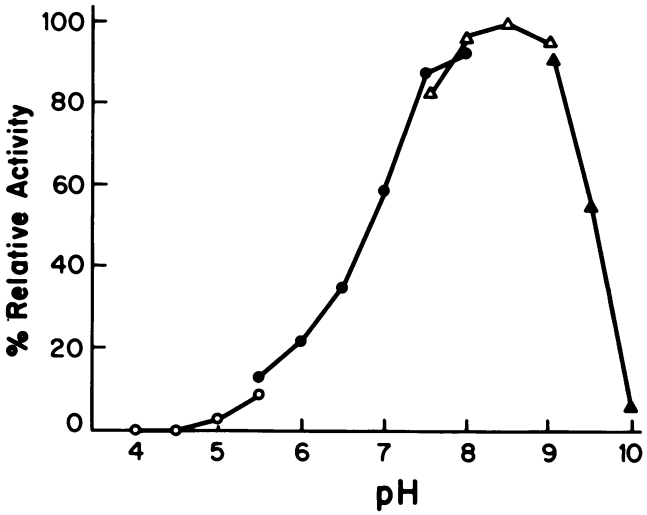

FIG. 5. Optimum pH for the hydrolysis of BAPNA by the *T. denticola* enzyme. The enzyme activity was measured in the standard reaction mixture without $CaCl_2$ by using 50 mM acetate buffer (pH 4.0 to 5.5), potassium phosphate buffer (pH 5.5 to 8.0), Tris hydrochloride buffer (pH 7.5 to 9.0), and bicarbonate buffer (pH 9.0 to 10.0).

inactivated between pH 4 and 5. The enzyme lost all activity upon heating for 10 min at 60°C.

The enzyme activity at various pH values was measured under the standard assay condition by use of BAPNA as the substrate with 50 mM acetate buffer (pH 4.0 to 5.5), phosphate buffer (pH 5.5 to 8.0), Tris hydrochloride buffer (pH 7.5 to 9.0), and bicarbonate buffer (pH 9.0 to 10.0). The pH optimum for the hydrolysis of BAPNA was around 8.5 (Fig. 5).

The purified enzyme was incubated with a 2 mM concentration of various divalent cations. The activity of the enzyme was inhibited by Mn^{2+}, Zn^{2+}, Hg^{2+}, and Co^{2+}, whereas Ca^{2+}, Mg^{2+}, and Ba^{2+} increased the activity (Table 2). Monovalent cations such as Na^+ and K^+ showed no effect on enzyme activity at the concentration of 0.2 M. Metal chelators and sulfhydryl reagents did not affect the enzyme activity (Table 3). Diisopropyl fluorophosphate (DFP), a serine protease inhibitor, completely inactivated the enzyme, whereas phenylmethylsulfonyl fluoride, another serine protease inhibitor, did not affect enzyme activity, even when added at a 5 mM concentration. *N*-α-*p*-Tosyl-L-lysine chloromethyl ketone, which alkylates the histidine residue at the active site of trypsin, caused significant inhibition of the enzyme. *N*-Tosyl-L-phenylalanine chloromethyl ketone, which alkylates a histidine residue at the active site of chymotrypsin, had no effect on enzyme activity. The enzyme was inhibited by antipain and leupeptin but not by α-1-antitrypsin or soybean trypsin inhibitor.

The purified enzyme hydrolyzed other synthetic substrates of trypsin such as BAEE, BANA, *N*-α-*p*-tosyl-L-arginine methyl ester, and *N*-α-*p*-tosyl-L-lysine methyl ester. No aminopeptidase activity was detected on various L-amino acid 2-naphthylamides, including alanine, arginine, glycine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, tryptophan, and valine. Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide, which is a synthetic substrate of chy-

TABLE 2. Effect of divalent cations on hydrolysis of BAPNA by purified enzyme[a]

Cation	Relative activity (%)
None	100
$CaCl_2$	133
$MgCl_2$	125
$BaCl_2$	130
$CuCl_2$	101
$CdCl_2$	96
$MnCl_2$	33
$ZnCl_2$	3
$HgCl_2$	2
$CoCl_2$	0

[a] The cations at 2.0 mM in 1 ml of 100 mM Tris hydrochloride buffer (pH 8.5) were incubated with 0.18 μg of pure enzyme for 15 min at 37°C before the addition of substrate.

TABLE 3. Effect of group-specific reagents and inhibitors on hydrolysis of BAPNA

Group and reagents	Concn		Relative enzyme activity (%)[a]
	mM	μg/ml	
Control, none			100
Chelators			
EDTA	10		102
1,10-Phenanthroline	1		100
Sulfhydryl agents			
2-Mercaptoethanol	10		103
L-Cysteine	10		100
DTT	10		105
Serine protease inhibitors			
DFP	1		0
Phenylmethylsulfonyl fluoride	1		98
	5		100
Protease inhibitors			
N-*p*-Tosyl-L-phenylalanine chloromethyl ketone	1		100
	5		97
N-α-*p*-Tosyl-L-lysine chloromethyl ketone	1		47
	5		22
Antipain		1	10
		5	0
Leupeptin		1	12
		5	0
α-1-Antitrypsin		100	100
Soybean trypsin inhibitor		100	100

[a] Enzyme activity was measured in a standard assay mixture as described in Materials and Methods. Each of the inhibitors at the final concentration indicated was incubated with 1.8 μg of the purified enzyme for 10 min at 37°C before the addition of BAPNA.

TABLE 4. Comparison of kinetic parameters of the enzyme from *T. denticola* and bovine trypsin upon BAPNA and BAEE

Substrate	*T. denticola* enzyme		Trypsin[a]	
	K_m (mM)	V_{max} (μmol/min per mg)	K_m (mM)	V_{max} (μmol/min per mg)
BAPNA	0.05	83	0.71	7.1
BAEE	0.12	91	0.0091	76.0

[a] Kinetic data for trypsin were determined under experimental conditions similar to those used for *T. denticola* enzyme as described in Materials and Methods.

motrypsin, was not hydrolyzed. The hydrolysis of heat-denatured casein, urea-denatured hemoglobin, azocasein, azocoll, bovine serum albumin, and gelatin was not detectable even after long incubation. BAPNA and BAEE were hydrolyzed, respectively, 11 and 1.2 times faster by the *T. denticola* enzyme than by trypsin (Table 4). The apparent K_m values of the enzyme from *T. denticola* for BAPNA and BAEE were estimated to be 0.05 and 0.12 mM, respectively, which differed from that of trypsin.

DISCUSSION

In the present study, we describe the purification and the properties of an enzyme from a strain of *T. denticola* capable of hydrolyzing synthetic substrates of trypsin. The enzyme was purified 310-fold, and seems to be homogeneous, as indicated by PAGE in the presence and absence of SDS. However, the presence of several faint, barely discernable bands in Fig. 4 raises the possibility that the final enzyme preparation was not 100% pure. The molecular weight of this enzyme as estimated by gel filtration was 40,000 to 50,000, whereas the estimate by SDS-PAGE analysis was 69,000. This discrepancy was not due to denaturing of the enzyme into subunits by SDS-PAGE, since the value obtained by SDS-PAGE was higher than that obtained by gel filtration. There is the possibility that the enzyme has a different shape in these separation media, which accounts for the different values. Other studies are needed to clarify the exact molecular weight of this enzyme. There was an unexpected loss of enzyme activity on the Mono Q HR5/5 prepacked ion-exchange column (Table 1), a phenomenon we have observed with other microbial proteases. It is possible that the strong interactions between the enzyme and the hydrophobic resin expose the enzyme active site to an inactivating environment, possibly by removing protective peptides from the vicinity of the active site.

The enzyme was not affected by either metal chelators or sulfhydryl reagents, but activity was increased by Ca^{2+}, Mg^{2+}, and Ba^{2+} ions. Ca^{2+} stimulates the activity of trypsin by causing a conformational change which leads to a more compact protein (41). The *T. denticola* enzyme might behave similarly in the presence of Ca^{2+}. The BAPNA-hydrolyzing activity was lost in the presence of DFP, indicating that a serine residue may have been involved at the catalytic site of the enzyme. The fact that phenylmethylsulfonyl fluoride did not inhibit the reaction may reflect that, because phenylmethylsulfonyl fluoride is a larger molecule than the DFP, it could not gain access to this serine residue. Similar results have been obtained with two serine proteases from *Escherichia coli* (35, 36). The inhibition of the *T. denticola* enzyme by *N*-α-*p*-tosyl-L-lysine chloromethyl ketone suggests that a histidine residue may be important for the activity of the enzyme.

The purified enzyme from *T. denticola* hydrolyzed several synthetic trypsin substrates, indicating that it catalyzed preferentially the hydrolysis of ester, amide, and peptide bonds involving the carboxyl group of arginine and lysine. In fact, it hydrolyzed BAPNA and BAEE faster than did trypsin (Table 4). This enzyme had neither aminopeptidase-like nor chymotrypsinlike activity, though some *T. denticola* strains have been reported to possess these activities (17). Unlike trypsin, the *T. denticola* enzyme did not hydrolyze protein substrates as tested under the experimental conditions. Also unlike trypsin, its activity against BAPNA was not affected by soybean trypsin inhibitor and α-1-antitrypsin. Consequently, the *T. denticola* enzyme may differ considerably from trypsin (10, 42).

Yoshimura et al. (43) characterized a trypsinlike protease from *B. gingivalis* using BAPNA as the substrate. This enzyme was membrane bound, and its activity was unaffected by DFP but enhanced by sulfhydryl reagents, suggesting that it was a thiol protease. Therefore, this enzyme is different from trypsin and the *T. denticola* enzyme.

A cytoplasmic endopeptidase from *E. coli*, named protease II, also hydrolyzed BAPNA and BANA, but it exhibited minimal proteolytic activity as measured on azocasein (35). Protease I, another enzyme isolated from *E. coli*, and which hydrolyzed *N*-acetyl-DL-phenylalanine 2-naphthyl ester, a synthetic substrate of chymotrypsin, did not hydrolyze casein (36). Kowit et al. (14) have suggested that protease I is probably not a protease. The BAPNA-hydrolyzing enzyme from *T. denticola* also does not seem to be an endopeptidaselike protease, as no activity against a wide array of proteins could be demonstrated. This fact does not preclude that the present enzyme contributes to the degradation of some peptides and has the potential to hydrolyze certain proteins. We have observed that crude enzyme extracts of *T. denticola* can digest casein, but this activity was present in the higher-molecular-weight fractions obtained from the Bio-Gel A-5m column (unpublished data). It may be that the present enzyme acts in concert with these proteases so as to effectively digest proteins into amino acid constituents which are then fermented by the organism.

Substrate amounts of arginine can be used by *T. denticola* to obtain energy by an arginine iminohydrolase pathway (2). Thus, the BAPNA-hydrolyzing enzyme should be able to break down any arginine-containing peptides that are present in the subgingival plaque and crevicular fluid and thereby provide L-arginine as an energy source for *T. denticola*. We are investigating the substrate specificity of this enzyme in detail and evaluating its ability to degrade peptides that are present in the gingival environment. In this regard, saliva is known to contain a tetrapeptide called sialin which contains both arginine and lysine residues (13) and which could serve as a substrate of the *T. denticola* enzyme.

T. denticola has been reported to hydrolyze gelatin (8) and to dissolve fibrin (33). The fibrinolytic enzyme was essentially extracellular, and it did not show activity on casein and hemoglobin, although it did liquify gelatin (33). This enzyme was different from the BAPNA-hydrolyzing enzyme, as it had a molecular weight of about 1,000,000 and was heat stable.

Certain bacterial enzymes which may contribute to periodontal pathogenicity are extracellular. Although we did not clarify the exact cellular localization of the BAPNA-hydrolyzing enzyme from *T. denticola*, the intact cells were able to hydrolyze any BAPNA that was present in the surrounding fluid. This observation suggests that the substrate was easily transported or diffused into the cells or that the enzyme might be located at or close to the cell surface. Therefore, this enzyme could, in concert with other enzymes possessed by either *T. denticola* or other organisms, contribute to the proteolytic activity of subgingival plaques taken from diseased sites (4, 25, 39, 40).

Recently, Boehring et al. (3) reported that sonic extracts of *T. denticola* caused inhibition of fibroblast proliferation, and they speculated that this might contribute to the loss of collagen in inflamed gingival tissues. The fibroblast-inhibitory factor of the sonic extract was fractionated as a peak corresponding to a molecular weight of approximately 50,000 by gel filtration on Sephadex G-150. The sonic extracts of *T. denticola* also contained a factor capable of suppressing lymphocyte proliferation (38), which was dis-

tinct from the fibroblast-inhibitory factor (3). These *T. denticola* factors have been suggested to be potential virulence factors in periodontal disease. Based on our observation, the fibroblast-inhibitory factor might include the BAPNA-hydrolyzing enzyme. Thus, it is possible that the ability of *T. denticola* to inhibit the growth of fibroblasts may be due to the enzyme studied in this study.

Although the subgingival bacteria play a major role in the pathogenicity of periodontal disease, the exact etiology is not known. Since the *Treponema* species are found in extremely high numbers and proportions in plaques taken from diseased sites and are both in close proximity to and actually invasive in the gingival tissue (21, 28), any potentially periodontopathic mechanisms of this organism should be clarified. In the present study, the BAPNA-hydrolyzing enzyme from *T. denticola* was shown to be unique in its biochemical characteristics, especially in view of its differences from trypsin. Further investigations are necessary to evaluate its possible role(s) in the pathogenesis of periodontal disease and its utility for the estimation of periodontal disease activity.

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