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Original Article

Rapid detection of Lys-gingipain using fluorogenic peptide substrate for diagnosis of periodontitis

Jeeyeon Park [a†], Seongsoo Kim [b†], Sang-Myung Lee [c], Hanseung Baek [d*], Dong-Sik Shin [a**]

[a] *Department of Chemical and Biological Engineering, Sookmyung Women's University, Seoul, Republic of Korea*
[b] *Biomaterials Research Center, Biomedical Research Division, Korea Institute of Science and Technology, Seoul, Republic of Korea*
[c] *Cantis Inc., Gyeonggi-do, Republic of Korea*
[d] *Oralbiome & Implant Care Center, Apple Tree Dental Hospital, Gyeonggi-do, Republic of Korea*

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KEYWORDS
Fluorogenic peptide substrate;
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Periodontitis;
Protease activity

Abstract *Background/purpose:* Early detection of periodontitis and its associated pathogens has become imperative in oral health. *Porphyromonas gingivalis* (*P. gingivalis*), a leading oral pathogen, is known to secrete Lys-gingipain (Kgp) enzyme. The aim of this study was to validate the detection of *P. gingivalis* in human saliva samples by measuring the fluorescence intensity generated by a Lys-specific peptide substrate after it is cleaved in the presence of Kgp.
Materials and methods: To confirm the biological activity of the fluorogenic His-Glu-Lys containing peptide substrate which is specific to the Kgp, we compared the fluorescence intensity of the fluorogenic peptide substrate with saliva samples obtained from both healthy individuals (n = 31) and periodontitis patients (n = 30).
Results: The results from the fluorogenic substrate were statistically compared with polymerase chain reaction (PCR) results of *P. gingivalis* quantification. Whereas the PCR test had an area under curve (AUC) value of 0.729, the normalized relative fluorescence unit (RFU) obtained from fluorogenic peptide samples had an AUC value of 0.756.
Conclusion: We anticipate that the fluorogenic peptide substrate will assist in detecting early stages of periodontitis and ultimately prevent further complications. This fluorogenic peptide substrate can be used as a basic material for various types of biosensors.

* Corresponding author. Oralbiome & Implant Care Center, Apple Tree Dental Hospital, 1450 Jungang-ro, Ilsanseo-gu, Goyang-si, Gyeonggi-do 10387, Republic of Korea.
** Corresponding author. Department of Chemical and Biological Engineering, Sookmyung Women's University, 100 Cheongpa-ro 47-gil, Yongsan-gu, Seoul 04310, Republic of Korea.
E-mail addresses: framingo@naver.com (H. Baek), dshin@sm.ac.kr (D.-S. Shin).
† These two authors contributed equally to this work.

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Introduction

The degree to which a pathogenic bacterium is involved in periodontitis is classified into five major color-coded complexes: Aa-complex, green, orange-associated, orange and red complex.[1] Among them, *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*) and *Treponema denticolar* (*T. denticolar*) bacterial species are grouped in the "red complex", which are known to be the strongest putative pathogens of periodontal diseases.[2,3] Lys-gingipain (Kgp) is one of the most prevalent cysteine proteinases that originates from *P. gingivalis*, a leading pathogen known to trigger the onset of periodontitis.[4–7] Periodontitis is a chronic oral infectious disease that has affected more than one billion people worldwide.[8] Common symptoms include swollen gums, deepened periodontal pockets and teeth loss among various age groups.[3,4,9] Such results derive from the host undergoing a defense mechanism against the foreign bacteria.[9–11]

Periodontitis may lead to swollen gum and increase risk for complications such as heart disease, stroke, rheumatoid arthritis, and Alzheimer's disease.[12–14] Especially, toxic proteins from gingipains were identified in the brain of Alzheimer's patients, and those from gingipains are correlated with tau and ubiquitin pathology.[13] Further, gingipains were neurotoxic *in vivo* and *in vitro*, exerting detrimental effects on tau, a protein needed for normal neuronal function.[14] A few of the conventional diagnostic methods include polymerase chain reaction (PCR) tests, radiological and clinical examinations.[15,16] However, these methods face limitations regarding quantitative measurement and selectivity of the target bacteria. Some attempts for the rapid detection of *P. gingivalis* were reported including a direct PCR assay and a nanoparticle-based lateral flow assay (LFA) associated with a nucleic acid amplification method.[17,18] However, their costly and time-consuming characteristics reflect their inherent drawbacks, especially to patients who are already in advanced stages of periodontitis. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)-based bioassays for molecular diagnosis have been explored, leading to the emergence of various detection platforms in the medical field.[19] For example, the removal of human genes by CRISPR enzymes may enhance the specificity of bacterial detection in PCR analysis. It is believed that this method could be used for highly sensitive detection of *P. gingivalis* from individual teeth. Meanwhile, the protease-based detection strategy for periodontitis focuses on a simple strategy for rapid detection, i.e., mixing the fluorogenic peptide with filtered saliva samples. This approach enables earlier preventive treatment and leads to more personalized and cost-effective therapies.

Proteases are widely used *in vitro* assays and imaging probe in the presence of chromogenic or fluorogenic substrates.[20–22] The synthetically designed substrate typically has a fluorophore (acceptor signal) and its respective quencher (donor signal).[23] When the fluorophore is quenched, no significant increase in fluorescence emission occurs; however, upon enzymatic hydrolysis, the fluorophore is independently released from its quencher and results in an increase in fluorescence emission.[24] This shift from excitation to emission wavelength is indicative of a successful peptide cleavage activity. In particular, Kgp bacterial protease has a unique catalytic characteristic in that it specifically cleaves Lys peptides.[25–27]

The change in fluorescence activity can then be detected using a microplate fluorescence plate reader set at a specific range of excitation and emission wavelength and indirectly indicate the level of enzymatic activity. The activated thiol group of Kgp cysteine protease acts as a nucleophile and attacks the carbonyl carbon of the amide group, cleaving the peptide bond (Fig. 1a).[28] Therefore, its enzymatic activity can be measured by analyzing its reaction with a peptide substrate containing a Lys residue.[29]

We hypothesize that the acquired fluorescence value from the Lys-specific substrate upon cleavage by Kgp can be applied to verify the effects of *P. gingivalis* in periodontitis. Hence, Dabcyl-His-Glu-Lys-Lys (FITC)-OH peptide with 4-[4-(dimethylamino)phenylazobenzoic acid] (Dabcyl) as a quencher and fluorescein-isothiocyanate (FITC) as a fluorophore served as a fluorogenic peptide substrate. Kgp recombinant protease was used as a simple and non-invasive biomarker to assess quantitatively its enzymatic activity (Fig. 1b). Based on this peptide cleavage specificity, Kgp may serve as a target enzyme for diagnostic as well as therapeutic studies of periodontal disease.

Materials and methods

Reagents and instruments

Fluorogenic peptide substrate Dabcyl-His-Glu-Lys-Lys (FITC)-OH was purchased from GL Biochem (Shanghai, China). Lys-gingiapin (Kgp) recombinant protein and Arg-gingipain (Rgp) recombinant protein were purchased from MyBioSource (San Diego, CA, USA). Cathepsin B from human liver buffered aqueous solution, cathepsin K active human recombinant, cathepsin S from human recombinant and trypsin from porcine pancreas were purchased form Sigma–Aldrich (St. Louis, MO, USA). Sterile syringe filter (25CS45AS) was purchased from ADVANTEC (Tokyo, Japan). Fluorescence data were obtained using a microplate reader (SpectraMax i3x, Molecular Devices, San Jose, CA, USA) at the Chronic and Metabolic Diseases Research Center for Sookmyung Women's University.

General measurement of fluorescence activity

Fluorescence value was measured at emission wavelength and excitation wavelength, at 485 nm and 535 nm, respectively. Relative fluorescence unit (RFU) value was obtained by subtracting the acquired fluorescence unit

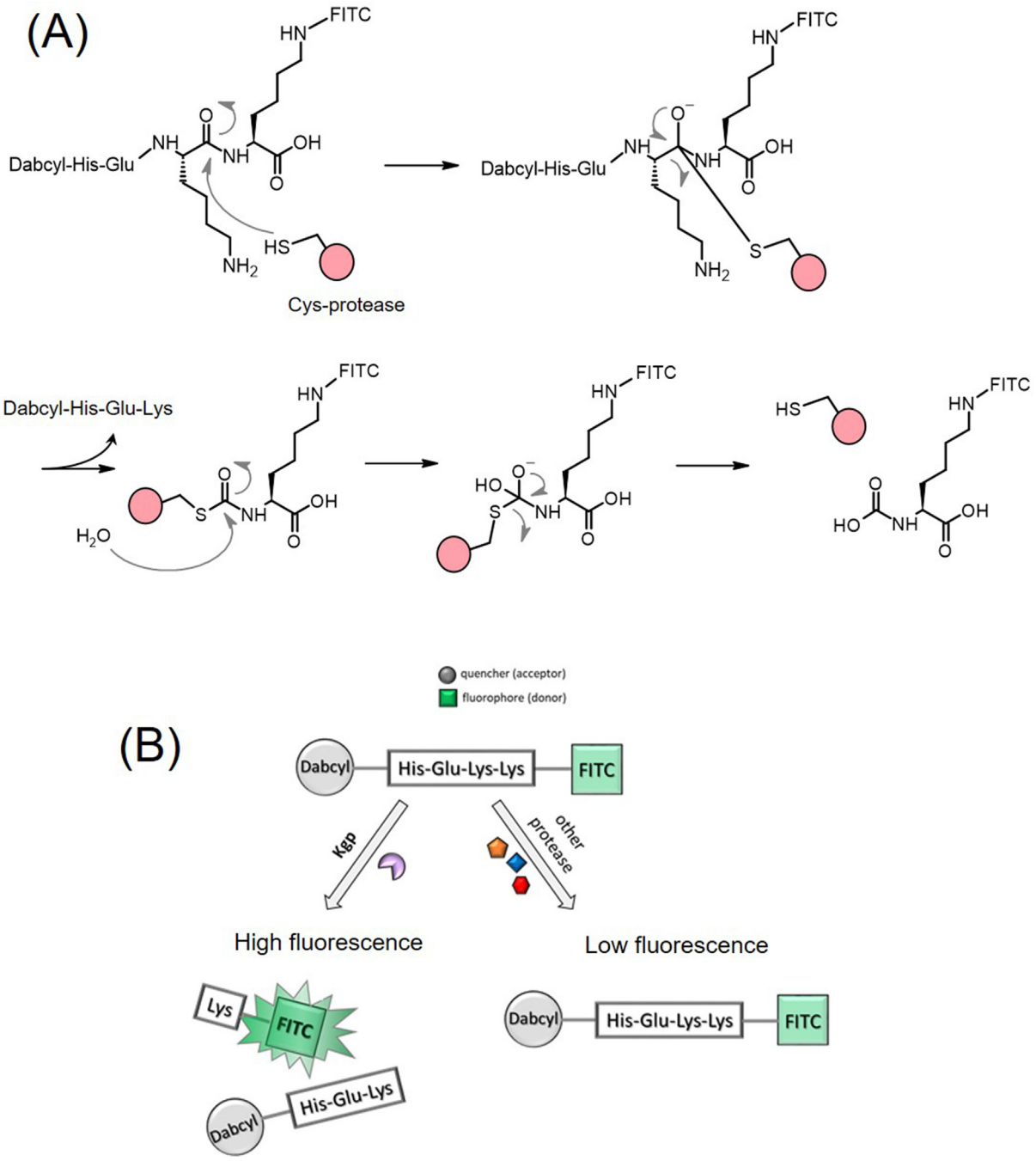

Figure 1 Mechanism of peptide cleavage upon Lys-gingipain (Kgp) enzyme reaction. (A) Fluorogenic peptide substrate cleavage in the presence of a cysteine protease. (B) Detection of Lys-gingipain (Kgp) enzymatic activity using a fluorogenic peptide substrate.

value in the presence of Kgp from its background fluorescence unit value when only the substrate was present.[30]

Optimization of reaction condition for the fluorogenic peptide substrate

The solutions of the fluorogenic peptide substrate were prepared in 100 μL PBS solution with the following concentrations: 0, 5, 10, 20, 40, 50, 100, 500 and 1000 μM. The RFU values were measured again after adding 100 μL of 90.88 nM Kgp solution diluted in phosphate buffered saline (PBS) solution. The experiments were performed in triplicates.

Calculation of Kgp enzymatic kinetics with the fluorogenic peptide substrate

RFU results of both the (L)-form and (D)-form fluorogenic peptide substrates were obtained after 8 min reaction of 90.88 nM Kgp. Prism 7.0 was used to perform non-linear regression analyses of Michaelis—Menten curves.

Fluorescence measurement assay for various Kgp concentrations

The normalized RFU values of the 50 μM fluorogenic peptide substrate reaction with various Kgp concentrations for 8 min were measured. Kgp concentrations were prepared at 0, 0.097, 0.19, 1.56, 3.25, 5, 12.5, and 25 μg/mL.

Measurement of Kgp activities from saliva samples

Human derivative study was approved by the Institutional Review Board at Sookmyung Women's University, and all subjects provided written informed consent (IRB No. SMWU-2109-BR-095-01). Real-time polymerase chain reaction (RT-PCR) data were acquired by Denomics (Busan, South Korea). Human saliva samples (1—2 mL) from 61 subjects (31 healthy individuals and 30 periodontitis patients) were collected in a Falcon tube and stored in an 80 °C freezer until further analyses. Using a sterilized syringe filter, each saliva sample was filtered, and at least 600 μL of the supernatant was extracted. The extracted samples were then diluted two-fold using a PBS solution. Next, 100 μL of the diluted saliva sample was added to a 96-well plate, followed by the addition of 100 μL of 50 μM fluorogenic peptide substrate solution to each well. Fluorescence activity data were acquired at 27 °C for 8-min incubation.

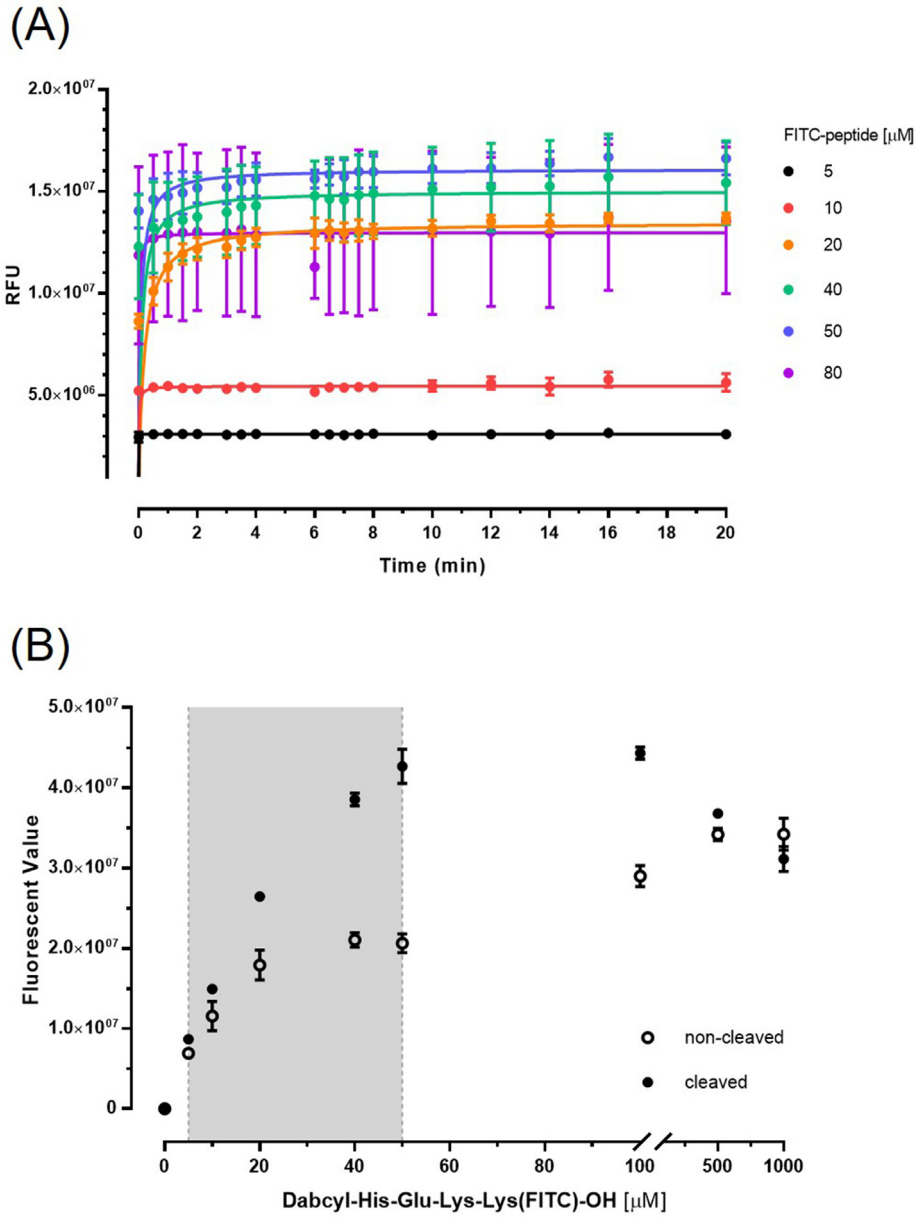

Figure 2 (A) Relative fluorescence unit (RFU) of fluorogenic peptide was measured at concentrations ranging from 5 to 80 μM in the presence of Lys-gingipain (Kgp). (B) Fluorescence values of the fluorogenic peptide substrate were measured at various concentrations after 8 min of incubation with and without Lys-gingipain (Kgp), representing the cleaved and non-cleaved states, respectively.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software (Boston, MA, USA). One-way ANOVA with Tukey's multiple comparisons, two-way ANOVA with Sidak's multiple comparisons, Mann—Whitney tests, and unpaired t-tests were used to compare all data. The type of analysis, number of samples, and *P*-values are provided in the figure captions of the corresponding graphs.

Results

Optimization of reaction condition for the fluorogenic peptide substrate

To optimize the substrate—enzyme reaction time, RFU values of the fluorogenic peptide substrate were measured in the presence of Kgp. The fluorescence activity results were obtained by measuring the increasing fluorogenic peptide substrate concentration against a constant Kgp concentration in real time. We found the fluorescence reached a plateau after 8 min (Fig. 2a). To optimize the concentration of the fluorogenic peptide substrate, RFU values of the substrate solutions with various concentrations were measured both in the absence and presence of Kgp. Among the various concentrations, the addition of Kgp to 50 μM substrate concentration resulted in the highest RFU value (Fig. 2b). Substrates with concentrations exceeding 50 μM resulted in a relatively lower RFU as its value started to decrease at 500 μM. This behavior, in which the RFU decreases as the substrate concentration increases, can be explained by the inner-filter effect.[30] The fluorescence activity of the peptide substrate cannot be properly measured when an excessive concentration of substrate is present due to its self-quenching effect from its fluorophore.[31,32] Hence, the fluorogenic peptide substrate was fixed at 50 μM for the further experiments.

Calculation of Kgp enzymatic kinetics with the fluorogenic peptide substrate

In order to quantitatively assess the enzyme—substrate interaction between Kgp protease and fluorogenic peptide substrate, enzyme kinetics were analyzed by performing assays. Substrate specificity was verified by comparing the RFU results of both the (L)-form and (D)-form fluorogenic peptide substrates. Since natural chiral amino acids have an (L)-form configuration (D)-form peptides should not be affected by proteases that selectively cleave (L)-form peptide substrates. The average RFUs of the (D)-form isomer were significantly lower than those of the original (L)-form fluorogenic peptide substrate at all concentrations upon the reaction of Kgp. This confirms that Kgp selectively cleaves the (L)-form substrate (Fig. 3a). Especially, the average RFU of 50 μM fluorogenic peptide and its (D)-form isomer were 1.59×10^7 and 2.04×10^6, respectively. In addition, non-linear regression analyses of Michaelis—Menten curves using Prism 7.0 was used to compare the K_m values of both substrates.[33] The initial velocity (3.5 min) was plotted against various substrate concentrations. A higher V_{max} (8.50×10^5 units min^{-1}) and lower

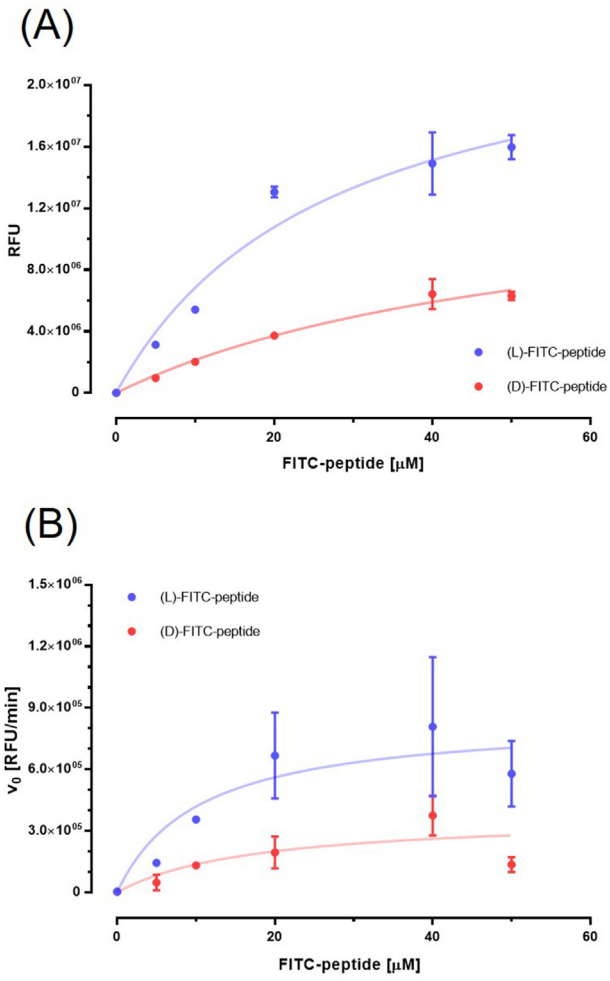

Figure 3 Relative fluorescence unit (RFU) of the fluorogenic peptide and its isomer was measured in the presence of Lys-gingipain (Kgp). (A) Relative fluorescence unit (RFU) was measured at various concentrations for both the (L)- and (D)-form substrates after 8-min reaction with Lys-gingipain (Kgp). (B) The initial velocity (v_0) was plotted against various concentrations of the fluorogenic peptide substrate.

K_m (10.32 μM) value for (L)-form fluorogenic peptide while a lower V_{max} (3.75×10^5 units min^{-1}) and higher K_m (17.51 μM) value for its (D)-isomer peptide substrate (Fig. 3b).

Fluorescence measurement assay for various Kgp concentrations

The normalized RFU curve of the fluorogenic peptide substrate upon various Kgp protease concentrations was measured to determine the appropriate Kgp concentration for the substrate. The RFU showed a plateaued curve when Kgp was prepared at a concentration higher than 4.0 μg/mL. However, a linear curve ($R^2 = 0.9854$) was observed when Kgp concentration was prepared at 0, 0.097, 0.19, 1.56, and 3.25 μg/mL (Fig. 4). As a result, 4.0 μg/mL of Kgp and 50 μM of the fluorogenic peptide substrate were set as the appropriate working concentrations.

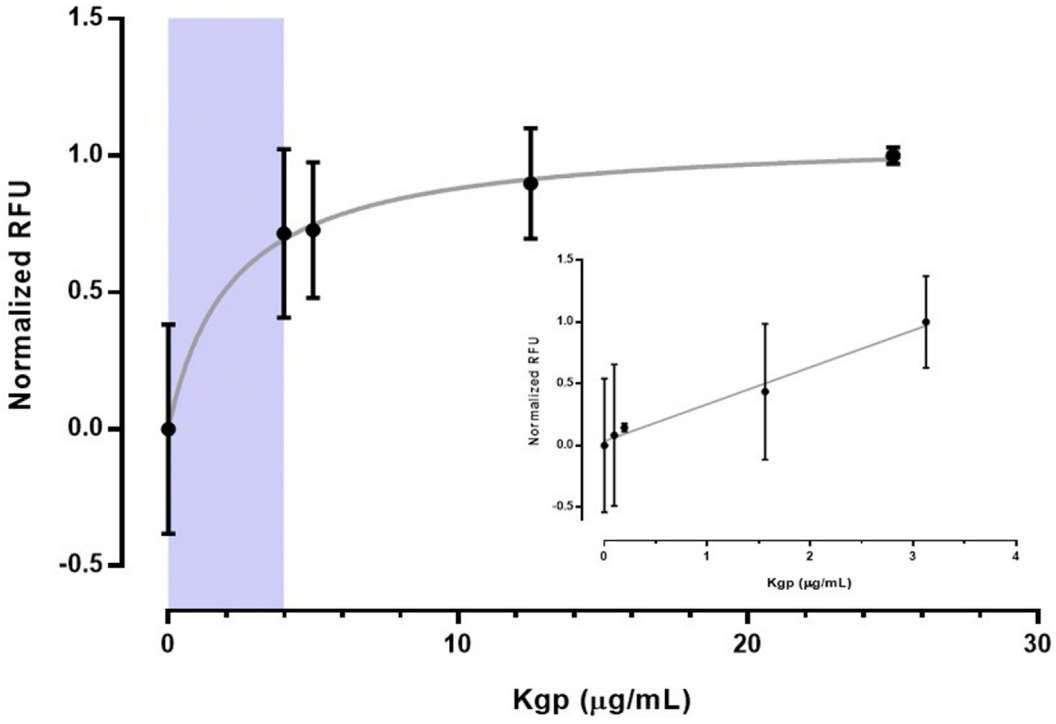

Figure 4 Normalized relative fluorescence unit (RFU) of fluorogenic peptide substrate at various Lys-gingipain (Kgp) concentrations.

Additionally, human proteases, including various types of cathepsins, were also tested with the fluorogenic peptide substrate to prove its specificity.[34–36] The normalized RFU of the fluorogenic peptide substrate in the presence of various proteases that are present in human saliva was compared with that of Kgp.[27] The following protease were used: Rgp, Cathepsin B, Cathepsin D, Cathepsin K, Cathepsin S, and Trypsin, with concentrations of 75.75, 83.28, 85.20, 4.0, and 84.0 nM, respectively. The RFU of various oral proteases was measured at 8 min of incubation and compared with that of 90.88, 80.0, 70.0, and 4.0 nM Kgp (Fig. 5). The data showed that the RFU of Kgp was the highest among the six proteases, regardless of the Kgp concentration. The RFUs of the Kgp samples imply that the fluorogenic peptide selectively reacted with Kgp, whereas it did not fully interact with other proteases.

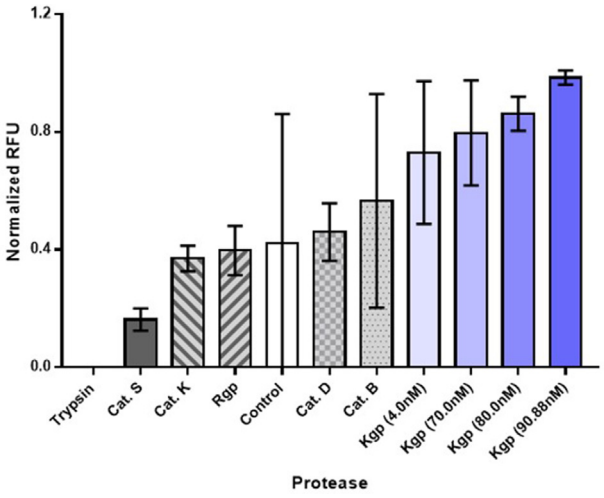

Figure 5 Normalized relative fluorescence unit (RFU) of fluorogenic peptide substrate in the presence of various oral proteases. Concentration of each protease sample was prepared at 70–90 nM.

Measurement of Kgp activities from saliva samples and statistical analysis

Human saliva samples were evaluated under optimized working concentration with the fluorogenic substrate. When the filtered saliva samples were reacted with the fluorogenic substrate, the mean value of normalized RFU for normal and patient subjects were 0.43 ± 0.041 and 0.60 ± 0.035, respectively (Fig. 6a). The normalized RFU data demonstrate a higher value among patient samples than that of normal samples, which further validates the high selectivity of the fluorogenic peptide substrate for Kgp protease. As a control, the number of DNA copies and types of periodontal pathogens for all 61 samples were obtained by RT-PCR test. Patient subjects had 2.5 folds more *P. gingivalis* DNA copies (5.95×10^6) than normal subjects (2.35×10^6) (Fig. 6b). These data show that both detection methods can distinguish between patient and normal samples with a *P*-value lower than 0.01. However, these methods need to be improved by optimizing reaction conditions for clinical samples to reduce variability, including the occurrence of some outliers.

For the individual characterization of normal or patient samples, an area under curve (AUC) receiver operating

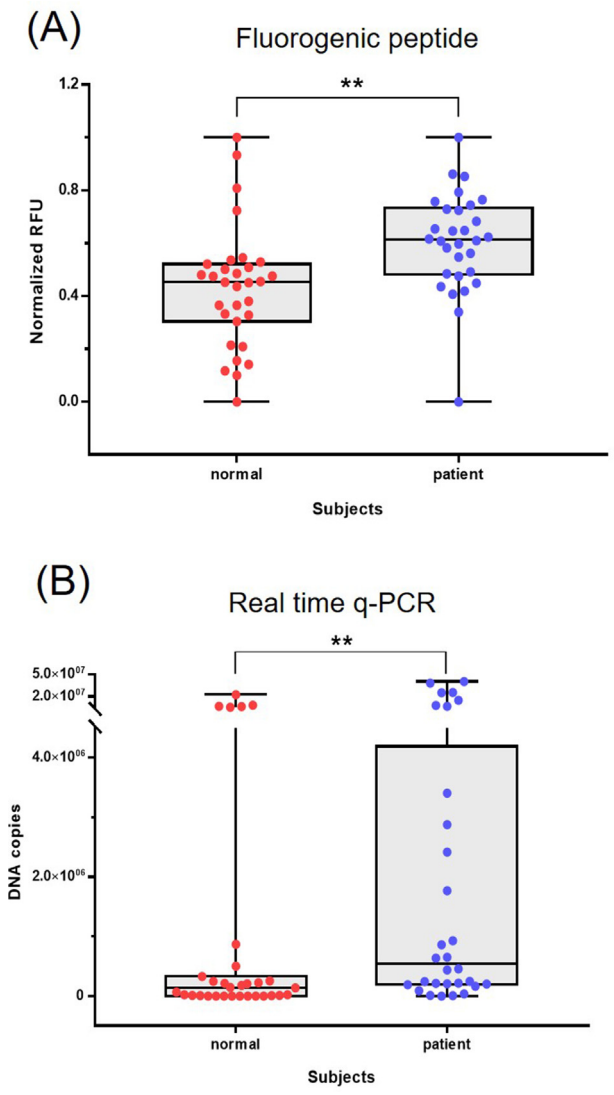

Figure 6 Box and whisker plots were generated from the data using the Lys-gingipain (Kgp) fluorogenic peptide substrate and DNA copies of *Porphyromonas gingivalis (P. gingivalis)* pathogen from all 61 subjects. The data were acquired from (A) the normalized relative fluorescence unit (RFU) value of all 61 subjects using the fluorogenic peptide, and (B) real-time quantitative polymerase chain reaction (RT-PCR).

characteristic (ROC) curve was generated to analyze the correlation between two independent group designs based on all 61 subjects. We compared the performance of the number of *P. gingivalis* DNA copies measured by real-time PCR and the normalized RFU of the fluorogenic peptide substrate after the addition of saliva samples detected by a microplate reader. The AUC values of PCR test and normalized RFU acquired from fluorogenic peptide samples were 0.729 and 0.756, respectively, with both *P*-values lower than 0.05 (0.0021 and 0.0006, respectively) (Fig. 7). Such calculated values by the ROC curve reflect an acceptable discrimination parameter as both the values are above 0.70. This confirms that the fluorogenic peptide substrate is a potential diagnostic biomarker for differentiating between normal and periodontitis samples.

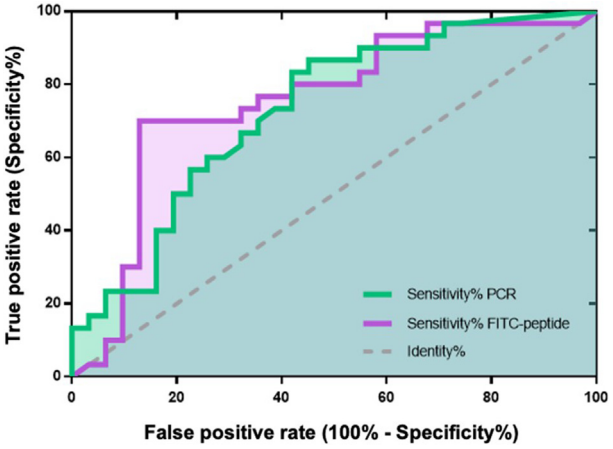

Figure 7 The receiver operating characteristic (ROC) curves were generated to compare the results of the real-time quantitative polymerase chain reaction (RT-PCR) test (in green) and the normalized relative fluorescence unit (RFU) of the fluorogenic peptide substrate (in magenta). The relative fluorescence unit (RFU) data from the fluorogenic peptide substrate were acquired after 8-min incubation.

To elucidate the correlation between PCR and the fluorescence data, the scales of the data were normalized because the dimensions between PCR and the fluorescence data were different. To compute the nonlinear correlation, the Spearman correlation coefficients (r_s) were calculated as 0.83, 0.81, and 0.70 for the normal and patient groups, and all subjects, respectively (Fig. 8). If the r_s value is between 0.60 and 0.79, the correlation is strong, while the r_s value between 0.80 and 1.00 indicates a very strong correlation.[37] These r_s values represent strong correlation for the PCR and the fluorescence data.

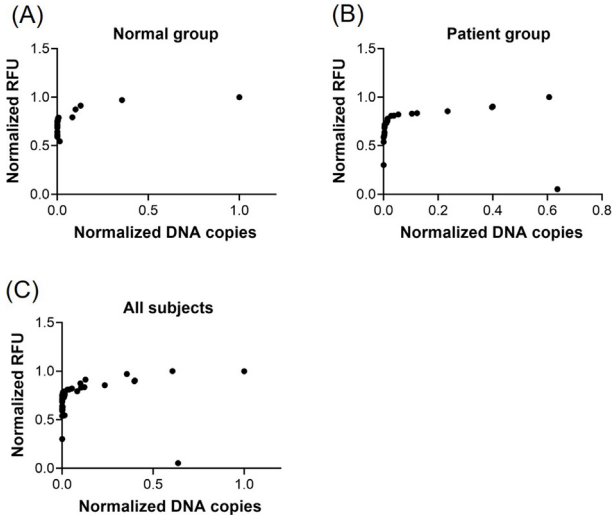

Figure 8 Correlations between the real-time quantitative polymerase chain reaction (RT-PCR) and the normalized relative fluorescence unit (RFU) were obtained from (A) normal group (B) patient group, and (C) all subjects.

Discussion

Lys-gingipain (Kgp) is a major virulence factor of the disease, yet it is challenging to make sensitive and convenient substrate. We designed simple and rapid early detection system of periodontitis by proteolyzing lysine-specific peptides in the presence of Kgp. Proteolysis capacity of fluorogenic peptide Dabcyl-His-Glu-Lys-Lys (FITC)-OH was determined by inner-filter effect in which the RFU decreases as the substrate concentration increases. The fluorescence activity of the peptide substrate cannot be properly measured when an excessive concentration of substrate is present due to its self-quenching effect from its fluorophore. From the comparison of fluorescent value of fluorescent substrate with and without Kgp cleavage, the apparent proteolytic activity of Kgp upon Lys—Lys in Dabcyl-His-Glu-Lys-Lys (FITC)-OH peptide was observed.

Many methods to detect periodontitis include DNA probe assays, enzyme assays, real-time PCR, and immunofluorescences. Real-time PCR is one of the accurate detection methods to identify nucleotide changes, but it is time-consuming and costly. Furthermore, there are conceivable probabilities of cross-reaction with other species and it does not reflect bacterial activity. Low and heterogeneous values obtained from real-time quantitative PCR indicate an insufficient distinctness between healthy and diseased subjects. However, Kgp-proteolyzed cleavage method of fluorogenic peptide substrate showed less heterogeneous values than real-time quantitative PCR. Taking the results for analysis of specificity and sensitivity into account, the benefit of RT-PCR appears questionable. Heterogeneous and low values indicate an insufficient distinctness between healthy and diseased conditions for RT-PCR.

ROC curves were drawn to determine the cut-off based on the proteolyzed fluorogenic peptide against the RT-PCR. The AUC was derived from the ROC curve that determined the ability of the fluorogenic peptide to discriminate between periodontitis patients and healthy subjects. The normalized RFU of fluorogenic peptide substrate (AUC value of 0.756) and the number of *P. gingivalis* DNA copies measured by RT-PCR (AUC value of 0.729) showed moderate discrimination. In the next study, *P. gingivalis*-focused tests will be performed, such as serially diluting *P. gingivalis* and optimizing its bacterial concentration for detection. In addition, even though the Spearman correlation coefficients are more than 0.70 for all subjects, they can be improved after testing a greater number of samples.

In conclusion, we designed a simple and non-invasive method of detecting periodontitis using a Kgp-sensitive fluorogenic peptide substrate. We optimized the substrate concentration by comparing the RFU and normalized RFU at various concentrations of the substrate. As a result, we found that 50 μM of the fluorogenic peptide substrate showed the highest normalized RFU. Among the various cysteine, aspartic, and serine proteases, Kgp exhibited the highest RFU, confirming its substrate specificity. Furthermore, saliva samples from 61 human subjects (31 healthy individuals and 30 periodontitis patients) were quantitatively analyzed to demonstrate that Kgp is a promising biomarker for the *P. gingivalis* pathogen. The AUC values acquired from both the RT-PCR test and our fluorescence test were 0.729 and 0.756, respectively. In addition, the Spearman correlation coefficients (r_s) were calculated as 0.83, 0.81, and 0.70 for the normal and patient groups, and all subjects, respectively. Even though this approach needs further optimization of reaction conditions for clinical samples, this design of the fluorogenic peptide substrate reacting with Kgp can be used as a simple diagnostic biomarker to detect *P. gingivalis*. We envision that this fluorogenic peptide substrate can be used as a basic material for various types of biosensors.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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