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Isolation and Biological Properties of Oral Spirochete, *Treponema denticola*

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Summary

Spirochetes were isolated from the lesions of periodontitis patients, identified them as *Treponema denticola* and their properties were investigated. These strains required serum for growth and demonstrated low sugar fermentation activity. Indole, hydrogen sulfide, acetic acid, propionic acid and butyric acid were detected in the metabolites of all the isolates. Proteases, esterase and neuraminidase were also produced by the isolates, of which, proteolytic enzymes appeared to contain at least three different kinds. These proteases were possible to separate from each other by column chromatography.

Introduction

Even though the spirochetes are the minor group of the normal subgingival microbial flora[1~3], their numbers in the lesions of adult periodontitis and acute necrotizing ulcerative gingivitis increase drastically, the proportion of the spirochetes accounts for 30 to 50% of all the microscopically countable microbial population[3,4]. *Treponema denticola*, a medium-sized spirochete is regarded as a potent periodontopathogen as well as Gram negative rods[5,6]. The recent progress of the cultivation methods facilitated in vitro growth of the oral spirochetes and resulted in increasing accumulation of the taxonomic studies[7] and the reports on the pathogenic factors including protease[8~13]. The aim of the present investigation was the isolation and the biological characterization of the spirohetes from the adult periodontitis patients.

Materials and Methods

The samples taken using paper points from the lesions of 8 patients with adult periodontitis (36 to 45 years of age) were suspended in TYGS broth[11] and aliquots of the suspension were introduced onto the sterile filter membranes (cellulose nitrate Type TM-4, pore size 0.2 μm, Toyo Roshi Co., Tokyo) placed on the TYGS agar plates. The plates were incubated at 37℃ in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, USA) filled with an mixture of gases (N_2-H_2-CO_2 ; 85 : 10 : 5) for 5 days. Eight spirochetes strains isolated by this procedure were cloned from the colonies formed beneath the membranes by the ordinary methods.

Morphological studies of the isolates were performed light and electron microscopically[14]. The

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biological properties of the isolated 8 spirochetes strains were determined by the methods of Holdeman[15] using the basal PY medium supplemented with 10% rabbit serum, 0.5% TPP solution and VFA solution.

Identification of the metabolic products was carried out by gas chromatography (Shimadzu Co. Ltd., Kyoto) according to Fukumoto et al.[7]. Phosphatases, esterases, lipases, arylamidases, trypsin, chymotrypsin, galactosidases, glucuronidase, glucosidases, glucosaminidase, mannosidase and fucosidase were detected using API ZYM system (API System S. A., Motalieu Vercieu, France). Neuraminidase activity was estimated by the methods of Potier[16] using a fluorescence substrate, 2'-(4-methylumbelliferyl)-α-D-N-acetylneuramic acid[17] (MUA, Sigma Chem. Co. St. Louis, USA). Trypsin and chymotrypsin activity in the culture supernatant and cell extracts of an isolate strain (TN-2) were also measured using benzoyl-arginyl-*p*-nitroanilide[18] (BAPNA, Sigma) and succinyl-alanyl-alanyl-prolyl-phenylalnyl-*p*-nitroanilide[19] (SAAPNA, Sigma), respectively. Collagenase activity was examined using 4-phenylazobenzyloxy-carbonyl-prolyl-leucyl-glycyl-prolyl-arginine (Pz-PLGPA, Sigma).

Results and Discussion

Eight isolated strains formed rough and opaque colonies on TYGS agar plates. The morphological and biochemical characteristics are summarized in **Table 1**. All the strains exhibited blunt end and spiral structure with 0.20 to 0.25 μm in width **(Fig. 1)**. All the strains required serum for the growth, however, volatile fatty acids were not essential. Acid production from 8 carbohydrates was not observed. Hydrolysis of esculin and gelatin was positive, indicating the spirochetes elaborate esteolytic and proteolytic enzymes. Indole and hydrogen sulfide production were positive. All the strains yielded acetic acid, propionic acid and butyric acid. Lactic acid and succinic acid were detected from various strains. Production of neuraminidase, trypsin-like protease, chymotrypsin-like protease and collagenase in the spent medium and cell sonicate by one of the isolates (strain TN-2) is compared in **Table 2**. Neuraminidase was found in the culture supernatant, however, proteolytic enzymes located only in the cell extracts. All the strains produced acid phosphatase, alkaline phosphatase, C-8 esterase, leucine arylamidase, phosphoamidase and trypsin-like enzyme. C-4 esterase, varine arylamidase, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucosidase, chymotrypsin-like enzyme and neuraminidase were produced by the many strains. C-14 lipase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were negative in all the strains.

The morphological and biochemical properties of the isolated spirochetes from the periodontitis patients resembled the observations by Fukumoto et al.[7]. These isolates were identified as *Treponema denticola*. Proteolytic activities were not found in the supernatant fluid of the liquid culture obtained by centrifugation at 10,000 g for 20 min, they were detected in the cell sonicate. Therefore, proteases locate in the cytoplasmic fraction or in the cell surface. In the synthetic protease substrates, BAPNA was most actively hydrolyzed and then SAAPNA and Pz-PLGPA followed. MUA (neuraminidase substrate) was degraded by both culture supernatant and cell sonicate, but the former fraction contained about 14-fold total activity of the latter fraction. These findings suggest that synthesized neuraminidase is actively secreted extracellularly.

The sample of cell sonicate, which had been dialyzed against 50 mM Tris-HCl buffer (pH 7.2) was applied to a column of Q-Sepharose equilibrated with the same buffer and eluted with a linear gradient concentration of NaCl. As illustrated in **Fig. 2**, collagenase and chymotrypsin-like enzyme

Table 1. Properties of the isolates of oral spirohetes

Width of cell(μm)	0.15−0.25
Cell ends	blunt
Requirement for growth	
serum	+
volatile fatty acids	−
Fermentation	
arabinose	−
fructose	−
glucose	−
glycogen	−
rhamnose	−
ribose	−
starch	−
xylose	−
Esculin hydrolysis	+
Indole prodution	+
Gelatin liquefaction	+
H_2S production	+
Endproducts	
acetate	+
propionate	+
butyrate	+
lactate	+
succinate	+

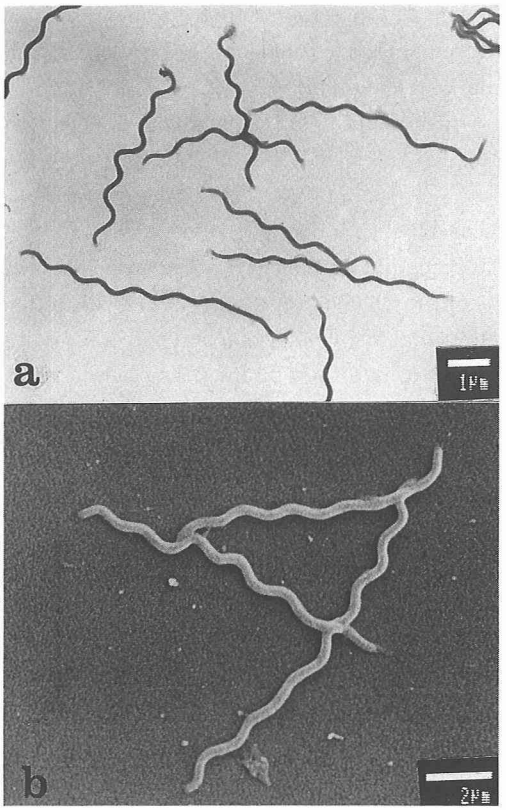

Fig. 1 : Electron micrographs of *T. denticola* TN-2
a : transmission electron micrograph
b : scanning electron micrograph

Table 2. Enzyme activities of *Treponema denticola* TN-2

Enzyme	Culture supernatant (1950 ml)	Cell sonicate from 10.4 g cell(165 ml)
Neuraminidase (MUA)	151(μM/ml/min)	126(μM/ml/min)
Trypsin-like (BAPNA)	N. D.	0.69(△ OD/ml/min)
Chymotrypsin-like (SAAPNA)	N. D.	1.78(△ OD/ml/min)
Collagenase (Pz-PLGPA)	N. D.	1.71(△ OD/ml/min)

N. D. ; not detected.

eluted in a single peak but trypsin-like enzyme appeared in a minor and a major peak. Collagenase, trypsin-like enzyme and chyomotrypsin-like enzyme eluted at NaCl concentrations of 150 to 200 mM, 220 to 250 mM and 300 to 400 mM, respectively. These three enzymes are being purified using Q-Sepharose chromatography as first step of procedure.

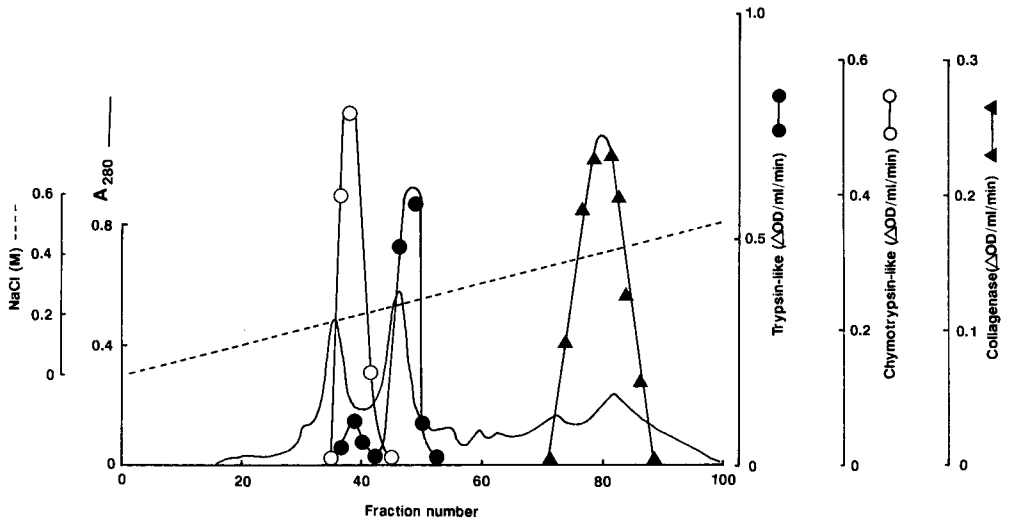

Fig. 2: Chromatography of proteases of *Treponema denticola* TN-2 on Q-Sepharose column

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References

1) Amitage, G. C., Dickson, W. R, Jenderseck, R. S., Levine, S. M. and Chambers, D. W. (1982) Relationship between the percentage of subgingival spirochetes and the severity of periodontal disease. J. Periodontol. **53**: 550—556.

2) Listgarten, M. A. and Hellden, L. (1978) Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans. J. Clin. Periodontol. **5**: 115—132.

3) Loesche, W. J., Syed, A. S., Schmidt, E. and Morrison, E. C. (1985) Bacterial profiles of subgingival plaques in periodontitis. J. Periodontol. **56**: 447—450.

4) Loesche, W. J., Syed, A. S., Laughon, B. E. and Stoll, J. (1982) The bacteriology of acute necrotizing gingivitis. J. Periodontol. **53**: 223—230.

5) Moore, W. E. C., Holdeman, L. V., Cato, E. P., Smibert, R. M., Burmeister, J. A., Palcanis, K. G. and Ranney, R. R. (1985) Comparative bacteriology of juvenile periodontitis. Infect. Immun. **48**: 507—519.

6) Simonson, L. G., Goodmann, C. H., Bial, J. J. and Morton, H. E. (1988) Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56**: 726—728.

7) Fukumoto, Y., Okuda, K., Kato, K., Ohta, T. and Takazoe, I. (1987) Taxonomic study of spirochetes isolated from human periodontal lesions. Oral Microbiol. Immunol. **2**: 82—87.

8) Makinen, K. K., Makinen, P. L. and Syed, S. A. (1992) Purification and substrate specificity of an endopeptidase from the human oral spirochete *Treponema denticola* ATCC 35405, active on furacryloyl-Leu-Gly-Pro-Ala and bradykinin. J. Biol. Chem. **267**: 14285—14293.

9) Shibata, Y., Fujimura, S. and Nakamura, T. (1992) Purification and characterization of an enzyme produced by *Treponema denticola* hydrolyzing synthetic collagenase substrates. Matsumoto Shigaku 8: 138—143.

10) Makinen, P. L., Makinen, K. K. and Syed, S. A. (1994) An endo-active proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. Infect. Immun. **62**: 4938—4947.

11) Ohta, K., Makinen, K. K. and Loesche, W. J. (1986) Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. Infect. Immun.

53: 213—220.
12) Rosen, G., Naor, R. and Sela, M. N. (1994) Characterization of fibrinolytic activities of *Treponema denticola*. Infect. Immun. **62**: 1749—1754.
13) Grenier, D., Uitto, V. J. and McBride, B. C. (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.
14) Rosen, G., Naor, R., Rahamim, E., Yiskal, R. and Sela, M. N. (1995) Proteases of *Treponema denticola* outer sheath and extracellular vesicles. Infect. Immun. **63**: 3973—3979.
15) Holdeman, L. V., Cato, E. P. and Moore, W. E. C. (1977) Anaerobe laboratory manual 4th ed. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.
16) Potier, H., Mameli, L., Belisle, M., Dallaire, L. and Melancon, S. B. (1979) Fluorometric assay of neuraminidase with a sodium (4-methyl-umbelliferyl-α-D-N-acetylneuraminate) substrate. Anal. Biochem. **94**: 287—296.
17) Shimura, R., Shibata, Y., Fujimura, S. and Nakamura, T. (1990) Purification and characterization of neuraminidase from oral *Propionibacterium acnes*. (in Japanese) Matsumoto Shigaku **16**: 276—284.
18) Fujimura, S. and Nakamura, T. (1987) Isolation and characterization of a protease from *Bacteroides ginigivalis*. Infect. Immun. **55**: 716—720.
19) Uitto, V. J., Grenier, D., Chan, E. C. S. and McBride, B. C. (1988) Isolation of a chymotrypsinlike enzyme from *Treponema denticola*. Infect. Immun. **56**: 2717—2722.

抄録：口腔スピロヘータ *Treponema denticola* の分離とその生物学的性状

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成人性歯周病患者の患部よりスピロヘータを分離し，それらは *Treponema denticola* と同定された．分離株の発育には血清を必要とし，炭水化物の分解能は低かった．インドール，硫化水素，酢酸，プロピオン酸，酪酸がすべての分離株の最終産物から検出された．プロテアーゼ，エステラーゼ，ニューラミニダーゼの産生も認められ，プロテアーゼについては少なくも3種類含まれることが分かった．これらのプロテアーゼはイオン交換クロマトグラフィーでそれぞれ分離されることも判明した．