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研究題目：Interaction between *Fusobacterium nucleatum* and salivary α-amylase

目　的：

Periodontal disease, one of the most common diseases, is a chronic inflammatory disorder caused by multiple periodontopathic pathogens. One of the etiological pathogens, *Fusobacterium nucleatum*, is a Gram-negative anaerobe predominantly isolated in lesions of patients with gingivitis and chronic periodontitis. It coaggregates with various oral bacteria including *Streptococcus* species, *Porphyromonas gingivalis, Treponema denticola*, and *Prevotella* species, resulting in the formation of a biofilm. In addition, *F. nucleatum* is invasive and one of the species frequently detected in amniotic fluid cultures from women with preterm labor and intact membranes. Several kinds of periodontopathic bacteria including *F. nucleatum* have been isolated from carotid endarterectomy specimens of patients undergoing surgical treatment for atherosclerosis.

While the coaggregation among oral bacteria has been well studied in relation to the formation of oral biofilm, a part is known about the effect of saliva. In this study, we examined the interaction between salivary α-amylase and *F. nucleatum*, in an effort to find new ways to control *F. nucleatum* from attaching to tooth and mucosal surfaces, and to prevent infection of oral cavity and causing diseases.

材料および方法：

F. nucleatum ATCC 10953 was used throughout the study. Whole saliva sample was collected from a healthy donor and was used after clarification by centrifugation. SDS-PAGE and Western blotting were performed to analyze salivary proteins. Anti-*F. nucleatum* serum was prepared by immunizing mice with a pipetted extract derived from *F. nucleatum* cells. TOF-mass spectrometric analyses were performed to identify the salivary proteins. Adherence assay of *F. nucleatum* to immobilized amylase was performed using microplates and anti-*F. nucleatum* serum. Copper concentration in saliva was determined spectrophotometrically using 3,5-DiBr-PAESA reaction. Statistical analyses for obtained data were performed by one way ANOVA followed by Dunnett, and P values of <0.05 were considered significant.

結果および考察：

First we identified the salivary components that possessed affinity for *F. nucleatum* by adsorption assay. Human saliva sample adsorbed with *F. nucleatum* cells was subjected to

SDS-PAGE（Fig. 1A）. When the adsorbed saliva was compared with the original saliva, a major band of approximately 110 kDa decreased considerably. This protein was reacted with the anti-amylase antibody in Western blotting（Fig. 1B）, and was identified as α-amylase by mass spectrometric analyses. These results indicate that the 110-kDa band is dimeric form of α-amylase. Next, we examined various conditions to produce a dimeric form of amylase. Salivary amylase(monomer)was purified from whole saliva by Superdex 200 chromatography, and was incubated in the presence of 1 mM of various metal ions and subjected to SDS-PAGE(Fig. 2). Only copper sulfate produced the dimeric form. The amylase was trapped on the bottom of wells in the presence of iron（III）sulfate. No metal ions tested except for copper and iron（III）ions affected the formation of the dimer, and there was only the monomeric form. When we changed the pH or concentration of NaCl in reaction mixture, no dimeric molecules were formed（data not shown）. To characterize the adherence of *F. nucleatum* to α-amylase, the purified α-amylase was coated on the surface of microplate wells and the adhered bacteria were measured by immuno-reaction. When 100 μl of 100 μg/ml α-amylase was used, approximately 18% of bacteria adhered to the monomeric form, and approximately 40% more *F. nucleatum* cells adhered to the dimeric than monomeric α-amylase(Fig. 3).

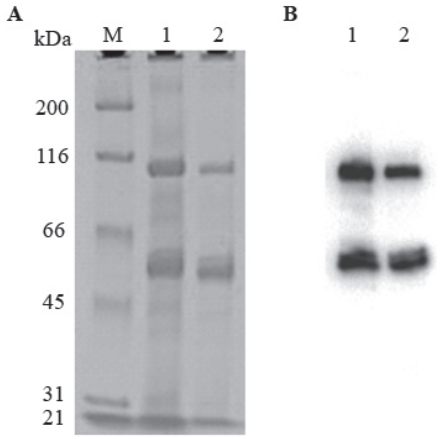

Fig. 1 Identification of salivary components with affinity for *F. nucleatum*.（A）Saliva samples after adsorption with an equalvolume of tris buffered saline（lane 1）or *F. nucleatum* cellsuspension（lane 2）were subjected to 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue. A portion ofmolecular mass standards was applied（lane M）.（B）Saliva samples as in（A）were subjected to 10% SDS-PAGE and transferred to a PVDF membrane. Salivary amylases were visualized using an anti-human amylase monoclonal antibody.

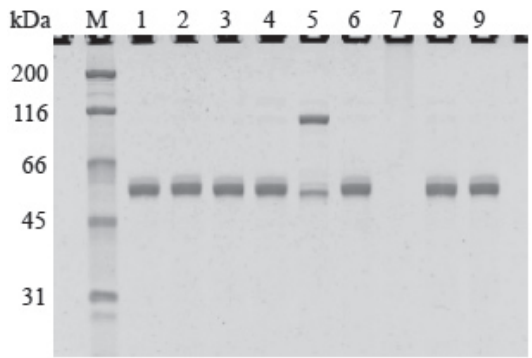

Fig. 2 Effect of metal ions on the formation of dimeric molecules of salivary <-amylase.（A）The purified <-amylase monomer was incubated in the presence of 1 mM of metal ions. Lanes：1, tris buffered saline；2, $CaCl_2$；3, $MgCl_2$；4, $MnCl_2$；5, $CuSO_4$；6, $NiCl_2$；7, $Fe_2(SO_4)_3$；8, $ZnSO_4$；9, EDTA；M, molecular mass standards.

These results indicate that formation of the dimer of α-amylase is predominantly associated with binding to *F. nucleatum*. To examine whether the adherence of *F. nucleatum* is specific to the human salivary α-amylase, we used three other α-amylases and a β-amylase. *F. nucleatum* adhered to all the α-amylases tested to a similar degree, but its adherence to β-amylase was considerably reduced (Fig. 4). To examine the association between copper concentration in saliva and the formation of α-amylase dimer, saliva sample was collected from 8 individuals. Subjects with higher copper concentration in saliva showed thicker bands of w-amylase dimer (Fig. 5).

In conclusion, we demonstrated interaction between *F. nucleatum* and salivary α-amylase. The dimeric form of α-amylase is predominantly associated with *F. nucleatum* binding and the copper ions in saliva might have significant role to form α-amylase dimer.

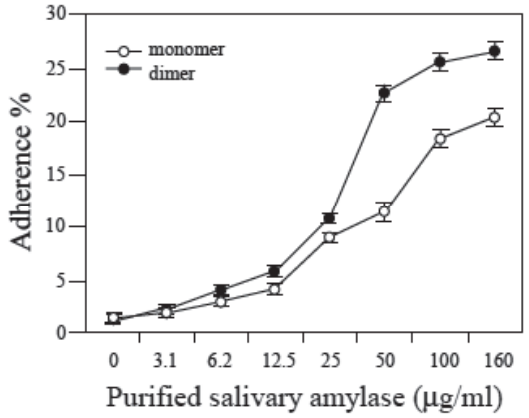

Fig. 3 Adherence of *F. nucleatum* to the salivary <-amylase immobilized on microplate wells. Various amounts of salivary <-amylase were added to microplate wells. After washing and blocking, *F. nucleatum* cells were added. The plate was incubated at room temperature for 30 min and numbers of bacteria adhered were measured using an immuno-reaction with anti-*F. nucleatum* serum. Values indicate a percentage of the number of bacteria applied. Data are expressed as means ± standard deviations of triplicate assays.

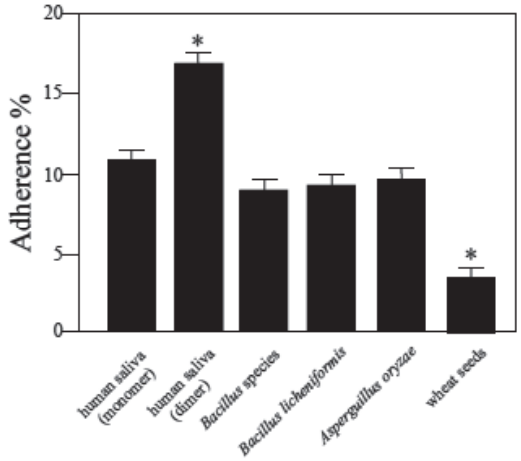

Fig. 4 Adherence of *F. nucleatum* to amylases derived from several sources. Amylases used were human salivary <-amylase, <-amylase from *Bacillus* species, <-amylase from *Bacillus licheniformis*, <-amylase from *Aspergillus oryzae* and Ⓡ-amylase from wheat seeds. Data are expressed as means ± standard deviations of triplicate assays. Asterisks indicate significant differences ($P < 0.01$) from the control value (adherence to human salivary <-amylase monomeric form)

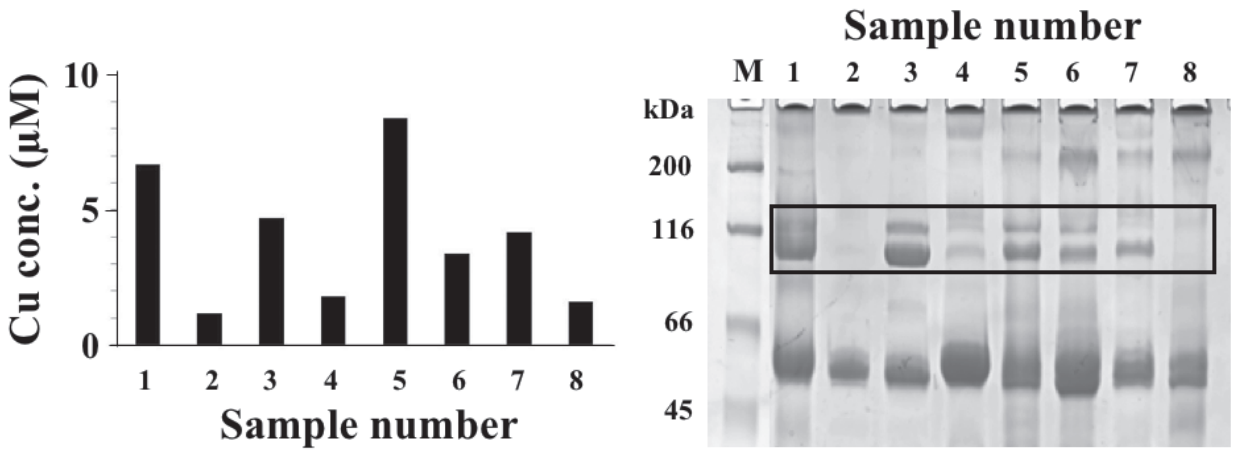

Fig. 5 Association between copper concentration in saliva and the formation of α-amylase dimer. Saliva sample was collected from 8 subjects and copper concentration was determined. The profile of α-amylase dimer was analyzed by SDS-PAGE and Coomassie brilliant blue staining（in rectangle）.

成果発表：

1．第 34 回九州口腔衛生学会総会　（平成 24 年 10 月 7 日，鹿児島市），一般発表。