SHORT COMMUNICATION

Isolation and partial characterization of neuraminidase of *Treponema denticola* strain TN-2

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Introduction

Neuraminidase (EC. 3.2.1.18) cleaves α -ketosidic linkages between N-acetylneuraminic acid and remaining carbohydrate chains of oligosaccharides, glycolipids and glycoproteins in body fluids, membrane structures and cell surfaces¹⁾. The enzyme is considered to enhance bacterial adhesion to cells^{2,3)}, indicating that neuraminidase plays a role in the early stage of infection. Accordingly, due to our interest in the production and properties of this enzyme of oral indigenous bacteria, We purified and characterized the neuraminidase of *Propionibacterium acnes*. Microbial neuraminidase has been isolated also from *Vibrio cholerae*⁵⁾, *Clostridium perfringens*^{6,7)}, the influenza virus⁸⁾, and *Actinomyces pyogenes*⁹⁾.

The spirochetes including *Treponema denticola* and other treponemes may be the etiological agents of periodontal disease¹⁰⁻¹⁴⁾, as treponemes are detected frequently in the inflamed pockets of periodontitis patients¹³⁾ and in the gingival soft tissues of patients with acute necrotic ulcerative gingivitis and in advanced periodontitis lesions¹⁵⁾.

Conceivable invading enzymes of *T. denticola*, such as the trypsin-like enzyme¹⁶⁾, chymotrypsin-like enzyme¹⁷⁾, and elastase-like enzyme¹⁸⁾, have been isolated and characterized. Recently, we found that a strain of *T. denticola* elaborated the trypsin-like

enzyme, chymotrypsin-like enzyme, and collagenase. These proteolytic enzymes were separated by column chromatography¹⁹⁾. Simultaneously, we detected production of neuraminidase of the same strain. In the present report the isolation and partial characterization of this enzyme of *T. denticola* are described.

Materials and Methods

1. Bacterial strain and culture conditions

T. denticola strain TN-2 was isolated from the lesion of an adult periodontitis patient in our laboratory¹⁹⁾. Organisms were grown in an anaerobic glove-box filled with a mixture of gases (85% N₂, 10% H₂, 5% CO₂) at 37°C for five days in the medium described by Ohta *et al*¹⁶⁾.

2. Assays

Neuraminidase activity was measured using 2'-4-methylumbelliferyl- α -D-N-acetylneuraminic acid (MUA, Sigma Chem. Co. St. Louis, M. O. USA.) as a substrate according to the method of Potier *et al*.²⁰⁾ Briefly, the reaction mixture contained 0.3 mM MUA, 0.03 M acetate buffer (pH 4.6), 0.15 mM CaCl₂, and 0.1 ml of enzyme solution in a total volume of 0.6 ml. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 2 ml of 0.133 M glycine buffer (pH 10.7) containing 0.06M NaCl and 0.083M Na₂CO₃. The amounts of free 4-methylum-

belliferone liberated from MUA were determined by spectrofluorometer, type 650-10 (Hitachi, Tokyo) using excitation light at 365 nm and fluorescence emission at 450 nm. One unit of neuraminidase was defined as the amount of enzyme which liberated one nmol of 4-methylumbelliferone per min.

Protein was determined by the method of Bradford²¹⁾ using a protein assay system (Bio-Rad Laboratories, Richmond C. A. USA.) with bovine serum albumin (Sigma Chem. Co.) as standard protein.

3. Purification of neuraminidase

All purification procedures described below were carried out at 4°C. *T. denticola* cells were removed by centrifugation at $12,000 \times g$ for 20 min, and ammonium sulfate was added to the culture supernatant to make a final concentration of 80% saturation. The resultant precipitate was collected by centrifugation at $12,000 \times g$ for 20 min and dissolved in 0.05M Tris-HCl buffer (pH 7.2), and dialyzed against the same buffer overnight. The dialyzed material was applied to a column (2.6 by 38 cm) of Q-Sepharose Fast Flow (Pharmacia Biotech. Inc. Uppsala, Sweden) equilibrated with 0.05M Tris-HCl buffer (pH 7.2). After the column was washed with the buffer, the enzyme was eluted with a linear NaCl gradient from 0 to 0.5 M in the same buffer. The fraction with the enzyme activity was pooled and dialyzed against 0.05 M Tris-HCl buffer (pH 7.2), followed by concentration using a rotary evaporator in vacuo. The concentrated neuraminidase fraction was further purified by gel filtration on Sephacryl S-300 (Pharmacia Biotech. Inc.) (2.4 by 90 cm), equilibrated with 0.05 M Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl, and the column was eluted with this buffered saline at a flow rate of 20 ml/h.

4. Estimation of molecular weight

The molecular weight of the enzyme was estimated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE)²²⁾. The gels were stained with silver.

5. Effects of metal ions and ethylenediamine-tetraacetic acid (EDTA)

Effect of Ca^{2+} , Mg^{2+} , Fe^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} , Cu^{2+} or EDTA on neuraminidase activity was examined at 1 mM of each agent. Activity was expressed as the percentage of the value for the control experiment, which did not include the test agents.

6. Effect of pH

Enzymatic activity at different pH levels was compared using acetate buffer (pH 4.0 to 7.0), Tris-maleate buffer (pH 7.0 to 7.5), and Tris-HCl buffer (pH 7.5 to 9.0), each at a final concentration of 0.15 M.

Results and Discussion

Neuraminidase was purified from the culture supernatant of *T. denticola* TN-2 by column chromatography and gel filtration. The purity of the enzyme increased 276-fold with a recovery of 30% (Table 1). The molecular weight was calculated as being 50 kDa (Fig. 1).

The effects of divalent ions and EDTA on neuraminidase activity is summarized in Table 2. Of the metal ions tested, Ca^{2+} markedly stimulated the activity. However, Mg^{2+} exhibited an inhibitory effect. About half the activity was inhibited by Fe^{2+} , Co^{2+} , or Mn^{2+} . Partial inhibition and complete inhibition were caused by Zn^{2+} and Hg^{2+} or Cu^{2+} , respectively. Substantial inhibition was observed by a chelator, EDTA.

Activity was considerably influenced by different pH values of the reaction mixtures (Fig. 2), and reached maximum level at pH 6.5. Activation by Ca^{2+} and slight inhibition by EDTA of the *T. denticola* TN-2 neuraminidase were also observed by the same enzyme isolated from an oral strain of *Propionibacterium acnes*⁴⁾. However, the enzyme from these microorganisms may be distinct from each other, since significantly different properties in molecular weights and optimum pH levels for the reaction were found.

The *in vivo* function of neuraminidase of *T. denticola* remains obscure, however, the activities of neuraminidase and the neuraminidase-related enzyme, considered to originate from oral bacteria, in the human crevicular fluid from patients with per-

Table 1 Purification of neuraminidase of *Treponema denticola* TN-2

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	2,435	62,470	26	1	100
Ammonium sulfate	862	45,084	52	2	72
Q-Sepharose Fast Flow	84	31,600	376	14	51
Sephacryl S-300	2.6	18,650	7173	276	30

Fig. 1 SDS-PAGE of the purified neuraminidase.

Lane A : standard proteins. Values indicate molecular masses (kDa).

Lane B : the purified neuraminidase from Sephacryl S-300.

iodontitis was substantially higher than that of healthy subjects^{23,24}). These observations suggest that neuraminidase from oral microbial residents may be implicated, if not directly, in the onset and/or development of periodontitis.

Table 2 Effect of divalent ions or EDTA on the neuraminidase activity

	Concentration (mM)	Relative activity (%)
None	—	100
Ca ²⁺	1.0	268
Mg ²⁺	1.0	64
Fe ²⁺	1.0	49
Co ²⁺	1.0	50
Mn ²⁺	1.0	49
Zn ²⁺	1.0	27
Hg ²⁺	1.0	0
Cu ²⁺	1.0	0
EDTA	1.0	43

Fig. 2 Relationship between pH and the enzyme activity.

● ; acetate buffer, ■ ; Tris-maleate buffer, ▲ ; Tris-HCl buffer.

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