Cell-bound peptidase activities of *Treponema denticola* ATCC 33520 in continuous culture

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The oral spirochaete *Treponema denticola* ATCC 33520 was grown at a mean generation time of 10 h in anaerobic continuous culture in a serum- and carbohydrate-free medium at pH 7.0. The extracellular proteolytic activities of this spirochaete were then investigated by incubating washed cells with 68 2-naphthylamide derivatives of the Extended API System. Chymotrypsin-like, trypsin-like, elastase-like and iminopeptidase activities were demonstrated. The phenylalanine peptidase or chymotrypsin-like activity of *T. denticola* ATCC 33520, estimated with *N*-succinyl-L-phenylalanyl-L-leucyl-L-phenylalanine-thiobenzyl ester (SPLP) had a pH optimum at pH 8.5, a specific activity of $36.6 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ and was inhibited only slightly by HgCl_2 . The trypsin-like activity, estimated with benzoyl-DL-arginine-7-amido-4-methylcoumarin (BAMC), had a pH optimum at pH 9, and a specific activity of $0.3 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$; inhibition by HgCl_2 indicated the involvement of active thiol groups. The activity should preferably be termed arginine peptidase activity, according to the carboxy-terminal amino acid of the test substrate. The extracellular proline peptidase activity, estimated with L-proline-7-amido-4-methylcoumarin. HBr (PRAMC), had an activity of $1.5 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$, an optimum at pH 8.5 and the properties of a thiol protease. The main cell-bound and extracellular active peptidase activities of fast-growing cells of *T. denticola* ATCC 33520 are phenylalanine peptidase, proline peptidase, arginine peptidase and an oligopeptide-dependent alanine peptidase activity. The cell-bound peptidase activities are of potential importance for invasion and multiplication in the junctional epithelium and the destruction process in periodontal pockets with an anaerobic and alkaline environment.

Introduction

Treponema denticola is one of the oral spirochaetes associated with periodontal disease (Listgarten & Hell-den, 1978; Armitage *et al.*, 1982; Loesche, 1988). *T. denticola* has endo- and exopeptidase activities and is the most proteolytic species of the genus *Treponema* (Mikx, 1991). Of the oral flora, *T. denticola* and *Porphyromonas gingivalis* show proteolytic activities, a characteristic probably related to their virulence and used in the diagnosis of periodontal disease (Laughon *et al.*, 1982; Syed *et al.*, 1984; Loesche *et al.*, 1990). *T. denticola* is able to degrade type IV collagen, gelatin, elastin and fibronectin (Uitto *et al.*, 1986). These proteolytic activities are considered of importance for the acquisition of nitrogenous growth substances, the perturbation of the host defenses, invasion and multiplication in the junctional epithelium (Saglie *et al.*, 1982; Uitto *et al.*, 1988a; Mikx *et al.*, 1990; Reijntjens *et al.*, 1986).

Trypsin-like, chymotrypsin-like, iminopeptidase and keratinolytic activities of *T. denticola* have been demonstrated by different investigators (Ohta *et al.*, 1986; Fiehn, 1986; Mäkinen *et al.*, 1986, 1987; Mikx & de Jong 1987; Syed *et al.*, 1988). Recently Grenier *et al.*, (1990) demonstrated the location of a chymotrypsin-like enzyme at the outside of the cell envelope of *T. denticola*.

Most studies of the different proteases of *T. denticola* have been performed with purified cell extracts of batch-grown spirochaetes. In the present study, the extracellular proteolytic activities of intact and relatively fast-growing *T. denticola* cells were investigated. A selection of synthetic substrates was made and the pH optima and specific activities of cell-bound arginine, phenylalanine and proline peptidases were estimated. In order to control growth and substrate effects, the spirochaetes were grown in continuous culture in a serum- and carbohydrate-free medium. In order to measure the activities of the same population, the cells were harvested, washed and tested simultaneously for the different activities.

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Methods

Continuous culture. *Treponema denticola* ATCC 33520 was grown in PTY broth, a serum- and carbohydrate-free medium, containing 1% (w/v) Proteose peptone no. 2, 0.5% yeast extract (both from Difco), 0.5% Trypticase peptone (BBL), 0.25% KCl, 0.05% L-cysteine, 25 mg thiamin pyrophosphate l⁻¹, 0.5 g NaH₂CO₄, and isobutyric, DL-2-methylbutyric, valeric and isovaleric acids (each 2.5 mg l⁻¹). The medium was stored at room temperature and connected to the culture vessel under a continuous stream of 4% (v/v) CO₂ and 5% (v/v) H₂ in oxygen-free nitrogen. The dilution rate of the anaerobic continuous culture was $D = 0.07 \text{ h}^{-1}$, the pH and redox potential were continuously recorded and the culture vessel was kept at 37 °C. The cell mass from 40 ml samples taken from the culture at steady state was estimated after washing the pellet twice in demineralized water by centrifugation (15 min at 15000 g) and drying at 100 °C. The fermentation products were measured by isotachopheresis in a LKB 2127 Tachophor. Samples for the enzyme activity measurements were taken directly from the culture vessel, and washed by centrifugation and resuspension in the indicated buffer.

***N*-acetyl arylamidase activities.** The Extended API System and API ZYM were used to test the activity of *T. denticola* ATCC 33520 cells on 68 different amino-, dipeptide- and oligopeptide naphthylamide derivatives. Suspensions of the spirochaetes (OD₅₅₀ 2) in 0.1 M-potassium phosphate buffer, pH 7.0, were added to the cupules of the API research kits, incubated aerobically for 4 h at 37 °C and evaluated according to the manufacturer's instructions as previously described (Mikx, 1991).

Arginine aminopeptidase activity. A mixture of 2 ml 0.25 mM-*N*-benzoyl-L-arginine-ethyl ester (BAEE; Sigma) and 2 ml of spirochaete suspension (OD₅₅₀ 0.85) in 0.1 M-potassium phosphate buffer, pH 7.5, was incubated at 37 °C for 90 min. After incubation and centrifugation the absorbance at 253 nm was measured in a Zeiss PMQ 3 spectrophotometer. In the control mixtures the substrate or the spirochaetes were omitted.

When testing different spirochaete densities, the reaction mixture was 2 ml of spirochaete suspension in 0.1 M-Tris/HCl buffer, pH 8.0, and 50 µl 0.1 M-*N*-benzoyl-DL-arginine-naphthylamide.HCl (BANA, Sigma) in dimethylsulphoxide (DMSO). Samples were taken at the start and after incubation at 37 °C for various times, centrifuged and measured in a Zeiss PMQ3/ZFM4 Spektralfuorometer, excitation 335 nm, emission 410 nm.

The optimum concentration of benzoyl-DL-arginine-7-amido-4-methylcoumarin.HCl (BAMC; Bachem, Bubendorf, Switzerland) was estimated with 1 ml of spirochaete suspension (OD₅₅₀ 0.128) in 0.1 M-Tris/HCl buffer, pH 8.0, and 1 to 80 µl of a stock solution of 32 mM-BAMC in DMSO. After 45 min incubation at 37 °C the reaction was stopped by transferring a 40 µl sample to 1 ml of demineralized water at 4 °C. The fluorescence was measured in a Perkin Elmer LS5 luminescence spectrophotometer, excitation 370 nm, emission 460 nm. The effect of different spirochaete densities on BAMC degradation was estimated with 0.32 mM-BAMC in 0.1 Tris/HCl buffer, pH 8.0.

The pH optimum was estimated in two experiments done in duplicate with 1 ml of spirochaete suspensions (OD₅₅₀ 0.05) in Teorell and Stenhagen's 1 mM-citrate/10 mM-phosphate/20 mM-borate buffer over the pH range pH 6.0 to pH 12 (Sober, 1970); 10 µl BAMC stock solution was added and the mixture was incubated at 37 °C for 60 min.

The specific activity for BAMC was estimated in two experiments done in triplicate by incubating a spirochaete suspension (OD₅₅₀ 0.05) in Teorell and Stenhagen's buffer, pH 9.0, containing 0.163 mM-BAMC for 30 min at 37 °C. The reactions were stopped in demineralized water at 4 °C as previously described. In the same set of tests the effect of 2 mM-dithiothreitol (DTT), 2 mM-phenylmethylsulphonyl fluoride (PMSF) or 0.5 mM-HgCl₂ in the reaction mixture was

measured. Calibration curves of coumarin and fluorescence and of spirochaete density (OD₅₅₀) and dry weight were used for calculating specific activities.

Phenylalanine aminopeptidase activity. The tests with 2.5 mM-benzoyloxycarbonyl-L-phenylalanine-2-naphthylamide (PHENA) and 0.32 mM-glutaryl-phenylalanine-7-amido-4-methylcoumarin (PHAMC) were carried out under the same conditions and in the same experiments as described for BANA and BAMC.

The pH optimum was estimated in two experiments done in duplicate with *N*-succinyl-L-phenylalanyl-L-leucyl-L-phenylalanine-thiobenzyl ester (SPLP; Bachem) according to Harper *et al.* (1981). The reaction mixture, comprising 0.9 ml 0.237 mM-SPLP, 75 µl 0.86 mM-dithiopyrimidine (DTP) and 50 µl of spirochaete suspension (OD₅₅₀ 0.5), all in Teorell and Stenhagen's buffer with 10% DMSO, was incubated at 20 °C for 30 min. The reaction product, 4-thiopyridone, was measured at 324 nm. In the same manner, the specific activity at pH 8.5 and the effect of 2 mM-PMSF and 0.5 mM-HgCl₂ were estimated. The specific activity was calculated using the results of two experiments in triplicate and the extinction coefficient for 4-thiopyridone ($\epsilon = 19800 \text{ M}^{-1} \text{ cm}^{-1}$).

Proline aminopeptidase activity. Benzoyloxycarbonyl-L-proline (PRONA; Bachem) (2.5 mM) and L-proline-7-amido-4-methylcoumarin.HBr (PRAMC; Bachem) (0.125 mM) were tested under the same conditions as described for BANA and BAMC. The specific activity for PRAMC was estimated in two experiments in triplicate with spirochaete suspensions (OD₅₅₀ 0.05) and 0.125 mM-PRAMC in Teorell and Stenhagen's buffer at pH 8.5 and 37 °C. The effects of 0.5 mM-HgCl₂, 2 mM-DTT and 2 mM-PMSF were tested as described for BAMC.

Results

Activities of the continuous culture

In the steady state, the continuous culture of *T. denticola* ATCC 33520 stabilized at pH 7.0, $E_h - 385 \text{ mV}$ and a dry weight of 215 mg l⁻¹. The main fermentation product was acetic acid, 1.86 g l⁻¹. Spirochaetes harvested from the culture vessel were used to test the hydrolysis of 68 different naphthylamide derivatives. The results of the peptidase activity tests were grouped according to the number of peptide bonds and the carboxy-terminal amino acid (Table 1). L-Alanine-related activities were only found by the use of the oligopeptides Ala-Phe-Pro-Ala-2NA and Phe-Pro-Ala-2NA, indicating an elastase-like activity. In addition we found a chymotrypsin-like activity involving phenylalanine, tyrosine and tryptophan derivatives, a trypsin-like activity involving arginine and lysine derivatives and an iminopeptidase activity involving proline, hydroxyproline and pyrrolidone derivatives.

Arginine aminopeptidase activity

Activity measurements with BAEE on the spirochaetes from the continuous culture were disturbed by the turbidity of the cell suspension in the incubation mixture.

Table 1. Cell-bound peptidase activities of *T. denticola* ATCC 33520

T. denticola was grown in continuous culture and peptidase activities were tested by the Extended API System. +, Activity present; —, activity absent; •, not tested. *a*, Elastase-like activity (substrates L-Ala-L-Phe-L-Pro-L-Ala-2NA and L-Phe-L-Pro-L-Ala-2NA); *b*, chymotrypsin-like activity; *c*, iminopeptidase activity; *d*, trypsin-like activity.

Carboxy-terminal amino acid in 2-naphthylamide derivative	Amino peptidase	Dipeptidase	Oligopeptidase
Glycine	—	—	•
L-Alanine	—	—	+ ^a
L-Valine	—	•	•
L-Leucine	—	•	•
L-Isoleucine	—	•	•
L-Phenylalanine	+ ^b	—	•
L-Proline	+ ^c	+ ^c	+ ^c
L-Serine	—	—	—
L-Threonine	—	•	•
L-Cysteine	—	•	•
L-Methionine	—	—	•
L-Glutamine	+	•	•
L-Hydroxyproline	+ ^c	•	•
L-Tyrosine	+ ^b	—	•
L-Tryptophane	+ ^b	+ ^b	•
L-Pyrrolidone	+ ^c	•	•
L-Aspartate	—	•	•
L-Glutamate	+	—	•
L-Arginine	+ ^d	+ ^d	+ ^d
L-Lysine	+ ^d	+ ^d	•
L-Histidine	—	—	—

The turbidity of the incubation mixture had no effect on fluorescence measurements using the fluorogenic substrate BAMC. A linear relation between fluorescence and time was observed with *T. denticola* ATCC 33520 densities (OD₅₅₀) ranging from 0.016 to 0.244 (Fig. 1*b*). In the figure the absolute fluorescence values are shown. A linear relation was also observed with the naphthylamide derivative BANA, but only with spirochaete densities above OD₅₅₀ 0.188 (Fig. 1*a*). The optimum BAMC concentration in the reaction mixture with *T.*

denticola cells was 0.32 mM; at higher concentrations a quenching of fluorescence was found (Fig. 2). The pH optimum for BAMC of *T. denticola* ATCC 33520 was pH 9.0 (Fig. 3*a*). The specific activity at pH 9.0 was 0.3 ± 0.01 nmol min⁻¹ (mg dry wt)⁻¹ and was lowered in the presence of 0.5 mM-HgCl₂ to 0.08 ± 0.01 nmol min⁻¹ (mg dry wt)⁻¹. The presence of 2 mM-DTT or -PMSF in the reaction mixture had no effect on the specific activity.

Phenylalanine aminopeptidase activity

Estimations of the phenylalanine aminopeptidase activities with PHENA were not successful due to the formation of a precipitate after addition of PHENA to the reaction mixture.

Incubating increasing densities of *T. denticola* cells with the fluorogenic substrate PHAMC gave some increase in fluorescence with time (Fig. 1*c*). The maximum increase in fluorescence was only 20 units after 80 min incubation. Finally, the phenylalanine peptidase activity was estimated with SPLP and gave an optimum at pH 8.5 (Fig. 3*b*). The specific activity at pH 8.5 was 36.6 ± 0.7 nmol min⁻¹ (mg dry wt)⁻¹. The activity was only slightly inhibited [32.2 ± 0.7 nmol min⁻¹ (mg dry wt)⁻¹] by the presence of 0.5 mM-HgCl₂ and was not affected by PMSF. The use of the substrate SPLP did not allow a test with DTT.

Proline aminopeptidase activity

The naphthylamide derivative of benzyloxycarbonyl-L-proline, PRONA, gave a precipitate in the reaction mixture that prevented successful estimation of peptidase activity. The fluorogenic substrate PRAMC gave a linear increase in fluorescence with increasing *T. denticola* densities (Fig. 1*d*). The proline peptidase activity estimated with 0.163 mM-PRAMC showed an optimum at pH 8.5 (Fig. 3*c*). The specific activity at

Fig. 1. Changes in fluorescence with time for different *T. denticola* ATCC 33520 densities (OD₅₅₀ indicated on the Figure itself) and amino acid derivatives in 0.1 M-Tris/HCl. (a) 2.5 mM-Benzoyl-DL-arginine-2-naphthylamide (BANA) at pH 8.0; (b) 0.32 mM-benzoyl-DL-arginine-7-amido-4-methylcoumarin (BAMC) at pH 8.0; (c) 0.32 mM-L-phenylalanine-7-amido-4-methylcoumarin (PHAMC) at pH 7.5; (d) 0.125 mM-L-proline-7-amido-4-methylcoumarin (PRAMC) at pH 7.5.

Fig. 2. Increase in fluorescence of benzoyl-DL-arginine-7-amido-4-methylcoumarin (BAMC) after 45 min incubation with *T. denticola* ATCC 33520 (OD₅₅₀ 0.128) in 0.1 M-Tris/HCl buffer at pH 8.0.

Fig. 3. pH optimum curves of (a) arginine peptidase with 0.32 mM-benzoyl-DL-arginine-7-amido-4-methylcoumarin (BAMC), (b) proline peptidase with 0.125 mM-L-proline-7-amido-4-methylcoumarin (PRAMC) and (c) phenylalanine peptidase with 0.231 mM-N-succinyl-L-phenylalanyl-L-leucyl-L-phenylalanine-thiobenzyl ester (SPLP), obtained by incubating the derivatives with *T. denticola* ATCC 33520 (OD₅₅₀ 0.5) in Teorell and Stenhagen's buffer. Results from two separate experiments (●, ○) are shown.

pH 8.5 was 1.5 ± 0.02 nmol min⁻¹ (mg dry wt)⁻¹; the activity was inhibited completely by 0.5 mM-HgCl₂ and slightly stimulated by 2 mM-DTT [2.19 ± 0.03 nmol min⁻¹ (mg dry wt)⁻¹]. PMSF had no effect on the proline aminopeptidase activity.

Discussion

The proteolytic activities of *T. denticola* have mainly been investigated with purified enzymes or extracts of sonicated spirochaetes (Mäkinen *et al.*, 1986, 1987; Uitto *et al.*, 1988a, b). In order to study the proteolytic activities which might play a role in tissue invasion and the persistence of *T. denticola* in the periodontal pocket, we investigated the extracellular peptidase activities of whole cells of *T. denticola* ATCC 33520, grown in a low redox potential (−385 mV), protein-rich medium without added carbohydrates at a mean generation time of 10 h, which is within the limits of their natural habitat (Socransky & Haffajee 1991; ter Steeg & van der Hoeven, 1990). It is realized, but unknown for *T. denticola*, that the composition of the culture medium might play a role in the induction of proteases. Because of these culture restrictions, our observations are limited to the simultaneous measurement of different activities of cells from the same relatively fast-growing culture.

Under the above conditions, *T. denticola* ATCC 33520 cells showed at least four different extracellular peptidase activities.

The alanine peptidase or elastase-like activity is considered to be a virulence factor because of its broad specificity towards several structural proteins and its presence in periodontal pockets showing active destruction (Eley & Cox, 1992). The activity in *T. denticola* ATCC 33520 was not observed with amino and dipeptide substrates but only with the carboxy-terminal alanine oligopeptide substrates, which indicates an endopeptidase activity that might be of nutritional importance, because numerous bacteria are dependent on small peptides for amino acid supply. Also, an effect of the test substrate may have played a role, since extension of the length of the peptide chain can lead to an increased sensitivity for peptidases (Zimmerman *et al.*, 1977). A similar increase in sensitivity might have been responsible for the relative high chymotrypsin-like activity observed with SPLP (Harper *et al.*, 1981).

A chymotrypsin-like enzyme has been isolated from *T. denticola* and shown to be attached to the outside of the cell envelope (Uitto *et al.*, 1988b; Grenier *et al.*, 1990). The enzyme has an optimum at pH 7.5 and is inhibited by PMSF and HgCl₂, indicating a serine protease with an active thiol group. The extracellular phenylalanine peptidase activity of *T. denticola* described here showed an optimum at pH 8.5, was not inhibited by PMSF and only slightly affected by HgCl₂. These differences cannot be explained by a substrate effect and indicate a difference in behaviour between the purified enzyme and the native cell-bound chymotrypsin-like activity, or the presence of more than one enzyme.

The trypsin-like activity of *T. denticola* ATCC 33520 showed a clear optimum at pH 9.0 with BAMC. A similar pH optimum was found with the substrate BAPNA by Mäkinen *et al.* (1986), who also indicated that the bulkier substrate BANA showed a less alkaline pH optimum. In addition, we found that BANA was a less sensitive substrate than BAMC. The trypsin-like activity of *T. denticola* has been stated to be a serine peptidase (Ohta *et al.*, 1986; Mäkinen *et al.*, 1986). The inhibition of the BAMC activity by HgCl₂ in the present study indicates that at least a part of the cell-bound trypsin-like activity contains an active thiol group. The absence of a stimulatory effect by DTT might be due to the reduced state of cells freshly harvested from the culture (with a redox potential of -385 mV). The cell-bound trypsin-like activity of *T. denticola* seems to be related to more than one enzyme or to a single enzyme with a broad or varying activity. A benzoyl-arginine peptidase (BANA) with a iminopeptidase activity (PRONA) has been isolated from cell extracts of *T. denticola* (Mäkinen *et al.*, 1987). For *P. gingivalis*, it has recently been shown that the membrane- or vesicle-bound BANA activity is a thiol peptidase and not a trypsin-like enzyme (Shah *et al.*, 1990). For these reasons, the proteolytic activities of *T. denticola* should be named after the substrate or the site of hydrolysis: for example, arginine peptidase activity instead of trypsin-like activity.

The cell-bound proline peptidase activity was totally blocked by the thiol reagent HgCl₂, only slightly stimulated by DTT and resembled the iminopeptidase Enzyme III of *T. denticola* purified by Mäkinen *et al.* (1987). Enzyme III is considered to be a thiol peptidase with a pH optimum between pH 7.0 and 7.5 for *N*-L-prolyl-2-naphthylamine. The more alkaline optimum, pH 8.5, might be due to the test substrate, PRAMC, but is also in agreement with the indicated difference between the cell-bound and purified chymotrypsin-like activity.

With the exception of the difference in pH optimum, the cell-bound and extracellular activities of *T. denticola* ATCC 33520 resemble the purified enzymes of extracts of *T. denticola*, which have been shown to degrade a variety of proteins such as proline-rich salivary peptides, immunoglobulins, transferrin, α -1-antitrypsin, fibrinogen and structural proteins (Uitto *et al.*, 1988a, b; Grenier *et al.*, 1990). Besides a role in nutrition, these cell-bound activities might play a role in tissue invasion by the spirochaetes. Migration of *T. denticola* through a basement-membrane-like material was found by Grenier *et al.* (1990). The alkaline optimum pH values of the investigated activities suggests that alkaline pH shifts in periodontal pockets (Eggert *et al.*, 1991) will favour the destructive potential of *T. denticola*. Although tissue

invasion by small spirochaetes including *T. denticola* is questionable (Mikx *et al.*, 1990; Riviere *et al.*, 1991), the different extracellular proteolytic activities of *T. denticola* are probably involved in the destruction process of the junctional epithelium in the anaerobic environment of the periodontal pocket, where spirochaetes including *T. denticola* frequently form the dominant group of bacteria.

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