สัญญาเลขที่ RSA5680010

รายงานวิจัยฉบับสมบูรณ์

ผลของจินจิเพนต่อการแสดงออกของทรีฟอยล์แฟคเตอร์สาม

โดย
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยขอนแก่น

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ สกว. และมหาวิทยาลัยขอนแก่นที่ให้การสนับสนุนทุนสำหรับโครงการวิจัยนี้และขอขอบคุณอาสาสมัครทุกคนที่ได้เข้าร่วมโครงการวิจัยนี้ คณะผู้วิจัยขอขอบคุณ ศาสตราจารย์ ดร. วัชระ กสินฤกษ์ ที่ได้ให้ความอนุเคราะห์ช่วยผลิตแอนติบอดีสำหรับนำมาใช้งานในการทดลองด้วยวิธีอีไลซ่า คณะผู้วิจัยขอขอบคุณ ทันตแพทย์หญิง อมลรุจี คำพิชัย และ Dr. Ranuch Tak ที่ช่วยเก็บตัวอย่างน้ำลายจากอาสาสมัคร และขอขอบคุณ คุณ วราภรณ์ พันธ์พรหม ที่ช่วยประสานงานในการจัดเตรียมตัวอย่างน้ำลายและอุปกรณ์วิจัย คณะผู้วิจัยขอขอบคุณ ฝ่ายวิจัย คณะทันตแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ที่อำนวยความสะดวกสำหรับการทำวิจัยในห้องปฏิบัติการ คณะผู้วิจัยขอขอบคุณ สถาบันจีโนม ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพที่ให้การสนับสนุนการทำวิจัยในห้องปฏิบัติการทางด้านโปรติโอมิกส์

บทคัดย่อ

สัญญาเลขที่ RSA5680010

ชื่อโครงการ ผลของจินจิเพนต่อการแสดงออกของทรีฟอยล์แฟคเตอร์สาม

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วัตถุประสงค์

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินผลของจินจิเพนต่อการแสดงออกของทรีฟอยล์แฟคเตอร์สาม วัตถุประสงค์ในระดับโมเลกุลเพื่อประเมินผลการย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามด้วยเอนไซม์จินจิเพน วัตถุประสงค์ในระดับเซลล์เพื่อประเมินผลการยับยั้งการแสดงออกของทรีฟอยล์แฟคเตอร์สามในเซลล์จากเนื้อเยื่อบุเหงือกด้วยเอนไซม์จินจิเพน วัตถุประสงค์ในระดับคลินิกเพื่อประเมินระดับโมเลกุลทรีฟอยล์แฟคเตอร์สามและเอนไซม์จินจิเพนในน้ำลายผู้ป่วยโรคปริทันต์อักเสบก่อนทำการรักษาและภายหลังการรักษาโรคปริทันต์

วัสดุและวิธีการดำเนินการวิจัย

เครื่องวัดมวลสารแมส-สเปคโตรเมตทรีถูกใช้เพื่อประเมินผลการย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามด้วยเอนไซม์จินจิเพน เรียลไทม์อาร์ทีพีซีอาร์และเวสเทิร์นบลอทถูกใช้เพื่อประเมินผลการยับยั้งการแสดงออกของทรีฟอยล์แฟคเตอร์สามในเซลล์จากเนื้อเยื่อบุเหงือกด้วยเอนไซม์จินจิเพน อีไลซ่าถูกใช้เพื่อวัดระดับโมเลกุลทรีฟอยล์แฟคเตอร์สามและเอนไซม์จินจิเพนในน้ำลายผู้ป่วยโรคปริทันต์อักเสบก่อนทำการรักษาและภายหลังการรักษาโรคปริทันต์ เรียลไทม์ทีพีซีอาร์ถูกใช้เพื่อวัดปริมาณเชื้อพีจินจิวาริส

ผลการศึกษา

การศึกษาในระดับโมเลกุลแสดงให้เห็นว่าเอนไซม์จินจิเพนสามารถย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สาม คณะผู้วิจัยพบว่าเครื่องวัดมวลสารชนิดมัลดิทอฟ-ทอฟ สามารถใช้ประเมินรูปแบบการย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามด้วยเอนไซม์จินจิเพนแต่ไม่สามารถระบุชัดเจนถึงชิ้นส่วนของโมเลกุลทรีฟอยล์แฟคเตอร์สามที่ถูกย่อยสลายมีลำดับกรดอะมิโนอะไรบ้าง ในการระบุลำดับกรดอะมิโนของชิ้นส่วนโมเลกุลทรีฟอยล์แฟคเตอร์สามที่ถูกย่อยสลายคณะผู้วิจัยเลือกใช้เครื่องวัดมวลสารชนิดแอลซีเอ็มเอสและพบว่าเอนไซม์จินจิเพนสามารถย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามในบริเวณที่เป็นทรีฟอยล์แฟคเตอร์โดเมน ผลการศึกษาสอดคล้องกับสมมุติฐานที่ได้ตั้งไว้ สำหรับข้อพิจารณาทางคลินิกมีประเด็นที่น่าสนใจสำหรับการทำวิจัยต่อไปคือภาวะในช่องปากซึ่งมีน้ำลายเป็นองค์ประกอบที่สำคัญเอนไซม์จินจิเพนจะย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามได้หรือไม่ สำหรับการศึกษาในระดับเซลล์คณะผู้วิจัยพบว่าเอนไซม์จินจิเพนยับยั้งการแสดงออกของเอ็มอาร์เอนเอของยีนทรีฟอยล์แฟคเตอร์สาม

ในเซลล์จากเนื้อเยื่อบุเหงือกโดยการยับยั้งนี้เกิดขึ้นที่บางความเข้มข้นของเอนไซม์จินจิเพนและผลการยับยั้งของเอนไซม์จินจิเพนไม่ได้เกี่ยวข้องกับกระบวนการตายของเซลล์เนื่องจากไม่ปรากฏการแสดงออกของโมเลกุลที่เกี่ยวข้องกับการตายของเซลล์ ได้แก่ คลิฟพาร์พและเคสเปสสาม ผลการศึกษาดังกล่าวสอดคล้องกับสมมุติฐานที่ได้ตั้งไว้ สำหรับการศึกษาในระดับคลินิกคณะผู้วิจัยพบว่าก่อนการรักษาโรคปริทันต์ผู้ป่วยโรคปริทันต์อักเสบมีระดับของโมเลกุลทรีฟอยล์แฟคเตอร์สามและเอนไซม์จินจิเพนในน้ำลายต่ำกว่าอาสาสมัครกลุ่มควบคุม ภายหลังการรักษาโรคปริทันต์เสร็จแล้วผู้ป่วยโรคปริทันต์อักเสบมีระดับของโมเลกุลทรีฟอยล์แฟคเตอร์สามลดลงแต่ระดับเอนไซม์จินจิเพนไม่แตกต่างจากเอนไซม์จินจิเพนก่อนที่ผู้ป่วยได้รับการรักษา นอกจากนี้คณะผู้วิจัยยังพบการมีอยู่ของเชื้อพีจินจิวาริสในน้ำลายของผู้ป่วยโรคเหงือกอักเสบและในน้ำลายของอาสาสมัครกลุ่มควบคุม ผลการศึกษาในระดับคลินิกไม่สอดคล้องกับสมมุติฐานที่ตั้งไว้โดยแสดงให้เห็นว่าการรักษาโรคปริทันต์อักเสบไม่มีผลต่อการเพิ่มระดับของโมเลกุลทรีฟอยล์แฟคเตอร์สามหรือลดระดับเอนไซม์จินจิเพนในน้ำลายของผู้ป่วยโรคปริทันต์อักเสบ

บทสรุปและวิจารณ์

จากผลการศึกษาตามวัตถุประสงค์ข้อแรกคณะผู้วิจัยพบว่าเอนไซม์จินจิเพนสามารถย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์สามและผู้วิจัยได้ทำการทดลองเพิ่มเติมและพบว่าเอนไซม์จินจิเพนสามารถย่อยสลายโมเลกุลทรีฟอยล์แฟคเตอร์หนึ่งและสองได้เช่นกัน ข้อมูลที่ได้ทั้งสองส่วนถูกใช้ในการเตรียมบทความต้นฉบับ(ตามเอกสารแนบโปรดดูในภาคผนวก) จากผลการศึกษาตามวัตถุประสงค์ข้อที่สองคณะผู้วิจัยพบว่าเอนไซม์จินจิเพนสามารถยับยั้งการแสดงออกของเอ็มอาร์เอนเอของยีนทรีฟอยล์แฟคเตอร์สามในเซลล์จากเนื้อเยื่อบุเหงือกโดยการยับยั้งนี้ไม่ได้เกี่ยวข้องกับกระบวนการตายของเซลล์ อย่างไรก็ตามคณะผู้วิจัยต้องการที่จะทำการศึกษาเพิ่มเติมเพื่อให้ได้มาซึ่งคำอธิบายเกี่ยวกับกลไกที่เอนไซม์จินจิเพนใช้ยับยั้งการแสดงออกของเอ็มอาร์เอนเอของยีนทรีฟอยล์แฟคเตอร์สาม จากผลการศึกษาตามวัตถุประสงค์ข้อที่สามคณะผู้วิจัยพบว่าเชื้อพีจินจิวาริสในน้ำลายของผู้ป่วยโรคเหงือกอักเสบและในน้ำลายของอาสาสมัครกลุ่มควบคุมและการรักษาโรคปริทันต์อักเสบไม่มีผลต่อการเพิ่มระดับของโมเลกุลทรีฟอยล์แฟคเตอร์สามหรือลดระดับเอนไซม์จินจิเพนในน้ำลายของผู้ป่วยโรคปริทันต์อักเสบดังนั้นการศึกษาต่อไปที่น่าสนใจคือการศึกษาบทบาทของเชื้อพีจินจิวาริสในน้ำลายจะแตกต่างจากเชื้อพีจินจิวาริสในร่องเหงือกและกระเป๋าปริทันต์ของผู้ป่วยโรคเหงือกอักเสบและผู้ป่วยโรคปริทันต์อักเสบหรือไม่

คำหลัก จินจิเพน; ผู้ป่วยโรคปริทันต์อักเสบ; น้ำลาย; ทรีฟอยล์แฟคเตอร์

Abstract

Project Code : RSA5680010

Project Title : Effects of gingipains on expression of trefoil factor 3

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Project Period : 3 years (June 2014-May 2016)

Objectives: The present study was aimed to determine the effects of gingipains on trefoil factor 3 (TFF3). At the molecular level, the objective was to investigate the proteolytic activity of gingipains on degradation of TFF3 peptides. At the cellular level, the objective was to investigate the inhibitory activity of gingipain on expression of TFF3 in primary cultured human gingival epithelial cells. At the clinical level, the objective was to investigate levels of TFF3 and gingipains in saliva from chronic periodontitis patients before, and after periodontal disease treatments.

Materials and Methods: The proteolytic effect of gingipains on TFF3 was determined by mass spectrometry (MS). The inhibitory effect of gingipain on expression of TFF3 in primary cultured human gingival epithelial cells was evaluated by real-time RT-PCR and Western blotting techniques. Levels of TFF3 and gingipains in saliva from chronic periodontitis patients before and after periodontal treatments were measured by ELISA. Number of *P.gingivalis* was evaluated by real time PCR.

Results: At the molecular study level, our observations provided new information that gingipains were able to digest human TFF3 peptides. MALDI-TOF/TOF MS could be used for evaluating patterns of TFF3 digestion by gingipains but not for identification of digested TFF3 fragments. LC-MS could identify digested TFF fragments. At the cellular study level, our findings demonstrated that gingipains inhibited the expression of TFF3 mRNA, and inhibited the expression of TFF3 peptides with a dose response manner in human gingival epithelial cells. To verify whether the reduction of *TFF3* mRNA expression is related to the apoptotic effects by gingipains, expression of apoptotic marker molecules such as cleaved PARP and caspase 3 were measured, using Western blotting technique. No detectable levels of these two apoptotic markers were observed from HGEP treated cells. These findings suggest that reduction of TFF3 mRNA was not influenced by apoptotic effects of gingipains. At the clinical study level, we observed that levels of salivary TFF3 and gingipains (RgpB) were significantly reduced in chronic periodontitis patients before periodontal treatment, compared with the control group.

Levels of salivary TFF3 in chronic periodontitis patients were also decreased after periodontal treatment, whereas salivary gingipains were not decreased. Our observations demonstrated that the presence of *P. gingivalis* could be detected in whole saliva samples of periodontally healthy, gingivitis, and periodontitis patients. Our findings demonstrated that periodontal treatments did not affect the levels of salivary RgpB in periodontitis and non-periodontitis subjects.

Conclusion and discussion: Regarding the first objective, we confirm the proteolytic activity of gingipains on degradation of TFF3 peptides. We also performed additional experiments by investigating the proteolytic effects of gingipains on TFF1 and TFF2 peptides. These results were combined with the findings from this present study for preparation of the first manuscript (please see the attached document in the appendix). Regarding the second objective, we confirm that the inhibitory activity of gingipains on expression of TFF3 mRNA in primary cultured human gingival epithelial cells is not influenced by an apoptotic mechanism. However, further experiments are needed to clarify how gingipains down-regulates the expression of TFF3. Regarding third objective, we demonstrate that periodontal treatments do not affect the levels of salivary TFF3 and RgpB in periodontitis and non-periodontitis subjects. In addition, we confirm the presence of *P. gingivalis* in whole saliva samples of periodontally healthy, gingivitis, and periodontitis patients. Thus, it would be of interest to do real time PCR to measure of number of *P. gingivalis* in whole saliva as compared with those in the periodontal pockets. Moreover, further studies are needed to identify the exact molecular mechanism of the regulation of TFF expression in response to chronic inflammation by periodontopathic bacteria.

Keywords : gingipains; periodontitis; saliva; trefoil factor

INTRODUCTION

Trefoil factors (TFFs) are small and stable molecules mainly derived from mucin-producing epithelial cells and belong to a family of short peptides with disulfide bonds that form a trefoil domain. Human TFFs consist of three members, TFF1, TFF2, and TFF3. TFFs are detected in various human tissues and secretions (1, 2), mainly in the gastrointestinal compartments. TFFs were involved in several biological functions such as cytoprotection against tissue damage, wound healing, and immune response (1, 2). Since the discovery of TFFs during 1980's, more than 700 published articles have reported information of TFFs (http://www.ncbi.nlm.nih.gov/sites/entrez), and there have been intensive studies about the roles of these molecules in human diseases such as gastrointestinal tract diseases and several cancers. However, information of TFFs in oral diseases is very limited. Less than 15 articles reported the information of TFFs in association with oral compartments or oral diseases. Previous studies demonstrated that, in oral compartments, TFFs originated mainly from salivary glands with some contribution from goblet cells of the parotid duct and oral mucosal epithelia (3-8). In saliva, TFF3 was predominant, followed by TFF1 and TFF2 (7). It was reported that TFF3 was a modifying factor for signaling pathways involved in cell survival, cell proliferation, and cell migration of oral keratinocytes (9, 10). Thus, expression of TFF3 peptides in saliva and oral epithelial cells may be an essential factor in protection against oral tissue damage.

In 2008, our research group was granted by Thailand Research Fund and Office of Higher Education Commission (RMU5180027) to investigate the expression of TFFs in saliva and gingival tissues of patients with chronic periodontits. We, for the first time, demonstrated that TFFs were detected in human gingival tissues (11). Our study demonstrated that TFF3 expression in saliva and gingival epithelia of chronic periodontitis patients was significantly decreased, as compared to control subjects. In addition, levels of salivary TFF3 concentrations were negatively correlated with periodontal pathology (such as bleeding on probing, pocket depth, and clinical attachment loss) and number of *P. gingivalis* (11). According to our observations, we have a new research question: which mechanisms cause the reduction of TFF3 in saliva and gingival epithelia of patients with chronic periodontitis? We hypothesize that *P. gingivalis* may have pathological effects on expression of TFF3 in saliva and gingival epithelia. However, there are no studies investigating the connection between *P. gingivalis* and expression of TFF3 in saliva and gingival epithelia. Therefore, our hypothesis is needed to be verified.

Periodontitis is considered to be a bacterially induced chronic inflammatory disease that destroys the tooth supporting tissues, resulting in tooth loss (12). Chronic periodontitis is a major oral health problem for the Thai population. *P. gingivalis* is a Gram-negative oral anaerobe that is highly associated with chronic periodontitis (13). *P. gingivalis* can invade periodontal tissues and evade the host defence mechanisms, utilizing a panel of virulence factors such as fimbrae, lipopolysaccharide, and proteolytic enzymes such as gingipains (14). Gingipains are cysteine proteases that have strict specificity for a residue which can be either arginine (Arg-X gingipains; gingipain R; or RgpA and RgpB) or lysine (Lys-X gingipain; gingipain K; or Kgp) (15). Gingipains account for 85% of the total proteolytic activity of *P. gingivalis* (16). These proteolytic enzymes have multiple effects on components of the immune system. At a molecular level, gingipains can cleave several T-cell receptors such as CD2, CD4, and CD8 (17). In addition, gingipains are capable of

degrading antimicrobial peptides such as human-β-defensin and LL-37 *in vitro* (18, 19). At a cellular level, gingipains are essential factors for *P. gingivalis* to induce apoptosis in human gingival epithelial cells (20). At a clinical level, measurements of gingipain concentrations and gingipain activities in oral fluid can be a potential approach in diagnosis of periodontal diseases (21, 22). According to our observations on TFF3 expression in saliva and gingival tissues combined with previous findings of gingipains in chronic periodontitis, the following scenario can be postulated.

[1]. Due to the fact that the molecule of TFF3 contains 3 arginine residues and 3 lysine residues (1, 2), and that gingipains have strict specificity for arginine or lysine residues (15), it is possible that, at a molecular level, *P. gingivalis* may utilize gingipains to cleave TFF3 peptides.

[2]. According to one previous study demonstrating that gingipains were essential factors for *P. gingivalis* to induce apoptosis in human gingival epithelial cells (20), it is possible that, at a cellular level, gingipains may be capable of inhibiting the expression of TFF3 from human gingival epithelial cells via apoptotic signaling pathways.

[3]. Based on our observations (11), levels of salivary TFF3 concentrations in chronic periodontitis patients were significantly decreased and negatively correlated with periodontal pathology (such as bleeding on probing, pocket depth, and clinical attachment loss) and number of *P. gingivalis*. Therefore, at clinical level, salivary TFF3 concentrations should be increased and number of *P. gingivalis* should be decreased in chronic periodontitis patients after receiving periodontal disease treatments.

MATERIALS AND METHODS FOR VALIDATION OF HYPOTHESES

[1] Proteolytic effect of gingipains on TFF3

Preparation of TFF peptides

Recombinant human TFF3 peptides were produced in *E. coli* (Raybiotech, Inc. Norcross, GA, USA). According to the company's information, recombinant human TFF3 peptides were conjugated with thioredoxin and histidine at the N-terminus with approximate molecular weight of 26.4 kDa. Recombinant human TFF3 peptides were purified by His-tag affinity purification using immobilized metal ion affinity chromatography (IMAC). Recombinant human TFF3 peptides were diluted in sterile milli Q water at 1,000 ng/μl, respectively.

Stock enzyme solutions

Gingipains, including RgpB and Kgp, were purified from the culture medium of the HG66 strain by a combination of gel filtration and ion-exchange chromatography as previously described [Potempa J, Nguyen KA (2007) Purification and characterization of gingipains. Curr Protoc Protein Sci. Chapter 21:Unit 21.20. doi: 10.1002/0471140864.ps2120s49]. RgpB and Kgp were prepared at 0.81, and 1.0 mg/ml respectively, in 20 mM Bis-Tris, 150 mM NaCl, 5 mM $CaCl_2$. 0.02% NaN_3, pH 6.5. Stock solutions of gingipains were kept at -20 °C until used. Trypsin (Promega, USA) was prepared at 20 ng/ml in 5 mM ammoniumbicarbonate in 50% acetonitrile and used as a cleavage reagent control.

An in vitro enzymatic digestion

The protocol for enzymatic digestion was as follows;1 µl of TFF peptides, and 5 µl of 10 mM dithiothreitol (DTT)/10 mM ammoniumbicarbornate was added into 1.5 ml microcentrifuge tube (performed in duplicate) followed by incubation at 56°C for 1 hr. Then, 20 µl of 100 mM iodoacetamide (IAA)/10 mM ammoniumbicarbornate was added. The tubes were kept in the dark and incubated at 37°C for 1 hr. Then 2 µl (20 ng/µl) of each gingipain (diluted in 10 mM ammoniumbicarbonate) was added into each tube, followed by incubation at 37°C for 3 hr. The 2 ul of trypsin (20 ng/ µl; diluted in 50% acetonitrite/10 mM ammoniumbicarbonate) was used as a cleavage reagent. To inactivate the enzymatic activities, gingipains and trypsin were boiled at 95°C for 1 hr. Then, boiled enzymes were used to compare the enzymatic activities with the intact enzymes. The 1 µl of 10% formic acid was added to stop each enzymatic reaction.

Analyses of digested TFF peptides by MALDI-TOF/TOF MS

Each TFF sample was acidified with 0.1% trifluoroacetic acid and 0.1%TritonX-100 to the final concentration of 0.1 ug/ml. The samples were mixed with MALDI matrix solution (10 mg sinapinic acid in 1 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid), directly spotted onto MALDI target (MTP 384 ground steel, Bruker Daltonik GmbH, Germany) and allowed to dry at room temperature. MALDI –TOF/TOF spectra were collected using Ultraflex III TOF/TOF (Bruker Daltonik GmbH, Germany) in a linear positive mode. Five hundred shots were accumulated with a 200 Hz laser for each sample. Mass spectra ranging from 500 to 30,000 Da were analyzed by FlexAnalysis and ClinproTool software (Bruker Daltonik GmbH, Germany). Cytochrome C was used as an external protein calibration.

Analyses of digested TFF fragments by LC MS

The digested peptides were injected into Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker Daltonik GmbH, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 um, 100A, 75 um id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 50% acetonitrile was run in 20 min. Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300—1500 *m*/*z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50—3000 *m*/*z*. Acquired LC-MS/MS raw data were quantitated based on the peptide ions signal intensities in MS mode using DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare, USA) [25, 26]. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK) [27]. The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (*Homo sapiens*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (1).

Statistical analyses

Statistical analyses were performed using SPSS program version 20. The Kolmogorov-Smirnov test and the Shapiro-Wilk test were used to assess the distribution of the investigated data. Comparisons of relative amount of digested TFF fragments from enzymatic digestions (Kgp, RgpB, and trypsin) were analyzed by one-way ANOVA. Two-tailed $p < 0.05$ was considered statistically significant.

[2] Inhibitory effect of gingipains on expression of TFF3 in human gingival epithelial cells

Cell culture

Primary human gingival epithelial cells (HGEP) purchased from CELLnTEC (Bern, Switzerland) were used in this study. HGEP cells were cultured in keratinocyte-SFM (serum-free medium) (Gibco®, NY, USA) containing 0.2 ng/mL human recombinant epidermal growth factor (rEGF) (Gibco®), 30 ug/mL bovine pituitary extract (BPE) (Gibco®), 100 units/mL penicillin, 100 ug/mL streptomycin and 250 ng/mL amphotericin B (Sigma-Aldrich, MO, USA), as recommended by the manufacturer's instruction. Cultures were incubated at 37^0C and 5% CO_2.

Purified gingipains

Mixed gingipains, containing RgpB and Kgp, were purified from the culture medium of the HG66 strain by a combination of gel filtration and ion-exchange chromatography as previously described [Potempa J, Nguyen KA (2007) Purification and characterization of gingipains. Curr Protoc Protein Sci. Chapter 21:Unit 21.20. doi: 10.1002/0471140864.ps2120s49]. RgpB and Kgp were prepared at 0.81, and 1.0 mg/ml respectively, in 20mM Bis-Tris, 150 mM NaCl, 5 mM $CaCl_2$. 0.02% NaN_3, pH 6.5. Stock solutions of gingipains were kept at -20^0C until used. The mixed gingipains were used at the final concentrations of 4, 8, and 16 ug/mL

Cell treatment and experimental groups

The third passage of HGEP cultures were harvested, seeded at 500,000 cells/well in 6-well plastic culture plates (Corning, MA, USA) and incubated in 5% CO_2 at 37^0C overnight. When reached the confluence (approximately 1,000,000 cells/well), HGEP cells were washed twice with fresh media and then were treated with mixed gingipains at various concentrations. Cells treated with keratinocyte growth factor (100 ng/mL) were used as a positive control. As a negative control, HGEP cells were cultured without gingipains. Incubation was performed at 1, 2, and 4 hours. Each experimental group was done in triplicate. Then, HGEP cells were harvested for RNA and protein extractions.

RNA extraction and detection of TFF3 mRNA by real-time RT-PCR

Total RNA was extracted by RNA extraction kits (RNeasy® Mini kit, Qiagen, Hilden, Germany). Extracted RNA was kept at -80^0C until used. Template cDNA was synthesized by PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) and a thermocycler (Biometra GmbH, Gottingen, Germany). The primers were used as follows:

Targets	Primers	Size (bp)	References
TFF3	Forward-5´-GTGCCAGCCAAGGACAG-3´	302	Wiede A, et al. Am J Respir Crit Care Med 1999; 159:1330–5.
	Reverse-5´-CGTTAAGACATCAGGCTCCAG-3´		
18S	Forward 5´-CTACCACATCCAAGGAAGGCA-3´	71	Denizot A, et al. Thyroid 2003; 13: 867-872.
	Reverse 5´-TTTTTCGTCACTACCTCCCCG-3´		

Measurement of TFF3 cDNA was performed by real time ABI PRISM 7500 Fast PCR System (Applied Biosystems, NY, USA). The PCR cycling conditions were as follows: 50^0C for 2 min; 95^0C for 10 min; 95^0C for 10 min; 95^0C for 15 sec; and 30 cycles of 60^0C for 1 min. The level of TFF3 mRNA in each group was normalized and quantified using a house keeping gene: 18S. Each specific PCR reaction was validated by the melting curve analysis. All reactions were run in triplicate with no-template controls and –RT controls were amplified to control for remaining DNA contamination.

Protien extraction and Western blotting analysis for expression of TFF3

HGEP cells from each experimental group were lysed by 500 uL of 1x lysis buffer with 0.1% phenylmethylsulfonyl fluoride and 1% protienase inhibitor cocktails (Amresco®, OH, USA). Protein lysate was separated on 15% sodium dodecylsulfate-polyacrylamide gel by electrophoresis (SDS-PAGE), and electronically transferred onto nitrocellulose membranes. The membrane was blocked with 5% skim milk in TBST (Tris buffered saline with 0.05% Tween20) at 4^0C overnight, and washed with TBST four times. The membrane was incubated with anti-human TFF3 mouse monoclonal (clone 298) antibody (a kind gift from Professor Watchara Kasinlerk) at room temperature for 1 hour. This antibody was prepared for final concentration at 5 ug/mL, diluted in 1xPBS with 2% skim milk. Recombinant human TFF3 (Raybiotech, Inc. Norcross, GA, USA) was used as a positive control. In addition, anti-human cleaved caspase-3 and cleaved PARP rabbit monoclonal antibodies (Cell signaling technology, MA, USA) were used to determine whether cells treated with gingipains were induced to cell apoptosis. After washing, the membrane was incubated with either secondary sheep anti-mouse antibody linked to horseradish peroxidase or secondary donkey anti-rabbit antibody linked to horseradish peroxidase (GE Healthcare Life Sciences, Buckinghamshire, UK) in TBST at room temperature for 1 hour. Immunodetection was performed by Amersham™ ECL™ Prime Blotting Detection Reagent and viewed by G:Box Chemi (Syngene, Cambridge, UK).

Enzyme-linked immunosorbent assay (ELISA) of TFF3 in cell culture medium and cell lysate

The quantitative sandwich ELISA of TFF3 was performed as follows. 5 μg/ml of anti-human TFF3 mouse monoclonal antibody clone 286: diluted in coating buffer containing 15 mM sodium carbonate and 35 mM sodium bicarbonate (anti-human TFF3 mouse monoclonal antibody clone 286A was a kind gift from Professor Watchara kasinlerk) was coated onto a 96-well plate. The plate was incubated for 3 hours at room temperature, and then washed with washing buffer. Recombinant human TFF3 (Raybiotech, Inc. Norcross, GA, USA) was used for setting up the standard curve. Supernatats and cell lysate were added into the wells and incubated for 12 hours at room temperature. The detecting antibody was prepared using anti-human TFF3 mouse monoclonal antibody clone 116A conjugated with FITC (anti-human TFF3 mouse monoclonal antibody clone 116A was a kind gift from Professor Watchara kasinlerk). 1μg/ml of anti-human TFF3 mouse monoclonal antibody with FITC diluted in 0.1% bovine-albumin PBS was added in the wells and incubated for 1 hour at room temperature. 1 ug/ml of sheep polyclonal anti mouse IgG antibody conjugated with HRP (GE Healthcare UK Limited Amersham Place, Buckinghamshire, UK) was added into the wells and incubated for 1 hour at room temperature. TMB substrate (Invitrogen Corporation, CA, USA) was added in to the wells and allowed to develop colored product for 15 minutes and the reaction was stopped by 1 M phosphoric acid. The plate was washed with washing buffer between each step. Absorbance was measured at 450 nm. All standards, cell culture medium, and cell lysate samples in the procedures of ELISA were done in triplicate,

and the data were calculated from the mean of three tests for each sample. Triplicate determinations for each sample were estimated for coefficient of variation. Concentrations of TFF3 in each sample were determined by a standard curve.

[3] Measurement of TFF3 and gingipains in saliva of chronic periodontitis patients

Study population

This research project was approved by clinical research program (Clinical trial/ Experimental study) for consideration of ethics in human research, KhonKaen University (HE581105). Volunteers were recruited from Periodontal Clinic, and Oral Diagnosis Clinic, Faculty of Dentistry, KhonKaen University. Inclusion criteria for recruitment of volunteers in this study were as follows: any individual who had at least 15 teeth remaining. Periodontitis individuals were included when they had > 30% of sites CAL $\geq$ 3 mm and BOP $\geq$ 20%. These criteria were modified from Wara-aswapati*et al*. [Wara-aswapati, N., Pitiphat, W., Chanchaimongkon, L., Taweechaisupapong, S., Boch, J. A., & Ishikawa, I. (2009). Red bacterial complex is associated with the severity of chronic periodontitis in a Thai population. Oral diseases*,*15(5), 354-9]. Gingivitis individuals were included when they had CAL = 0-1 mm with BOP $\geq$20% of sites. Healthy individuals who had no evidence of clinical attachment loss (CAL = 0 mm) and BOP < 20% of sites was included. Diagnosis of periodontal diseases was based on the 1999 International Workshop for classification of Periodontal Diseases and Conditions [Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. Annals of periodontology / the American Academy of Periodontology*,* 4(1), 1-6]. Exclusion criteria were as follows: history of any systemic diseases, salivary gland disease, aggressive periodontitis, smoking, current pregnancy or lactation, periodontal therapy or use of mouth rinse within the previous 3 months. One examiner performed the periodontal examination. PD, CAL and BOP were determined by using a periodontal probe. The periodontal assessments were performed on six sites of each remaining tooth.

Saliva Collection

All volunteers were refrained from eating or drinking one hour before saliva collection. Each individual was informed to rinse the mouth thoroughly with water, follow by expectorating whole saliva into a 50-ml centrifuge tube, and a final saliva volume of 3 to 5 ml was obtained. Saliva samples were collected in the morning at three different time points including: before treatment, 2 weeks after treatment, and 3 months after treatment. Each saliva sample was immediately place in ice container for transport to the laboratory. Saliva samples were vortexed for 3 minutes, following by centrifuging at 10,000 x g at 4^0C for 10 minutes. Supernatants were stored at -80^0C until further measurement of salivary gingipains concentrations, using ELISA assay.

Collection of periodontopathic Bacteria from Subgingival Plaque

Six selected teeth (#16, #11, #24, #36, #31, #44) from gingivitis group and healthy group were collected. If the selected teeth were missing, we planned to collect from adjacent tooth or the opposite site tooth. In the periodontitis group, subgingival plaque samples were collected from 4 deepest sites of periodontal pockets at each quadrant in the first visit. At the 2 weeks after treatment and 3 months post hygienic visits, subgingival plaque samples were obtained at the same site as the sites of GCF collection, and it was performed just after GCF collection had been done. Before subgingival plaque collection, the area was

dried with sterile cotton swabs, and supragingival plaque was removed by cotton pellets and air-drying. One sterile paper point was inserted into the bottom of the periodontal pocket or gingival crevice for 20 seconds to collect subgingival plaque from each selected tooth site. The paper points were kept in an empty eppendorf tube and stored at -80^{0}C for further analysis.

Assessment of number of P. gingivalis by real-time-PCR assay

Assessment of number of *P. gingivalis* was performed by using real-time-PCR assay as previously described [Chaiyarit et al. (2012). Trefoil factors in saliva and gingival tissues of patients with chronic periodontitis. Journal of periodontology, 83(9), 1129-38]. Total bacterial DNAs were extracted from subgingival plaque samples by using crude genomic DNA preparation kits (innuPrep DNA Mini Kit, analytikjena, Germany). Subgingival plaque samples were suspended in 1 ml sterile double-distilled water and centrifuged for 1 minute at 10,000 x g. The pellets were resuspended in 200ul of matrix, according to the manufacturer's instruction (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, USA). The suspension was incubated at 56^{0}C for 30minutes following by 100^{0}C for 8 minutes. After the incubation, the suspension was centrifuged at 10,000 x g for 3 minutes then 5 µl of the resulting supernatant was subjected to real-time PCR procedure. The real-time PCR Specific oligonucleotide (forward and reverse) primers for *P. gingivalis* are 5'- CTTGAC TTCAGTGGCGGCAG-3', and 5'-AGGGAAGACGGTTTTCACCA-3'. The reaction mixture contained 2x (PCR Master Mix PCR Master Mix, Applied Biosystems), 20 pmol of forward and reverse primers and 5ul of extracted DNA. The PCR reaction condition included 40 cycles of 95^{0}C for 10 seconds and 65^{0}C for 1 minute for *P. gingivalis*. After the reaction, a dissociation curve (melting curve) was constructed in a range from 60^{0}C to 95^{0}C. To determine the quantitative range of real-time PCR, DNA was prepared from 10 to 10^6 cells of cultured *P. gingivalis*. All data were analyzed using the software (StepOne Software, Applied Biosystems), according to the manufacturer's instruction. The establishment of a cut-off value for positive results was based on the standard curve and the melting curve. The sample was considered to be positive (presence of particular bacteria) when 10 copies or higher were detected. We attempted to report the number of *P. gingivalis* number count in the score number following score 0 $\leq$ 9 of *P. gingivalis*copies was detected or negative result, score 1 = 10 – 500 of *P. gingivalis*copies, score 2 = 501 – 25000 of *P. gingivalis*copies, score 3 = 25001 – 125000 of *P. gingivalis*copies, and score 4 > 125000 of *P. gingivalis*copies.

Measurement of total protein concentrations in whole saliva

Measurement of total protein concentrations in whole saliva samples was determined by using Bradford protein assay kit. The procedure was followed the company manual instruction. The Bradford protein assay dye was diluted with deionized distilled water (DDW) (1:4). In each plate, a standard protein concentration curve was prepared by using bovine serum albumin (BSA). Two hundred microliters of dye solution were added in each well. 2 µl of saliva was used, and diluted with 8 µl of 1X Tris- buffer. 200 µl of dye solution was mixed with diluted saliva samples. The plates were shaken for 5 minutes. Finally, the plates were read at 595nm absorbance in spectrophotometer machine. The total protein concentration in each sample was calculated through the standard curve equation after subtraction the blank OD value.

Measurement of salivary TFF3

The quantitative sandwich ELISA of TFF3 was performed as follows. 5 ug/ml of anti-human TFF3 mouse monoclonal antibody clone 286: diluted in coating buffer containing 15 mM sodium carbonate and 35

mM sodium bicarbonate (anti-human TFF3 mouse monoclonal antibody clone 286 was a kind gift from Professor Watchara kasinlerk) was coated onto a 96-well plate. The plate was incubated for 3 hours at room temperature, and then washed with washing buffer. Recombinant human TFF3 (Raybiotech, Inc. Norcross, GA, USA) was used for setting up the standard curve. Saliva samples were added into the wells and incubated for 12 hours at room temperature. The detecting antibody was prepared using anti-human TFF3 mouse monoclonal antibody clone 116A conjugated with FITC (anti-human TFF3 mouse monoclonal antibody clone 116A was a kind gift from Professor Watchara kasinlerk). 1ug/ml of anti-human TFF3 mouse monoclonal antibody with FITC diluted in 0.1% bovine-albumin PBS was added in the wells and incubated for 1 hour at room temperature. 1 ug/ml of sheep polyclonal anti mouse IgG antibody conjugated with HRP (GE Healthcare UK Limited Amersham Place, Buckinghamshire, UK) was added into the wells and incubated for 1 hour at room temperature. TMB substrate (Invitrogen Corporation, CA, USA) was added in to the wells and allowed to develop colored product for 15 minutes and the reaction was stopped by 1 M phosphoric acid. The plate was washed with washing buffer between each step. Absorbance was measured at 450 nm. All standards, and saliva samples in the procedures of ELISA were done in triplicate, and the data were calculated from the mean of three tests for each sample. Triplicate determinations for each sample were estimated for coefficient of variation. Concentrations of TFF3 in each sample were determined by a standard curve. Student's t tests were used for comparison of salivary TFF3 concentrations between chronic periodontitis and control groups.

Measurement of salivary gingipains

Saliva samples were diluted (1:2) with coating buffer (0.1M carbonate-bicarbonate buffer pH 9.6). Total protein concentrations from saliva were determined to test whether any differences in the levels of gingipains could be ascribed to differences in total salivary protein concentrations, employing the Bradford protein assay kit (Bio-Rad Laboratories, USA). Levels of salivary gingipains in each group were represented as mean OD values, which were normalized for the OD of total salivary protein concentrations. The indirect ELISA assay was optimized under checkerboard titration to determine the appropriate concentration of each used reagent. The aim of the optimization was to get the true positive reaction between the specific antigen (gingipains) and the antibody and to reduce the false positive reaction between non-specific antigen and the antibody. Purified RgpB gingipains were diluted with coating buffer (0.1M carbonate-bicarbonate buffer, pH 9.6) at the concentration of 5 μg/ml and then it was loaded into the well (50 μl/well) in the 96 wells plate (Costa® 3590, USA). Incubation was performed in moist chamber at 37 ^{o}C for 3 hours. The plates were washed thoroughly with washing buffer containing phosphate buffer saline and 0.05% Tween 20 four times. After washing, the plates were tapped to make them dry. Nonspecific bindings were blocked using 100 μl of 1% casein (Bio-Rad Laboratories, USA) and the plates were incubated for 1 h at 37 ^{o}C. The plates were tapped to remove all blocking solution. 50 μl of of monoclonal antibody against RgpB (a gift from Prof. Jan Potampa) diluted in washing buffer (4μg/ml) was added in each well, and the plates were incubated for 1h at 37 ^{o}C. The plates were washed four times. 50 μl of polyclonal antibody (sheep anti-mouse IgG antibody conjugated with horseradish peroxidase GPR, NXA931 lot 9586848, GE Healthcare UK limited) diluted with washing buffer (1:400) was added in each well and the plates were incubated for 1h at 37 ^{o}C, followed by washing four times. 3,3`, 5,5`-Tetramethylbenzidine (TMB) (Bio-Rad Laboratories, USA) substrate (50 μl) was added in

each well, and the plates were incubated at dark room for 30 minutes. Finally, (50 µl) 1M HCl was added to stop the reaction, and the reaction was read at 450nm absorbance in the spectrophotometer machine (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

Measurement of RgpB Gingipains in GCF

The optimized protein recovery condition was chosen from our previous study as shown in Fig 2. Total protein concentrations from GCF were determined to test whether any differences in the level of RgpBgingipains could be ascribed to differences in total GCF protein concentrations, employing the Bradford protein assay kit (Bio-Rad Laboratories, USA). Levels of RgpBgingipains in GCF from each group were represented as mean OD values which were normalized for the OD values of GCF total protein concentrations. The level of RgpBgingipains in GCF was measured by using the indirect ELISA assay. The procedure of ELISA assay used for GCF samples was similar to those in whole saliva samples, excepted that undiluted GCF was coated to the plate and for polyclonal antibody was 1:100. The indirect ELISA assay was optimized under checkerboard titration to determine the appropriate concentration of each used reagent. The aim of the optimization was to get the true positive reaction between the specific antigen (RgpB gingipains) and the antibody and to reduce the false positive reaction between non-specific antigen and the antibody. Purified RgpBgingipains were diluted with coating buffer (0.1M carbonate-bicarbonate buffer, pH 9.6) at the concentration of 5 µg/ml and then it was loaded into the well (50 µl/well) in the 96 wells plate (Costa® 3590, USA). Incubation was performed in moist chamber at 37 ^{o}C for 3 hours. The plates were washed thoroughly with washing buffer containing phosphate buffer saline and 0.05% Tween 20 four times. After washing, the plates were tapped to make them dry. Nonspecific bindings were blocked using 100 µl of 1% casein (Bio-Rad Laboratories, USA) and the plates were incubated for 1 h at 37 ^{o}C. The plates were tapped to remove all blocking solution. 50 µl of of monoclonal antibody against RgpB (a gift from Prof. Jan Potampa) diluted in washing buffer (4µg/ml) was added in each well, and the plates were incubated for 1h at 37 ^{o}C. The plates were washed four times. 50 µl of polyclonal antibody (sheep anti-mouse IgG antibody conjugated with horseradish peroxidase GPR, NXA931 lot 9586848, GE Healthcare UK limited) diluted with washing buffer (1:100) was added in each well and the plates were incubated for 1h at 37 ^{o}C, followed by washing four times. 3,3`, 5,5`-Tetramethylbenzidine (TMB) (Bio-Rad Laboratories, USA) substrate (50 µl) was added in each well, and the plates were incubated at dark room for 30 minutes. Finally, (50 µl) 1M HCl was added to stop the reaction, and the reaction was read at 450nm absorbance in the spectrophotometer machine (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

Statistical analysis

Characteristics of healthy control subjects, gingivitis patients, and chronic periodontitis patients were compared using the Chi-square test for categorical variables and analysis of variance (ANOVA) test for continuous variables. The Kruskal-Wallis test was performed to analyze differences in salivary and GCF RgpB gingipains. To control the effect of age and gender, multiple linear regression were used to evaluate the association of periodontitis with the levels of gingipains, using the log-transformed gingipains levels as the outcomes. Spearman correlation coefficient was performed to determine the correlations between the investigated gingipians parameters and number of *P. gingivalis* as well as clinical periodontal parameters.

Two-tailed $P< 0.05$ was considered statistically significant. All statistical analysis was calculated by using SPSS software (IBM SPSS statistics version 20, US).

RESULTS

[1] Proteolytic effect of gingipains on TFF3

Analyses of digested TFF peptides by MALDI-TOF/TOF MS

It should be noted that mass spectra by a MALDI-TOF/TOF method (operated at a linear mode) were acquired using the mass range between 500 to 30,000 Da. Therefore, the mass spectral data of gingipains (RgpB and Kgp) and trypsin were not demonstrated because their molecular masses were beyond the upper limit. Patterns of mass signals of TFF3 were demonstrated in Figure 1A. We could not detect the mass signal representing TFF3 peptides (approximate molecular weight of recombinant TFF3 is 26.4 kDa). According to mass signal analysis, it was indicated that there was the impurity in preparation of recombinant TFF3 peptides. Nevertheless, the enzymatic reactions by RgpB, Kgp, and trypsin showed different fragmentation patterns (Figure 1A). The enzymatic reactions by boiled RgpB, Kgp, and trypsin demonstrated different patterns of mass signals (Figure 2B) as compared with the results from those with the unboiled enzymes.

Analyses of digested TFF peptides by LC MS

Identification of digested TFF3 peptides by LC MS was demonstrated in table 1. It was demonstrated that TFF3 domains were cleaved by Kgp, RgpB, and trypsin. Digested TFF3 fragments covered 51.06% of TFF3 peptide sequence. Relative amount of digested fragments of TFF3 from enzymatic digestions by RgpB, Kgp, and trypsin was not significantly different. It should be addressed that there were no identified fragments of TFF3 from enzymatic reactions by boiled RgpB, Kgp, and trypsin.

Table 1 Analyses of digested TFF3 peptides by LC-MS

			Relative amount[c] of TFF3 (after digestion by enzymes)		
TFF3 peptide sequences[a]	**Score[b]**	**Mass (Da)**	**Kgp**	**RgpB**	**Trypsin**
PHVTPK	19.4	679.48	17.23	25.11	19.04
KDRVDC	16.02	792.67	22.00	21.90	22.11
VGLSANQCA	14.49	920.76	18.94	21.74	22.47
LLSSSSAE	12.02	794.36	21.36	23.79	21.77
VVMAARAL	10.72	846.69	24.67	23.13	23.05
EPTVVMAARA	10.58	1058.94	20.44	20.65	25.25
ALCMLGLVLALLSS	10.02	1476.02	16.74	15.80	21.61

[a]Peptide sequences cover 51.06% of TFF3 and matched peptide sequences are demonstrated in bold. Amino acid sequences with underline demonstrate a TFF3 domain:

MKRVLSCVP**EPTVVMAARALCMLGLVLALLSSSSAE**EY**VGLSANQCA**

VPA**KDRVDC**GY**PHVTPK**ECNNRGCCFDSRIPGVPWCFKPLQEAECTF

[b]Score obtained from Mascot for each match represents degree of similarity between the theoretical and experimental data. [c]Relative amount of TFF3 peptide(s) was calculated from peptide signal intensity of MS spectra.

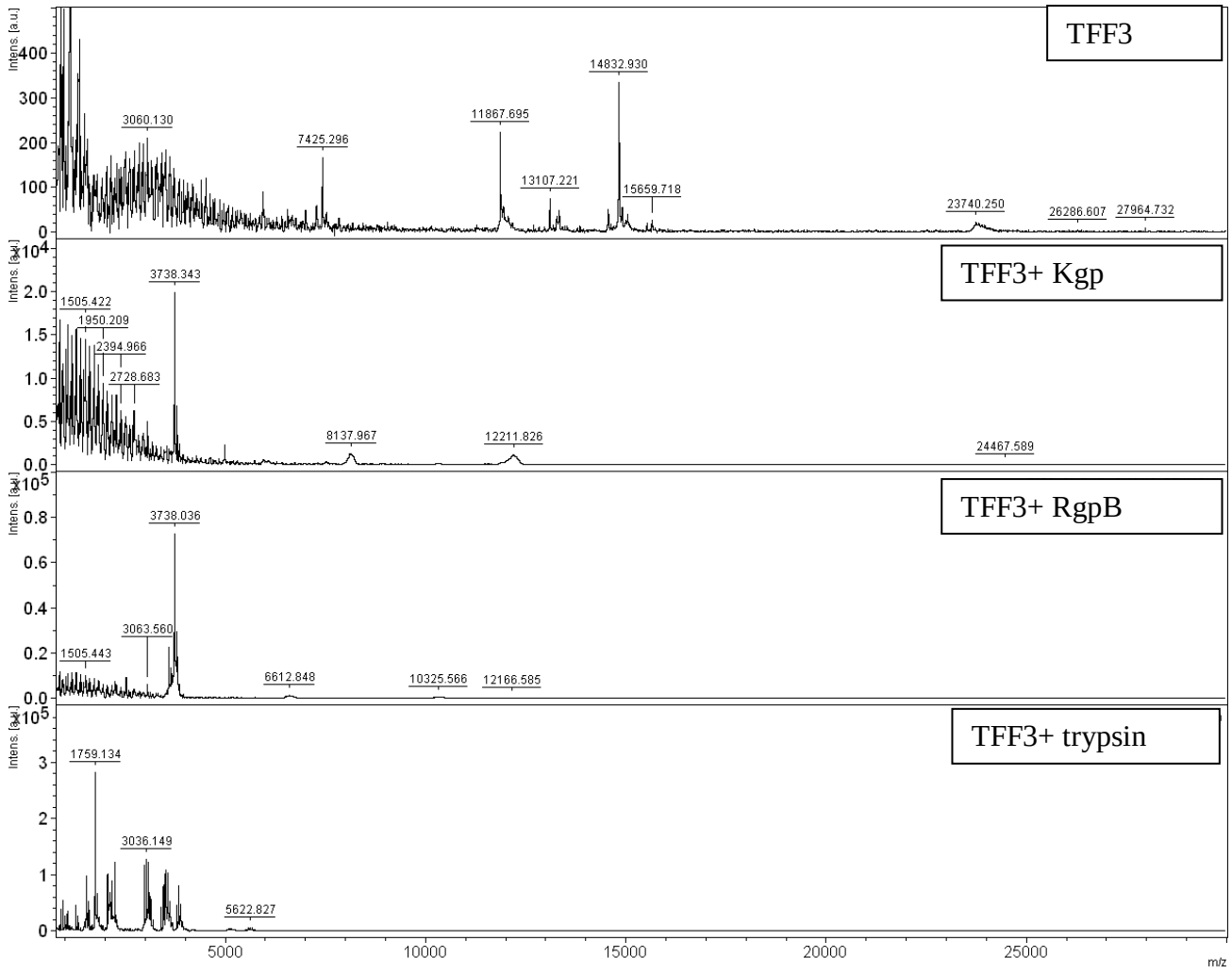

Figure 1A Pattern of TFF3 mass signals when TFF3 peptides were digested by Kgp, RgpB, and trypsin, respectively.

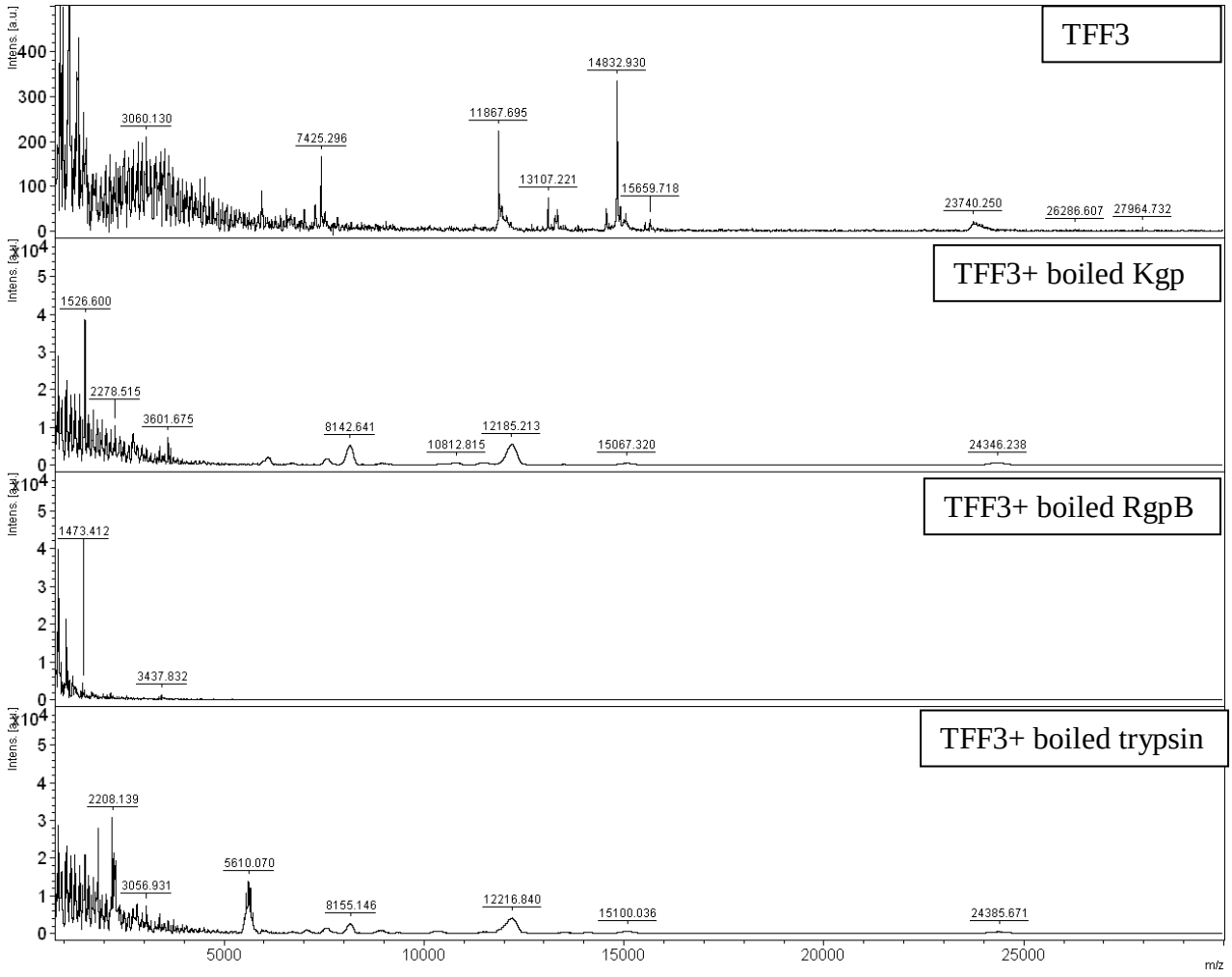

Figure 1B Pattern of TFF3 mass signals when TFF3 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

[2] Inhibitory effect of gingipains on expression of TFF3 in human gingival epithelial cells

Real-time RT-PCR analyses of the expression of TFF3 mRNA

Our study demonstrated that gingipains inhibited the expression of TFF3 mRNA, and inhibited the expression of TFF3 peptides with a dose response manner in human gingival epithelial cells (Figure 2).

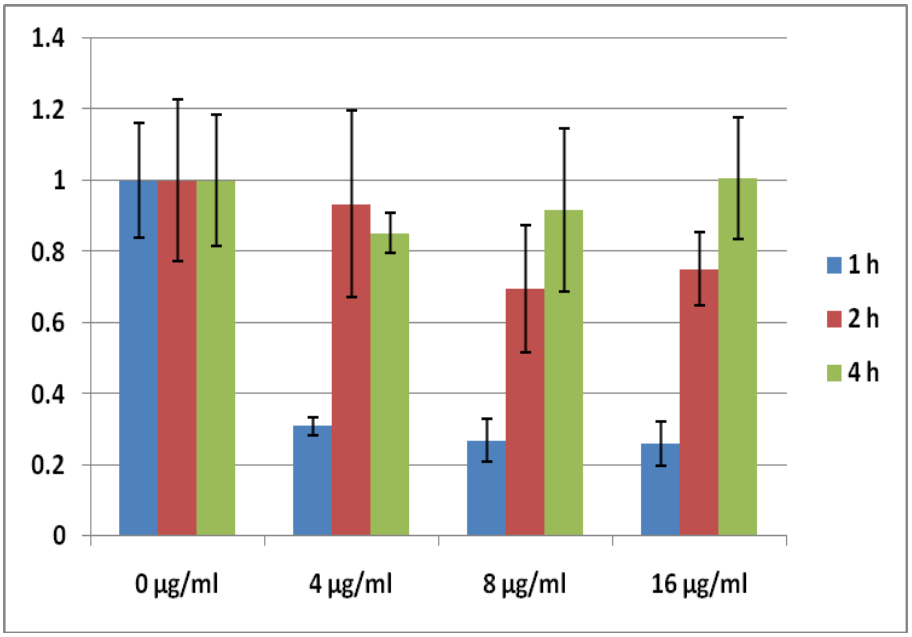

Figure 2 Inhibitory effects of gingipains on the expression of TFF3 in human gingival epithelial cells

SDS-PAGE and Western blotting analyses of the expression of TFF3

We performed SDS-PAGE (Figure 2) and Western blotting (Figures 3-5) to investigate the protein patterns in cell lysate and supernatant. We could not detect TFF3 peptides in the cell lysate and culture media from all experimental groups.

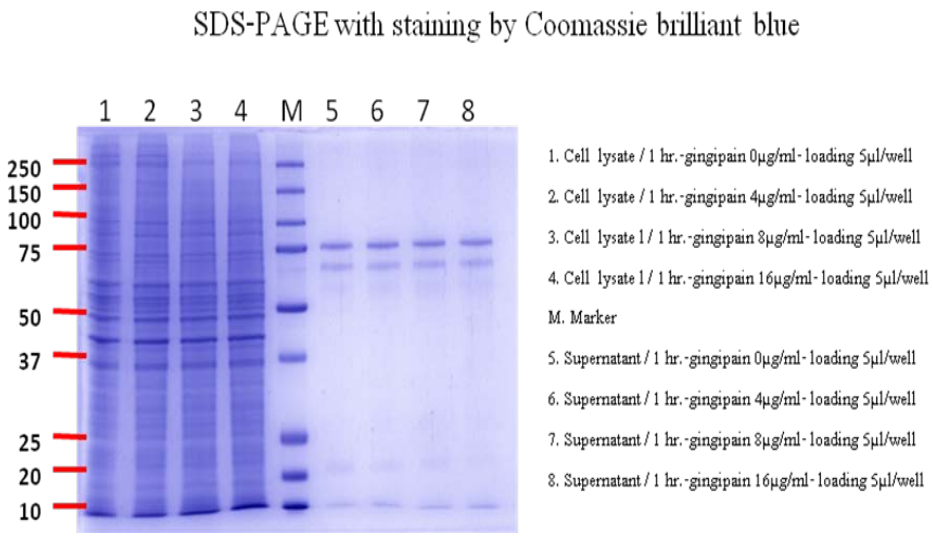

Figure 3 the protein patterns in cell lysate and supernatant

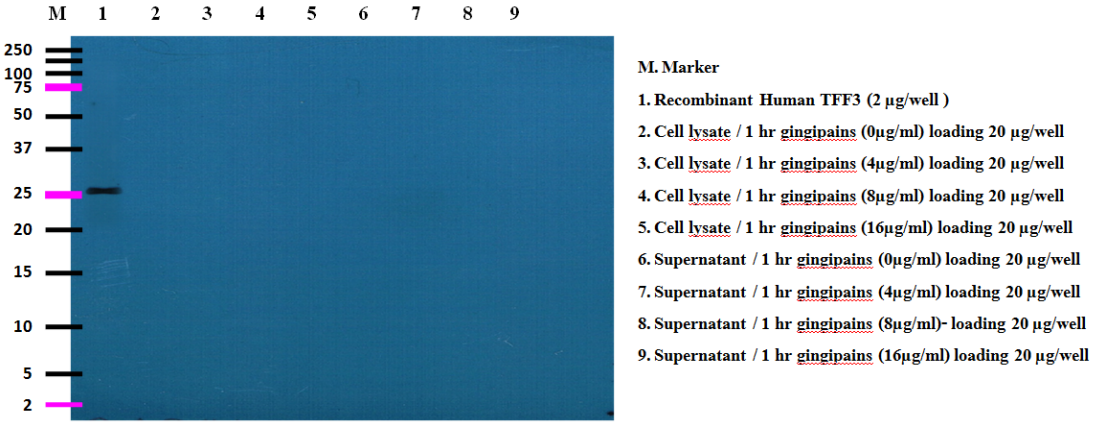

Figure 4 Westeren blotting of TFF3 expression at incubation for 1 hours

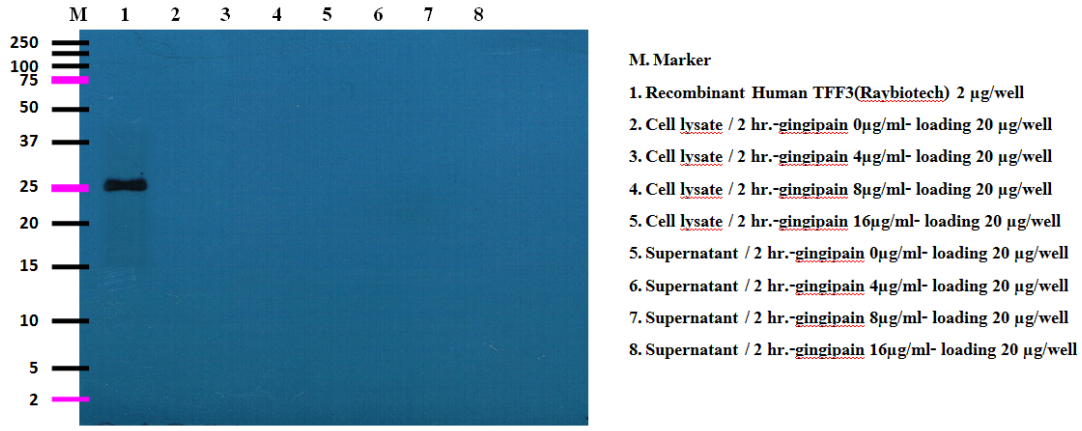

Figure 5 Westeren blotting of TFF3 expression at incubation for 2 hours

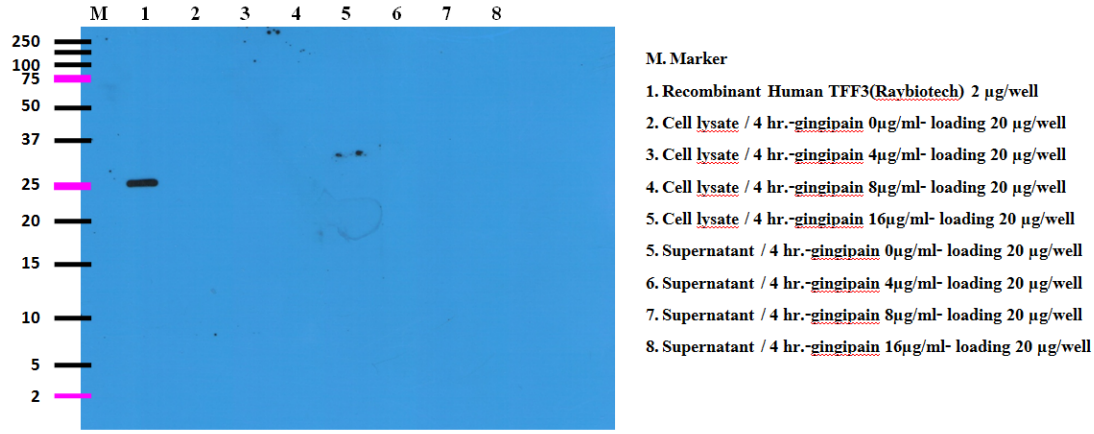

Figure 6 Westeren blotting of TFF3 expression at incubation for 4 hours

Western blotting analyses of the expression of apoptotic markers

Figure 7 Western blotting of apoptotic makers (cleaved PARP and capase-3)

[3] Measurement of TFF3 and gingipains in saliva of chronic periodontitis patients

Table 1 Demographic data and clinical parameters of study subjects: before periodontal disease treatment

	Healthy group	Gingivitis group	Periodontitis group	*p*-value
Age (mean ± SD)	32.13 ± 9.92	32.19 ± 8.99	44.18 ± 13.43	
Gender				
Male	5	5	9	
Female	25	26	13	
Sampling sites				
- Pocket depth (mean ± SD)	1.86 ± 0.2	1.91 ± 0.16	2.89 ± 0.68	< 0.001
- Clinical attachment level				
(mean± SD)	0.54 ± 0.16	0.54 ± 0.13	2.73 ± 1.04	< 0.001
- Bleeding on probing (%)	16.02 ± 6.55	39.07 ± 10.71	53.22 ± 17.11	< 0.001
Full mouth				
- Pocket depth (mean ± SD)	1.89 ± 0.2	1.94 ± 0.16	2.65 ± 0.6	< 0.001
- Clinical attachment level				
(mean± SD)	0.52 ± 0.17	0.55 ± 0.12	2.42 ± 1.1	< 0.001
- Bleeding on probing (%)	15.42 ± 3.57	38.28 ± 9.98	51.73 ± 15.65	< 0.001

: Patients with gingivitis and periodontitis demonstrated incleased levels of clinically periodontal parameters including bleeding on probing, formation of periodontal pockets, and clinicall attachment loss.

Table 2 Clinical parameters of study subjects: before and 2 weeks after periodontal disease treatment

	Before treatment	2 weeks after treatment	*p*value
Gingivitis group			
Sampling sites			
- Pocket depth (mm ± SD)	1.91 ± 0.16	1.86 ± 0.16	= 0.16
- Clinical attachment level (mm± SD)	0.54 ± 0.13	0.56 ± 0.15	= 0.48
- Bleeding on probing (%)	39.07 ± 10.71	23.57 ± 7.75	< 0.001
Full mouth			
- Pocket depth (mean ± SD)	1.94 ± 0.16	1.86 ± 0.14	= 0.02
- Clinical attachment level (mean± SD)	0.55 ± 0.12	1.86 ± 0.14	< 0.001
- Bleeding on probing (%)	38.28 ± 9.98	23.02 ± 6.83	< 0.001
Periodontitis group			
Sampling sites			
- Pocket depth (mm ± SD)	2.89 ± 0.68	2.29 ± 0.37	< 0.001
- Clinical attachment level (mm± SD)	2.73 ± 1.04	2.37 ± 0.9	= 0.001
- Bleeding on probing (%)	53.22 ± 17.11	32.48 ± 14.6	< 0.001
Full mouth			
- Pocket depth (mean ± SD)	2.65 ± 0.6	2.15 ± 0.28	< 0.001
- Clinical attachment level (mean± SD)	2.42 ± 1.1	2.1 ± 0.9	= 0.003
- Bleeding on probing (%)	51.73 ± 15.65	30.59 ± 12.94	< 0.001

: Levels of clinically periodontal parameters including bleeding on probing, formation of periodontal pockets, and clinical attachment loss were significantly decreased in patients with gingivitis and periodontitis after periodontal treatment by 2 weeks.

Table 3 Clinical parameters of study subjects: before and 3 months after periodontal disease treatment

	Before treatment	3months after treatment	*p*value
Gingivitis group			
Sampling site			
- Pocket depth (mm ± SD)	1.91 ± 0.16	1.84 ± 0.18	= 0.02
- Clinical attachment level (mm± SD)	0.54 ± 0.13	0.72 ± 0.21	< 0.001
- Bleeding on probing (%)	39.07 ± 10.71	19.89 ± 11.14	< 0.001
Full mouth			
- Pocket depth (mean ± SD)	1.94 ± 0.16	1.87 ± 0.16	= 0.02
- Clinical attachment level (mean± SD)	0.55 ± 0.12	0.69 ± 0.18	= 0.001
- Bleeding on probing (%)	38.28 ± 9.98	20.62 ± 9.02	< 0.001
Periodontitis group			
Sampling sites			
- Pocket depth (mm ± SD)	2.89 ± 0.68	2.38 ± 0.451	= 0.001
- Clinical attachment level (mm± SD)	2.73 ± 1.04	2.44 ± 0.97	= 0.12
- Bleeding on probing (%)	53.22 ± 17.11	37.41 ± 17.68	= 0.001
Full mouth			
- Pocket depth (mean ± SD)	2.65 ± 0.6	2.24 ± 0.32	= 0.001
- Clinical attachment level (mean± SD)	2.42 ± 1.1	2.22 ± 0.81	= 0.31
- Bleeding on probing (%)	51.73 ± 15.65	33.28 ± 17	< 0.001

: Levels of clinically periodontal parameters including bleeding on probing, formation of periodontal pockets, and clinical attachment loss were significantly decreased in patients with gingivitis and periodontitis after periodontal treatment by 3 months.

Table 4 Clinical parameters of study subjects: 2 weeks and 3 months after periodontal disease treatment

	2 weeks after treatment	3months after treatment	*p*value
Gingivitis group			
Sampling site			
- Pocket depth (mm ± SD)	1.86 ± 0.16	1.84 ± 0.18	= 0.38
- Clinical attachment level (mm± SD)	0.56 ± 0.15	0.72 ± 0.21	= 0.001
- Bleeding on probing (%)	23.57 ± 7.75	19.89 ± 11.14	= 0.07
Full mouth			
- Pocket depth (mean ± SD)	1.86 ± 0.14	1.87 ± 0.16	= 0.93
- Clinical attachment level (mean± SD)	1.86 ± 0.14	0.69 ± 0.18	< 0.001
- Bleeding on probing (%)	23.02 ± 6.83	20.62 ± 9.02	= 0.1
Periodontitis group			
Sampling sties			
- Pocket depth (mm ± SD)	2.29 ± 0.37	2.38 ± 0.451	= 0.12
- Clinical attachment level (mm± SD)	2.37 ± 0.9	2.44 ± 0.97	= 0.22
- Bleeding on probing (%)	32.48 ± 14.6	37.41 ± 17.68	= 0.17
Full mouth			
- Pocket depth (mean ± SD)	2.15 ± 0.28	2.24 ± 0.32	= 0.05
- Clinical attachment level (mean± SD)	2.1 ± 0.9	2.22 ± 0.81	= 0.09
- Bleeding on probing (%)	30.59 ± 12.94	33.28 ± 17	= 0.43

: Levels of clinically periodontal parameters including bleeding on probing, and clinical attachment loss were significantly decreased in patients with gingivitis, whereas levels of periodontal pocket depth were not significantly different when compared between 2 weeks and 3 months after periodontal treatment.

: Levels of clinically periodontal parameters including bleeding on probing, clinical attachment loss, and periodontal pocket depth were not significantly different in patients with periodontitis, when compared between 2 weeks and 3 months after periodontal treatment.

Table 5 Levels of salivary TFF3 and gingipains from study subjects: before periodontal disease treatment

	Healthy group	Gingivitis group	Periodontitis group	*p*-value
TTF3 (ng/ml)	115.39± 56.78	39.58± 22.08	51.12± 17.85	< 0.001
salivary gingipains (OD)	0.22 ± 0.23	0.18 ± 0.19	0.06 ± 0.06	0.001
salivary proteins (OD)	0.12 ± 0.07	0.12 ± 0.05	0.14 ± 0.17	< 0.001

: Salivary TFF3 concentrations and levels of salivary gingipains were significantly lower in patients with gingivitis and chronic periodontitis.

Table 6 Levels of salivary TFF3 and gingipains from study subjects: before and 2 weeks after periodontal disease treatment

	Before treatment	2 weeks after treatment	*p*-value
Gingivitis group			
TTF3 (ng/ml)	39.58 ± 22.08	111.8 ± 64.7	< 0.001
salivary gingipains (OD)	0.18 ± 0.19	0.13 ± 0.11	0.14
salivary proteins (OD)	0.12 ± 0.05	0.13 ± 0.04	0.35
Periodontitis group			
TTF3 (ng/ml)	51.12 ± 17.85	22.54 ± 21.96	< 0.001
salivary gingipains (OD)	0.06 ± 0.06	0.08 ± 0.1	0.27
salivary proteins (OD)	0.14 ± 0.17	0.15 ± 0.12	0.57

: Salivary TFF3 concentrations in patients with gingivitis were significantly increased after periodontal treatment by 2 week, whereas salivary TFF3 concentrations in patients with chronic periodontitis were decreased after periodontal treatment by 2 week.

: Levels of salivary gingipains in patients with gingivitis and chronic periodontitis were not significantly different between before periodontal treatment and after 2 weeks of treatment.

Table 7 Levels of salivary TFF3 and gingipains from study subjects: before and 3 months after periodontal disease treatment

	Before treatment	3 months after treatment	*p*-value
Gingivitis group			
TTF3	39.58 ± 22.08	18.71 ± 9.48	< 0.001
salivary gingipains (OD)	0.18 ± 0.19	0.07 ± 0.05	0.13
salivary proteins (OD)	0.12 ± 0.05	0.12 ± 0.08	0.96
Periodontitis group			
TTF3	51.12 ± 17.85	7.83 ± 3.5	< 0.001
salivary gingipains (OD)	0.06 ± 0.06	0.08 ± 0.06	0.3
salivary proteins (OD)	0.14 ± 0.17	0.21 ± 0.02	0.01

:Salivary TFF3 concentrations in patients with gingivitis and periodontitis were significantly decreased after periodontal treatment by 3 months.

: Levels of salivary gingipains in patients with gingivitis and chronic periodontitis were not significantly different between before periodontal treatment and after 3 months of treatment.

Table 8 Levels of salivary TFF3 and gingipains from study subjects: 2 weeks and 3 months after periodontal disease treatment

	2 weeks after treatment	3 months after treatment	*p*-value
Gingivitis group			
TTF3 (ng/ml)	111.8 ± 64.7	18.71 ± 9.48	< 0.001
salivary gingipains (OD)	0.13 ± 0.11	0.07 ± 0.05	= 0.01
salivary proteins (OD)	0.13 ± 0.04	0.12 ± 0.08	0.57
Periodontitis group			
TTF3 (ng/ml)	22.54 ± 21.96	7.83 ± 3.5	0.08
salivary gingipains (OD)	0.08 ± 0.1	0.08 ± 0.06	0.38
salivary proteins (OD)	0.15 ± 0.12	0.21 ± 0.02	0.06

: Salivary TFF3 concentrations in patients with gingivitis after periodontal treatment by 2 weeks were significantly decreased when compared with those by 3 months after treatment

: Salivary TFF3 concentrations in patients with chronic periodontitis were not significantly different after periodontal treatment by 2 week.

: Levels of salivary gingipains in patients with gingivitis after periodontal treatment by 2 weeks were significantly decreased when compared with those by 3 months after treatment

: Levels of salivary gingipains in patients with chronic periodontitis were not significantly different after periodontal treatment by 2 week.

Table 9 Levels of GCF gingipains and *P. gingivalis* count from study subjects: before periodontal disease treatment

	Healthy group (n = 30)	Gingivitis group (n = 31)	Periodontitis group (n = 22)	*p*-value
GCF gingipains	5	1	14	< 0.001
P. gingivalis count	3	6	6	0.09

: Gingipains were more detectable in GCF of patients with periodontitis as compared with patient with gingivitis and healthy individuals.

: *P. gingivalis* count was somewhat more detected in patients with gingivitis and periodontitis as compared with healthy individuals.

:

Table 10 Levels of GCF gingipains and *P. gingivalis* count from study subjects: before and 2 weeks after periodontal disease treatment

	Before treatment	2 weeks after treatment	*p*-value
Gingivitis group (n = 31)			
- GCF gingipains	1	13	>0.05
- *P. gingivalis* count	6	1	>0.05
Periodontitis group(n = 22)			
- GCF gingipains	14	13	>0.05
- *P. gingivalis* count	6	4	>0.05

: In gingivitis and priodontitis patients, there were no significant differences in levels of GCF gingipains and *P. gingivalis* count between before and after 2 weeks of treatment.

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Table 11 Levels of GCF gingipains and *P. gingivalis* count from study subjects: before and 3 months after periodontal disease treatment

	Before treatment	3 months after treatment	*p*-value
Gingivitis group (n = 31)			
- GCF gingipains	1	22	0.04
- *P. gingivalis* count	6	6	>0.05
Periodontitis group(n = 22)			
- GCF gingipains	14	12	>0.05
- *P. gingivalis* count	6	0	>0.05

: GCF gingipains were more detectable in gingivitis patients after 3months of treatment.

: In priodontitis patients, there were no significant differences in levels of GCF gingipains and *P. gingivalis* count between before and after 3 months of treatment.

Table 12 Levels of GCF gingipains and *P. gingivalis* count from study subjects: 2 weeks and 3 months after periodontal disease treatment

	2 weeks after treatment	3 months after treatment	*p*value
Gingivitis group (n = 31)			
- GCF gingipains	13	22	0.001
- *P. gingivalis* count	1	6	0.1
Periodontitis group(n = 22)			
- GCF gingipains	13	12	0.76
- *P. gingivalis* count	4	0	0.02

: GCF gingipains were more detectable in gingivitis patients after 3months of treatment.

: In priodontitis patients, there were no significant differences in levels of GCF gingipains, whereas *P. gingivalis* count was significantly decreased in paatients after 3 months of treatment.

DISCUSSION

[1] Proteolytic effect of gingipains on TFF3

Previous studies by isoelectric focusing, immunoreactivity, and N-terminal sequence analyses demonstrated that TFF peptides were protease resistant due to their compact configuration (25). However, degradation of TFF peptides was observed when adding reducing agents in the enzymatic reactions (26). The present study, using mass spectrometric techniques, demonstrated that human TFF peptides were digested by oral bacterial proteases called gingipains. These findings were in agreement with previous evidence that gingipains were capable of degrading host defense proteins (17-19). According to our MALDI-TOF/TOF MS findings of TFF3 digestion, the impurity in peptide substrates was a limiting factor that affected the interpretation of mass signals by MALDI-TOF/TOF analyses (27). Thus, LC-MS was performed to identify digested fragments of TFF3, and the results confirmed that TFF domains were cleaved by Kgp, and RgpB. Our observations are in agreement with previous studies demonstrating that TFF peptides were digested by bacterial proteases present in the caecum and colon (28). In opposite to MALDI-TOF/TOF analyses, LC-MS did not show the identified fragments of digested TFF peptides from the enzymatic reactions by boiled Kgp, RgpB, and trypsin. The physio-chemical characteristics of the peptides detected by the MALDI and ESI techniques are different. ESI prefers to detect smaller, more acidic peptides while MALDI is better at the detection of larger, more basic peptides (29-31). It is, therefore, good to analyze peptide samples by both techniques.

According to our observations that gingipains cleaved TFF domains, the proteolytic effects of gingipains on TFF peptides in the oral cavity should be considered. Our previous study demonstrated that reduction of salivary TFF3 concentrations were observed in chronic periodontitis patients, and that levels of salivary TFF3 were negatively correlated with *P. gingivalis* (11). Taking these findings into account, reduction of salivary TFF3 may be due to the proteolytic effects of gingipains. Nevertheless, it should be noted that our investigation on digestion of TFF3 peptides by gingipains was performed by the *in vitro* experiment. These results may be different from those in the oral environment. Therefore, further investigations on TFF3 digestion by gingipains in saliva are needed to be validated. Several studies introduced the ELISA based approach for detection of gingipains in saliva (32, 33). Thus, it would be of interest to evaluate the correlations between levels of salivary gingipains and TFF peptides in chronic periodontitis patients. The present study, using mass spectrometric approaches, provides new information that gingipains are able to digest human TFF3 peptides. MALDI-TOF/TOF MS can be used for evaluating patterns of TFF3 digestion by gingipains but not for identification of digested TFF3 fragments. LC-MS can identify digested TFF fragments. For clinical considerations, the proteolytic effects of gingipains on TFF3 peptides may be a possible explanation for reduction of salivary TFF peptides in chronic periodontitis patients.

[2] Inhibitory effect of gingipains on expression of TFF3 in human gingival epithelial cells

Gingipains are capable of degrading antimicrobial peptides such as human-β-defensin and LL-37 *in vitro* (18, 19). At a cellular level, gingipains are essential factors for *P. gingivalis* to induce apoptosis in human gingival epithelial cells (20). In this study, we demonstrated an inhibitory effect of gingipains on TFF3 mRNA

expression in dose and time response manners. It should be noted that we could not detect TFF3 expression at the protein level. Several possibilities can be explained as follows: First, the amount of TFF3 produced by HGEP cells was below the minimal sensitivity of our Western blotting system. Second, our in-house anti-human TFF3 antibodies might not recognize the native form of TFF3 peptides. To validate these possibilities, immunoprecipitation should be added in the future investigation. In addition, a highly sensitive method should be utilized to confirm the expression of TFF3 peptides. Another explanation is that, in the cell culture environment, without any stimulators or inducing signals, *TFF3* gene could not be expressed. To confirm this speculation, stimulating molecules such as keratinocyte growth factors (KGF) should be added in the cell culture medium, and levels of TFF3 mRNA and TFF3 peptides should be re-evaluated.

It should be addressed that the association between protein and mRNA levels was complex, depending on several factors such as steady state, long-term state changes, and short-term adaptation, during dynamic transitions (34). It was suggested that the spatial and temporal variations of mRNAs, as well as the local availability of resources for protein biosynthesis, could affect the relationship between mRNA and protein levels, and transcript levels of *TFF3* were not sufficient to predict protein levels of TFF3 peptides in this cell culture condition (34).

To verify whether the reduction of *TFF3* mRNA expression is related to the apoptotic effects by gingipains, expression of apoptotic marker molecules such as cleaved PARP and caspase 3 were measured, using Western blotting technique. No detectable levels of these two apoptotic markers were observed from HGEP treated cells. These findings suggest that reduction of TFF3 mRNA occurred without the influence by apoptotic effects of gingipains. However, the molecular mechanisms that reduce the *TFF3* mRNA are not understood. Additional studies on the control of *TFF3* mRNA expression are required to confirm our results.

[3] Measurement of TFF3 and gingipains in saliva of chronic periodontitis patients

Our observations demonstrated that periodontitis patients had the highest level of clinical parameters such as PD, CAL, and BOP, as compared with the control and gingivitis groups. As the prevalence of chronic periodontitis is more likely to occur in elderly people, the mean age of periodontitis individuals was significantly older than the control and gingivitis subjects. The numbers of *P. gingivalis* from chronic periodontitis individuals were more positively detected than those in the control and gingivitis groups. According to our results, *P. gingivalis* was positively detected in subgingival plaque samples from 7 chronic periodontitis patients, and 4 individuals had greater than $1* 10^5$ of *P. gingivalis* copies. It should be noted that the number of *P. gingivalis* could be observed in the control and gingivitis groups, but was not much as compared with the periodontitis group. Our observations are in agreement with one previous study showing the presence of *P. gingivalis* in whole saliva samples of periodontally healthy, and gingivitis individuals (35). It would be of interest for future investigations on the determination of level of *P. gingivalis* in whole saliva in relation to periodontal diseases.

The only *P. gingivalis* strain that produced the soluble type of gingipains was HG66 strain (36, 37). In this study we measured the level of secreted arginine gingipain B (RgpB) in whole saliva using the indirect ELISA assay. The level of RgpB in whole saliva was detected in all three investigated groups. In periodontitis patients, reduction of RgpB was observed in whole saliva, whereas RgpB was increased in GCF. However,

our findings demonstrated that periodontal treatments did not affect the levels of salivary RgpB in periodontitis and non-periodontitis subjects. Our observations are in agreement with one previous study showing an increase in GCF RgpB of patients with chronic periodontitis (38). According to our results, the presence of RgpB in whole saliva samples implies that secreted RgpB may be released from infected sites of periodontal pockets into whole saliva. Previous studies combined with our observations confirm that *P. gingivalis* can be found in an aerobic environment such as whole saliva. Moreever, this bacterium was also detected in the buccal mucosa swab specimen, tongue and buccal gingiva (39, 40). These findings raise several new research questions. First, does secreted RgpB found in whole saliva play any important role in the progression of periodontal disease? Second, whether or not *P. gingivalis* found in an aerobic environment such as whole saliva is less aggressive than those in the anaerobic environment such as in the GCF of periodontal pockets. It remains unclear why decreased levels of RgpB in whole saliva were observed in periodontitis patients as compared with non-periodontitis individuals. We speculate that RgpB can be released into whole saliva in the normal and pathological conditions but, during the progression of the disease, reduction of secreted RgpB in whole saliva of periodontitis patients may be associated with the unidentified mechanisms that *P. gingivalis* utilizes for invading periodontal tissue and evading host immune defense. However, further investigations are needed to confirm this hypothesis.

Previous studies demonstrated that, in oral compartments, TFFs originated mainly from salivary glands with some contribution from goblet cells of the parotid duct and oral mucosal epithelia (3-8). In saliva, TFF3 was the most predominant TFF peptide, followed by TFF1 and TFF2 (8). Regarding the measurement of salivary TFF3, the present study extended our previous observations (11) by investigating the levels of salivary TFF3 concentrations before periodontal disease treatment and after treatment in gingivitis and periodontitis patients. Before periodontal treatments, salivary TFF3 of chronic periodontitis patients was significantly decreased, as compared to control subjects. Previously, we speculated that reduction of salivary TFF3 might be associated with the proteolytic effect of gingipains in saliva. However, our present results showed that, after two weeks of periodontal treatment, the levels of salivary TFF3 were increased in gingivitis patients, whereas salivary TFF3 were declined in periodontitis patients. These findings imply that it is unlikely that decreased levels of salivary TFF3 in chronic periodontitis patients were caused by the directly proteolytic effect of gingipains in saliva (13). Several studies in a rat model reported the pathological effects of periodontal diseases on salivary gland functions (41-44). It remains unclear how *P. gingivalis* may affect the production of TFF3 by salivary glands. Previous observations showed that C/EBPb and NFΚB signaling pathways were associated with the downregulation of TFF expression (45). Base on these findings, we speculate that chronic inflammation from host immune response to periodontopathic bacteria could transfer unidentified signals to the salivary glands resulting in down regulation of TFF3 expression. However, further studies are needed to understand the exact molecular mechanism of the regulation of TFF expression in response to chronic inflammation by periodontopathic bacteria.

CONCLUSION

At the molecular study level, our observations provided new information that gingipains were able to digest human TFF3 peptides. MALDI-TOF/TOF MS could be used for evaluating patterns of TFF3 digestion by gingipains but not for identification of digested TFF3 fragments. LC-MS could identify digested TFF fragments. For clinical considerations, it would be of interest to further investigate whether gingipains can digest TFF3 peptides in saliva, leading to reduction of salivary TFF peptides in chronic periodontitis patients. At the cellular study level, our findings demonstrated that gingipains inhibited the expression of TFF3 mRNA with a dose response manner. Our western blotting analyses showed the negative results for the expression of TFF3 peptides in human gingival epithelial cells. At the clinical study level, we observed that levels of salivary TFF3 and gingipains (RgpB) were significantly reduced in chronic periodontitis patients before periodontal treatment, compared with the control group. Levels of salivary TFF3 in chronic periodontitis patients were also decreased after periodontal treatment, whereas salivary gingipains were not decreased. Our observations demonstrated that the presence of *P. gingivalis* could be detected in whole saliva samples of periodontally healthy, gingivitis, and periodontitis patients. Our findings demonstrated that periodontal treatments did not affect the levels of salivary RgpB in periodontitis and non-periodontitis subjects.

SUGGESTION

According our results, there is a potential to get the prepared manuscripts published in the international journals. Regarding the first objective, we performed additional experiments by investigating the proteolytic effects of gingipains on TFF1 and TFF2 peptides. These results were combined with the findings from this present study for preparation of the first manuscript (please see the attached document in the appendix). Regarding the second objective, we need to perform several additional experiments to get more results to confirm the inhibitory effects of gingipains on expression of TFF3, such as immune precipitation to detect the synthesized TFF3 from cell lysate and secreted TFF3 peptides in cultured medium. We also need to do real time RT-PCR and western blotting to re-evaluate the expression of TFF3 mRNA and TFF3 peptides under the condition of KGF activation. Regarding third objective, comparison between number of *P. gingivalis* in whole saliva and those in the periodontal pockets should be analyzed. In addition, further studies are needed to clarify the exact molecular mechanism in regulation of TFF expression in response to chronic inflammation by periodontopathic bacteria.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. บทความต้นฉบับเพื่อตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Proteolytic effects of gingipains on trefoil factor family peptides
(สถานะของการตีพิมพ์: อยู่ในระหว่างขั้นตอนการเตรียมส่งบทความ: เอกสารแนบในภาคผนวก)

1.2 Inhibitory effect of gingipains on expression of TFF3 in human gingival epithelial cells
(สถานะของการตีพิมพ์: อยู่ในระหว่างขั้นตอนการเขียนบทความ)

1.3 Levels of salivary TFF3 and ginipains in association with periodontal disease treatment
(สถานะของการตีพิมพ์: อยู่ในระหว่างขั้นตอนการเขียนบทความ)

1.4 Production, characterization of mouse monoclonal antibodies against human trefoil factor 3 (TFF3) peptides, and assay applications for detection of TFF3 peptides in oral tissues and saliva
(สถานะของการตีพิมพ์: อยู่ในระหว่างขั้นตอนการเขียนบทความ)

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์

-

2.2 เชิงนโยบาย

-

2.3 เชิงสาธารณะ

มีการสร้างเครือข่ายความร่วมมือเกี่ยวกับการวิจัยทรีฟอยล์แฟคเตอร์
(เอกสารแนบในภาคผนวก)

2.4 เชิงวิชาการ

มีการนำข้อมูลความรู้เกี่ยวกับการวิจัยจินจิเพนไปพัฒนาการเรียนการสอนในระดับบัณฑิตศึกษา
(เอกสารแนบในภาคผนวก)

2.5 อื่นๆ

- ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ
(เอกสารแนบในภาคผนวก)
- การเสนอผลงานในที่ประชุมวิชาการ
(เอกสารแนบในภาคผนวก)

ภาคผนวก

[1]. บทความต้นฉบับเพื่อตีพิมพ์ในวารสารวิชาการนานาชาติ

Proteolytic effects of gingipains on trefoil factor family peptides

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Short running title:
Proteolytic effects of gingipains on trefoil factor family peptides

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Abstract

Objectives The present study was aimed to determine whether trefoil factor family (TFF) peptides which are generally considered to be protease resistant could be digested by oral bacterial enzymes called gingipains.

Materials and Methods Recombinant human TFF1, TFF2, and TFF3 peptides were used as substrates. Gingipains including arginine gingipain (RgpB), and lysine gingipain (Kgp) were used as enzymes. Trypsin was used as a cleavage reagent control. Matrix-assisted laser desorption/ionization with time-of-flight/time-of-flight (MALDI-TOF/TOF) and liquid chromatography (LC) mass spectrometry (MS) were used for analyzing peptide mass signals and amino acid sequences of digested TFF peptides.

Results MALDI-TOF/TOF analyses demonstrated that Kgp, RgpB, and trypsin were able to cleave TFF1 and TFF2 peptides, resulting in different patterns of digested fragments. However, inhibition of enzymatic activities by boiling was failed to prevent TFF peptides from being digested. Impurity in recombinant TFF3 peptide substrates affected the interpretations of enzymatic reaction by MALDI-TOF/TOF. LC-MS analyses demonstrated that identified fragments of TFF1, TFF2, and TFF3 from digestion by gingipains were similar to those by trypsin. However, fragments of TFF1, TFF2, and TFF3 from digestion by boiled gingipains and trypsin were not identified by LC-MS.

Conclusions Although TFF peptides are considered to be protease resistant, the present study demonstrates that gingipains containing trypsin-like activities are able to digest TFF peptides.

Clinical Relevance Further investigations on the correlations between levels of salivary gingipains and TFF peptides in chronic periodontitis patients would be of interest to determine whether gingipains may pathologically affect TFF peptides in the oral cavity.

Keywords: gingipains; mass spectrometry; proteolysis; proteomic; trefoil factor

Introduction

Trefoil factor family (TFF) peptides are secretory products of mucin-producing epithelial cells, and human TFF peptides consist of three members: TFF1; TFF2; and TFF3 [1]. These peptides share a commonly molecular motif called TFF domain. The unique TFF domain demonstrates a three loop structure containing 42 amino acids with six cysteine residues and intramolecular disulfide bonds [2]. TFF1, in the monomeric form, contains 60 amino acids with approximately 6.5 kDa. TFF1 can be found in a dimeric form with a molecular weight of 14 kDa. TFF2 contains 106 amino acids (12 kDa) with two trefoil domains. TFF3 contains 59 amino acids with a molecular weight of 6.6 kDa in a monomeric form or 13 kDa in a dimeric form [2]. TFF peptides are mainly produced in the alimentary tract and can be detected in a variety of body fluids [3]. The complex pattern of TFF domain is beneficial for resistance against proteolytic degradation [1]. TFF peptides have several biological actions including cytoprotective effects, wound healing, and immune response [3]. Previous studies reported the roles of TFF peptides in human diseases such as gastrointestinal tract diseases [4, 5] and several cancers [6].

In the oral compartments, TFF peptides are mainly produced from salivary glands [7-9] with some contribution from goblet cells of the parotid duct and oral mucosal epithelia [10]. In saliva, TFF3 is the most predominant TFF peptide, followed by TFF1 and TFF2 [11]. It was demonstrated that TFF3 peptides involved in cell survival, cell proliferation, and cell migration of oral keratinocytes [12, 13]. Therefore, TFF peptides expressed in saliva and oral epithelial cells may be an important factor in protection against oral tissue damage. However, information of TFF peptides in oral diseases is very limited [14-17]. Our previous study demonstrated that salivary TFF1 and TFF3 concentrations were significantly decreased in chronic periodontitis patients [14]. In addition, levels of salivary TFF3 peptides were negatively correlated with number of *Porphyromonas gingivalis* which was considered as a

major periodontopathic pathogen [14]. It remains unclear whether *P. gingivalis* has any pathological effects on the reduction of salivary TFF peptides in chronic periodontitis patients. Gingipains are proteolytic enzymes and considered to be a major virulence factor produced by *P. gingivalis*. It was reported that gingipains were the essential proteinases responsible for the trypsin-like activity [18]. Gingipains belong to a cysteine proteinase family consisting of three members: arginine specific gingipain with high molecular weight (RgpA: approximately 70-110 kDa); arginine specific gingipain with low molecular weight (RgpB: approximately 50 kDa); and lysine-specific gingipain (Kgp: approximately 60 kDa) [19]. RgpA and Kgp have four principal fragments including: a signal peptide; an N-terminal propeptide; an Arg-specific or Lys-specific catalytic domain; and a C-terminal fragment with haemagglutinin/adhesion domain, whereas RgpB contains a signal peptide; an N-terminal propeptide; an Arg-specific catalytic domain; and a small C-terminal fragment without haemagglutinin/adhesion domain [20]. Gingipains exist in several forms such as membrane-bound and soluble forms [18]. Regarding biological functions, gingipains were capable of degrading several T-cell receptor molecules such as CD4, and CD8, and antimicrobial peptides such as human-β-defensin and LL-37 [21-23].

TFF peptides were thought to be resistant against proteolytic degradation due to the complex pattern of TFF domains. It would be of interest to evaluate whether gingipains are able to cleave TFF peptides. According to previous studies demonstrating that TFF peptides contained several arginine and lysine residues [1, 2], we hypothesized that gingipains could cleave TFF peptides. The aim of the present study was to determine the proteolyic effects of gingipains on TFF peptides, using matrix-assisted laser desorption/ionization with time-of-flight/time-of-flight (MALDI-TOF/TOF) and liquid chromatography coupled with mass spectrometry (LC-MS) for analyzing mass signals and sequences of digested TFF peptides.

Materials and Methods

Preparation of TFF peptides

Recombinant human TFF1, TFF2, and TFF3 peptides were produced in *E. coli* (Raybiotech, Inc. Norcross, GA, USA). According to the company's information, recombinant human TFF1 was a non-glycosylated homodimer containing 120 amino acids with approximate molecular weight of 13.2 kDa. Recombinant human TFF2 was a non-glycosylated polypeptide chain containing 106 amino acids with approximate molecular weight of 12 kDa. Recombinant human TFF1 and TFF2 peptides were purified by propriety chromatographic techniques. Recombinant human TFF3 peptides were conjugated with thioredoxin and histidine at the N-terminus with approximate molecular weight of 26.4 kDa. Recombinant human TFF3 peptides were purified by His-tag affinity purification using immobilized metal ion affinity chromatography (IMAC). Recombinant human TFF1, TFF2, and TFF3 peptides were diluted in sterile milli Q water at 500, 500, and 1,000 ng/µl, respectively.

Stock enzyme solutions

Gingipains, including RgpB and Kgp, were purified from the culture medium of the HG66 strain by a combination of gel filtration and ion-exchange chromatography as previously described [24]. RgpB and Kgp were prepared at 0.81, and 1.0 mg/ml respectively, in 20 mM Bis-Tris, 150 mM NaCl, 5 mM $CaCl_2$. 0.02% NaN_3, pH 6.5. Stock solutions of gingipains were kept at -20°C until used. Trypsin (Promega, USA) was prepared at 20 ng/µl in 5 mM ammoniumbicarbonate in 50% acetonitrile and used as a cleavage reagent control.

An in vitro enzymatic digestion

The protocol for enzymatic digestion was as follows;1 µl of TFF peptides, and 5 µl of 10 mM dithiothreitol (DTT)/10 mM ammoniumbicarbornate was added into 1.5 ml microcentrifuge tube (performed in duplicate) followed by incubation at 56°C for 1 hr. Then,

20 µl of 100 mM iodoacetamide (IAA)/10 mM ammoniumbicarbornate was added. The tubes were kept in the dark and incubated at 37°C for 1 hr. Then 2 µl (20 ng/µl) of each gingipain (diluted in 10 mM ammoniumbicarbonate) was added into each tube, followed by incubation at 37°C for 3 hr. The 2 ul of trypsin (20 ng/ µl; diluted in 50% acetonitrite/10 mM ammoniumbicarbonate) was used as a cleavage reagent. To inactivate the enzymatic activities, gingipains and trypsin were boiled at 95°C for 1 hr. Then, boiled enzymes were used to compare the enzymatic activities with the intact enzymes. The 1 µl of 10% formic acid was added to stop each enzymatic reaction.

Analyses of digested TFF peptides by MALDI-TOF/TOF MS

Each TFF sample was acidified with 0.1% trifluoroacetic acid and 0.1%TritonX-100 to the final concentration of 0.1 µg/µl. The samples were mixed with MALDI matrix solution (10 mg sinapinic acid in 1 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid), directly spotted onto MALDI target (MTP 384 ground steel, Bruker Daltonik GmbH, Germany) and allowed to dry at room temperature. MALDI –TOF/TOF spectra were collected using Ultraflex III TOF/TOF (Bruker Daltonik GmbH, Germany) in a linear positive mode. Five hundred shots were accumulated with a 200 Hz laser for each sample. Mass spectra ranging from 500 to 30,000 Da were analyzed by FlexAnalysis and ClinproTool software (Bruker Daltonik GmbH, Germany). Cytochrome C was used as an external protein calibration.

Analyses of digested TFF fragments by LC MS

The digested peptides were injected into Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker Daltonik GmbH, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 50% acetonitrile was run in 20 min. Peptide

fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300−1500 *m*/*z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50−3000 *m*/*z*. Acquired LC-MS/MS raw data were quantitated based on the peptide ions signal intensities in MS mode using DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare, USA) [25, 26]. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK) [27]. The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (*Homo sapiens*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (1).

Statistical analyses

Statistical analyses were performed using SPSS program (version 20). The Kolmogorov-Smirnov test and the Shapiro-Wilk test were used to assess the distribution of the investigated data. Comparisons of relative amount of digested TFF fragments from enzymatic digestions (Kgp, RgpB, and trypsin) were analyzed by one-way ANOVA. Two-tailed $p < 0.05$ was considered statistically significant.

Results

Analyses of digested TFF peptides by MALDI-TOF/TOF MS

It should be noted that mass spectra by a MALDI-TOF/TOF method (operated at a linear mode) were acquired using the mass range between 500 to 30,000 Da. Therefore, the mass spectral data of gingipains (RgpB and Kgp) and trypsin were not demonstrated because their molecular masses were beyond the upper limit. Patterns of mass signals of TFF1 were demonstrated in Figure 1A. An intense mass signal of 13,394 Da representing TFF1 peptides was shown. RgpB, Kgp, and trypsin were able to digest TFF1, resulting in similar mass

signals of 3,364 and 6,726 Da. However, inhibition of enzymatic activities by boiling was failed to prevent TFF peptides from being digested (Figure 1B). Boiled RgpB, Kgp, and trypsin were able to digest TFF1, resulting in similar mass signals of 3,361 and 6,719 Da.

Patterns of mass signals of TFF2 were demonstrated in Figure 2A. An intense mass signal of 12,035 Da representing TFF2 peptides was observed. RgpB, Kgp, and trypsin were able to digest TFF2, but showing different mass signal patterns. Boiled RgpB, Kgp, and trypsin were also able to digest TFF2 (Figure 2B), showing different patterns of mass signals from those by unboiled enzymes. Patterns of mass signals of TFF3 were demonstrated in Figure 3A. We could not detect the mass signal representing TFF3 peptides (approximate molecular weight of recombinant TFF3 is 26.4 kDa). According to mass signal analysis, it was indicated that there was the impurity in preparation of recombinant TFF3 peptides. Nevertheless, the enzymatic reactions by RgpB, Kgp, and trypsin showed different fragmentation patterns (Figure 3A). The enzymatic reactions by boiled RgpB, Kgp, and trypsin demonstrated different patterns of mass signals (Figure 3B) as compared with the results from those with the unboiled enzymes.

Analyses of digested TFF peptides by LC MS

Identification of digested TFF1, TFF2, and TFF3 peptides by LC MS was demonstrated in tables 1, 2, and 3, respectively. It was demonstrated that TFF domains were cleaved by Kgp, RgpB, and trypsin. Digested TFF1 fragments covered 89.29% of TFF1 peptide sequence. Digested TFF2 fragments covered 64.34% of TFF2 peptide sequence, whereas digested TFF3 fragments covered 51.06% of TFF3 peptide sequence. Relative amount of digested fragments of TFF1, TFF2, and TFF3 from enzymatic digestions by RgpB, Kgp, and trypsin was not significantly different. It should be addressed that there were no identified fragments of TFF1, TFF2, and TFF3 from enzymatic reactions by boiled RgpB, Kgp, and trypsin.

Discussion

Previous studies by isoelectric focusing, immunoreactivity, and N-terminal sequence analyses demonstrated that TFF peptides were protease resistant due to their compact configuration [28]. However, degradation of TFF peptides was observed when adding reducing agents in the enzymatic reactions [29]. The present study, using mass spectrometric techniques, demonstrated that human TFF peptides were digested by oral bacterial proteases called gingipains. These findings were in agreement with previous evidence that gingipains were capable of degrading host defense proteins [21-23]. According to our MALDI-TOF/TOF MS findings, patterns of mass signals derived from TFF1digestion by Kgp, RgpB, and trypsin were similar. These findings imply that Kgp and RgpB cleave TFF1 peptides through the same enzymatic reactions by trypsin. It is well known from the literature that Kgp and RgpB are cysteine proteases containing trypsin like activities [18-20]. Moreover, the results from enzymatic reactions by boiled Kgp, and RgpB are similar to those by intact enzymes. These findings imply that gingipains are thermostable. In contrast to TFF1, patterns of mass signals derived from TFF2 digestion were different among enzymatic reactions by Kgp, RgpB, and trypsin. It could be possible that Kgp and RgpB might cleave TFF2 by different mechanisms from those enzymatic reactions by trypsin. Regarding TFF3 digestion, the impurity in peptide substrates was a limiting factor that affected the interpretation of mass signals by MALDI-TOF/TOF analyses [30].

Several candidate mass signals of digested fragments of TFF1and TFF2 were demonstrated by MALDI-TOF/TOF. However, our attempts to identify these candidate masses directly by MALDI-TOF/TOF were not successful. This is probably due to the low quality of b- and y-ion series resulting in sequence tags that gave the low confident identification [31]. Thus, LC-MS was performed to identify digested fragments of TFF1, TFF2, and TFF3, and the results confirmed that TFF domains were cleaved by Kgp, and

RgpB. Our observations are in agreement with previous studies demonstrating that TFF peptides were digested by bacterial proteases present in the caecum and colon [32]. In opposite to MALDI-TOF/TOF analyses, LC-MS did not show the identified fragments of digested TFF peptides from the enzymatic reactions by boiled Kgp, RgpB, and trypsin. The physio-chemical characteristics of the peptides detected by the MALDI and ESI techniques are different. ESI prefers to detect smaller, more acidic peptides while MALDI is better at the detection of larger, more basic peptides [33-35]. It is, therefore, good to analyze peptide samples by both techniques.

According to our observations that gingipains cleaved TFF domains, the proteolytic effects of gingipains on TFF peptides in the oral cavity should be considered. Our previous study demonstrated that reduction of salivary TFF1 and TFF3 concentrations were observed in chronic periodontitis patients, and that levels of salivary TFF3 were negatively correlated with *P. gingivalis* [14]. Taking these findings into account, reduction of salivary TFFs may be due to the proteolytic effects of gingipains. Nevertheless, it should be noted that our investigation on digestion of TFF peptides by gingipains was performed by the *in vitro* experiment. These results may be different from those in the oral environment. Therefore, further investigations on TFF digestion by gingipains in saliva are needed to be validated. Several studies introduced the ELISA based approach for detection of gingipains in saliva [36, 37]. Thus, it would be of interest to evaluate the correlations between levels of salivary gingipains and TFF peptides in chronic periodontitis patients.

In conclusion, the present study, using mass spectrometric approaches, provides new information that gingipains are able to digest human TFF peptides. MALDI-TOF/TOF MS can be used for evaluating patterns of TFF1 and TFF2 digestion by gingipains but not for identification of digested TFF fragments. LC-MS can identify digested TFF fragments. For

clinical considerations, the proteolytic effects of gingipains on TFF peptides may be a possible explanation for reduction of salivary TFF peptides in chronic periodontitis patients.

Acknowledgements

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Compliance with ethical Standards

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Ethical approval

-

Informed consent

-

Conflicts of Interest Statement

The authors declare that they have no conflicts of interest.

Description of author's contribution to the research and its publication

P Chaiyarit performed the experimental design and research plan, and contributed to statistical analysis and interpretation of data. S Roytrakul, J Jaresitthikunchai, and N Phaonakrop contribute to MALDI-TOF/TOF and LC-MS analyses. B Potempa and J Potempa contribute to preparation of gingipains. All authors sufficiently participated in writing this manuscript.

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Table 1 Analyses of digested TFF1 peptides by LC-MS

TFF1 peptide sequences[a]	Score[b]	Mass (Da)	Relative amount[c] of TFF1 (after digestion by enzymes)		
			Kgp	RgpB	Trypsin
QNCGFPGVTPSQCANK	63.14	1764.78	16.11	18.51	21.46
PEEECEF	28.1	940.09	16.04	17.21	16.95
LVSMLALG	24.98	820.35	20.50	20.31	21.65
VSMLALGT	22.25	807.67	16.92	16.56	16.64
MENKVICALVLVSMLA	18.22	1821.97	15.24	15.28	20.38
EAQTETCTVAPRER	17.84	1648.48	18.88	19.68	16.32
MATMENKV	17.61	923.31	16.91	17.92	20.90
YPNTIDVP	16.48	920.12	16.20	18.16	17.48
NKVICA	12.47	704.31	16.88	16.91	17.34
TPSQCANKGCCFDD	11.85	1661.58	18.44	18.51	19.16
SMLALGTLAEAQT	11.57	1303.72	19.69	19.16	20.29
PNTIDVPPEEECE	11.43	1527.21	22.31	23.05	22.85
TETCTVAPRE	10.55	1163.51	22.75	22.47	21.29
GVTPSQCAN	10.12	934.59	17.26	15.28	19.21

[a]Peptide sequences cover 89.29% of TFF1 and matched peptide sequences are demonstrated in bold. Amino acid sequences with underline demonstrate a TFF1 domain: **MATMENKVICALVLVSMLALGTLAEAQTE<u>TCTVAPRERQN</u>**

<u>CGFPGVTPSQCANKGCCFDD</u>TVRGVPWCF**<u>YP</u>NTIDVPPEEECEF**

[b]Score obtained from Mascot for each match represents degree of similarity between the theoretical and experimental data.

[c]Relative amount of TFF1 peptide(s) was calculated from peptide signal intensity of MS spectra.

Table 2 Analyses of digested TFF2 peptides by LC-MS

TFF2 peptide sequences[a]	Score[b]	Mass (Da)	Relative amount[c] of TFF2 (after digestion by enzymes) Kgp	RgpB	Trypsin
TNCGFPGITSDQCFDNG	54.29	1888.63	20.50	20.97	21.00
LCALAGSE	15.90	820.66	22.87	22.62	22.38
AGSEKPSPC	15.36	929.40	17.76	17.02	18.55
LVLGLCA	14.87	746.63	19.21	18.27	20.40
LPKQES	14.78	701.28	20.04	19.82	20.10
CGFPGIT	14.44	750.36	17.91	17.26	17.44
ALAGSEKP	14.02	772.46	18.28	18.43	17.70
DNGCCF	13.85	770.75	18.47	17.75	17.43
HPLPKQESD	13.44	1050.21	16.80	17.14	18.38
RRNCGYPGISPEE	12.8	1533.06	21.04	21.41	20.32
RTNCGFPGITSD	12.43	1326.01	16.93	17.04	17.95
LAGSEKPSPCQCSR	11.65	1577.14	20.08	20.80	20.58
KPSPCQCSRLSPH	11.61	1554.87	21.60	22.04	21.17
VMEVSDRRNCGYPG	11.61	1639.91	22.87	22.62	22.38
ISPEECASR	10.84	1049.93	18.52	19.60	19.09
CFFPKS	10.83	784.61	18.89	21.46	21.66
GFPGITSD	10.16	792.56	19.01	18.16	17.62

[a]Peptide sequences cover 64.34% of TFF2 and matched peptide sequences are demonstrated in bold. Amino acid sequences with underline demonstrate TFF2 domains:

MGRRDAQLLAAL**LVLGLCALAGSEKPSPC**<u>**QCSRLSPH**</u>N<u>**RTNCGFPGITSD**</u>

<u>**QCFDNGCCF**</u>DSSVTGVPWCF<u>**H**</u>**PLPKQESD**QC<u>**VMEVSDRRNCGYPGISPEE**</u>

<u>**CASR**</u>KCCFSNFIFEVPW<u>**CFFPKS**</u>VEDCHY

[b]Score obtained from Mascot for each match represents degree of similarity between the theoretical and experimental data

[c]Relative amount of TFF2 peptide(s) was calculated from peptide signal intensity of MS spectra.

Table 3 Analyses of digested TFF3 peptides by LC-MS

			Relative amount[c] of TFF3 (after digestion by enzymes)		
TFF3 peptide sequences[a]	**Score[b]**	**Mass (Da)**	**Kgp**	**RgpB**	**Trypsin**
PHVTPK	19.4	679.48	17.23	25.11	19.04
KDRVDC	16.02	792.67	22.00	21.90	22.11
VGLSANQCA	14.49	920.76	18.94	21.74	22.47
LLSSSSAE	12.02	794.36	21.36	23.79	21.77
VVMAARAL	10.72	846.69	24.67	23.13	23.05
EPTVVMAARA	10.58	1058.94	20.44	20.65	25.25
ALCMLGLVLALLSS	10.02	1476.02	16.74	15.80	21.61

[a]Peptide sequences cover 51.06% of TFF3 and matched peptide sequences are demonstrated in bold. Amino acid sequences with underline demonstrate a TFF3 domain:

MKRVLSCVP**EPTVVMAARALCMLGLVLALLSSSSAE**EY**VGLSANQCA**

VPA**KDRVDC**GY**PHVTPK**ECNNRGCCFDSRIPGVPWCFKPLQEAECTF

[b]Score obtained from Mascot for each match represents degree of similarity between the theoretical and experimental data.

[c]Relative amount of TFF3 peptide(s) was calculated from peptide signal intensity of MS spectra.

Figure legends

Figure 1A Pattern of TFF1 mass signals when TFF1 peptides were digested by Kgp, RgpB, and trypsin, respectively.

Figure 1B Pattern of TFF1 mass signals when TFF1 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

Figure 2A Pattern of TFF2 mass signals when TFF2 peptides were digested by Kgp, RgpB, and trypsin, respectively.

Figure 2B Pattern of TFF2 mass signals when TFF2 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

Figure 3A Pattern of TFF3 mass signals when TFF3 peptides were digested by Kgp, RgpB, and trypsin, respectively.

Figure 3B Pattern of TFF3 mass signals when TFF3 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

Figure1A Pattern of TFF1 mass signals when TFF1 peptides were digested by Kgp, RgpB, and trypsin, respectively.

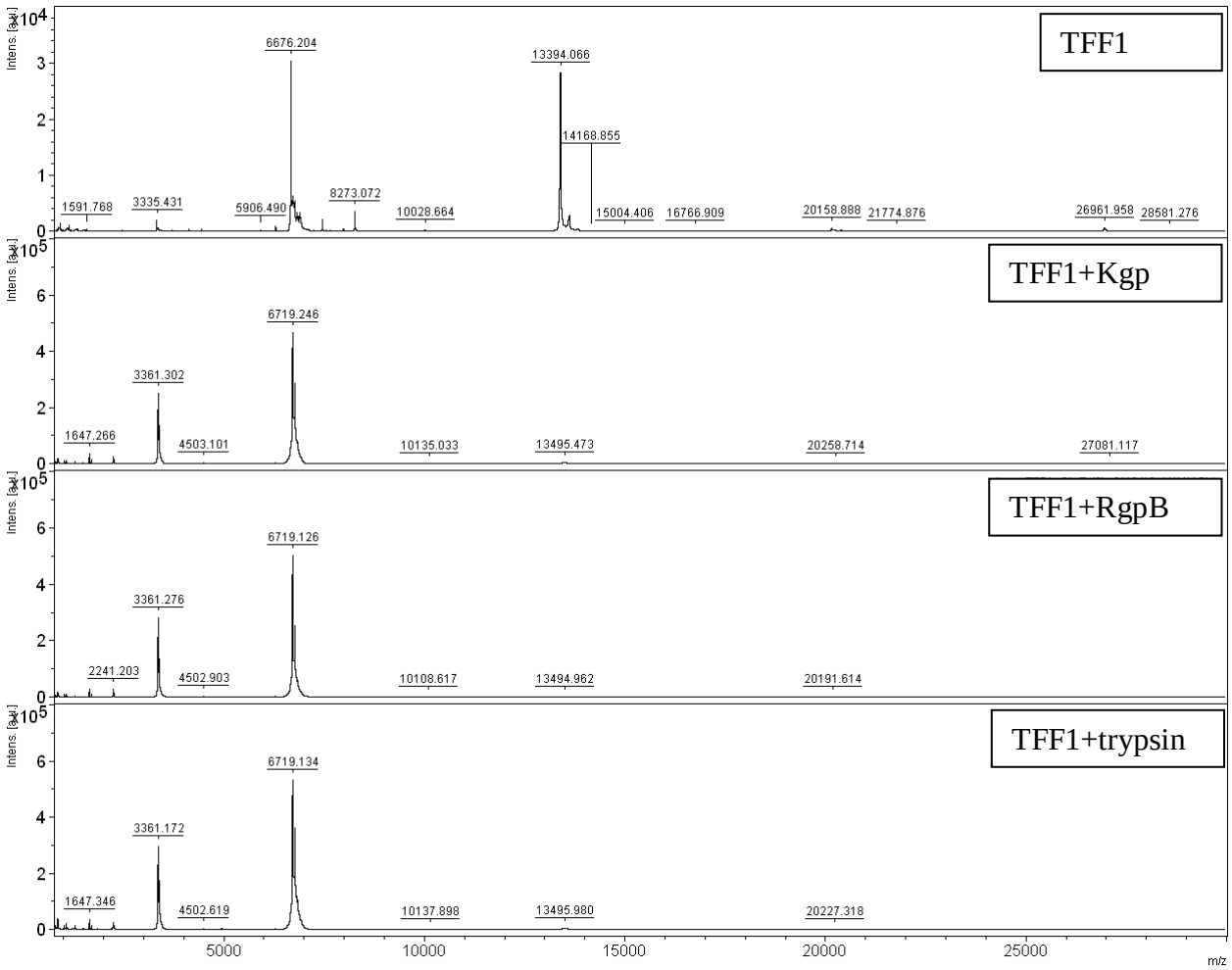


Figure1B Pattern of TFF1 mass signals when TFF1 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

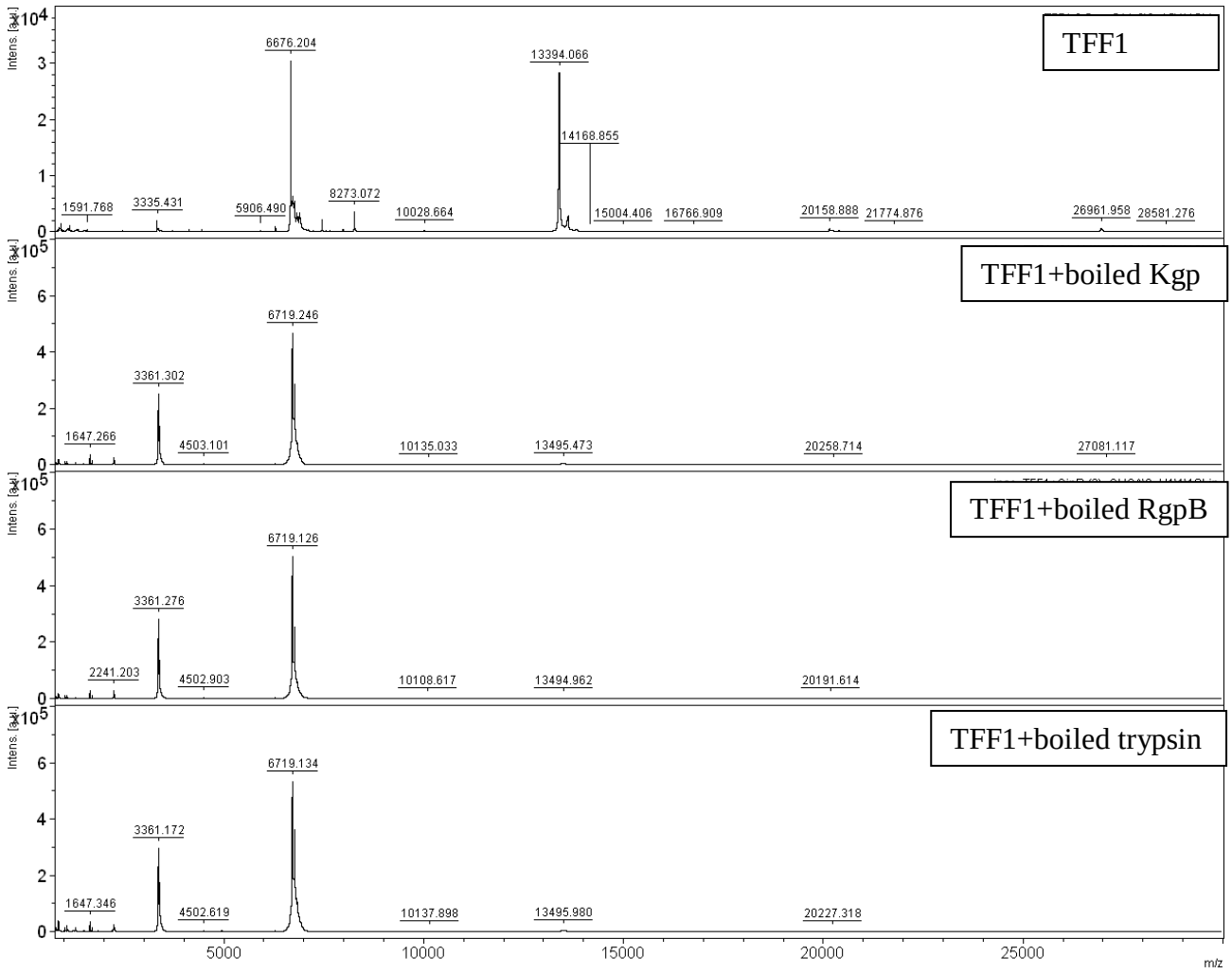


Figure 2A Pattern of TFF2 mass signals when TFF2 peptides were digested by Kgp, RgpB, and trypsin, respectively.

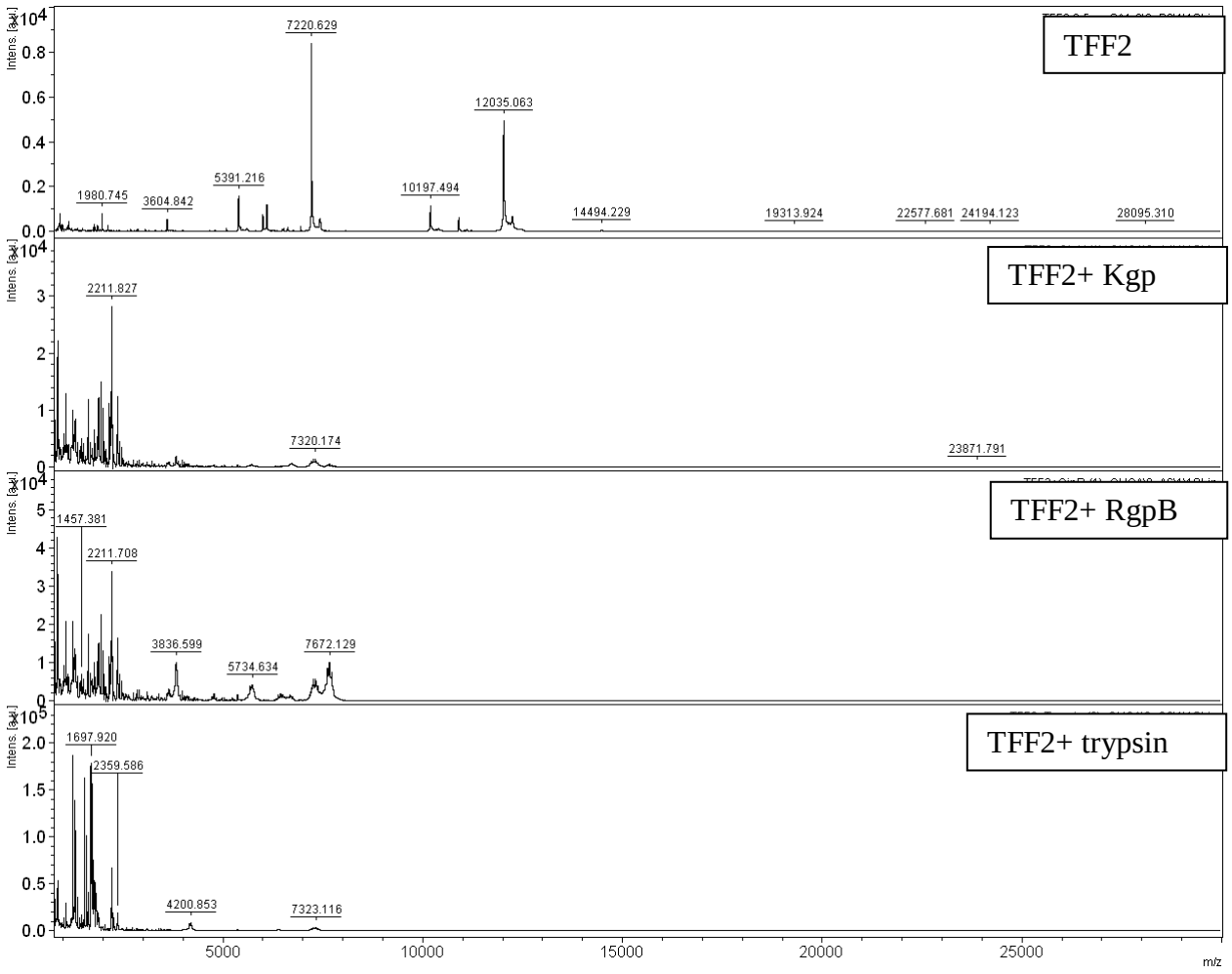


Figure 2B Pattern of TFF2 mass signals when TFF2 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.

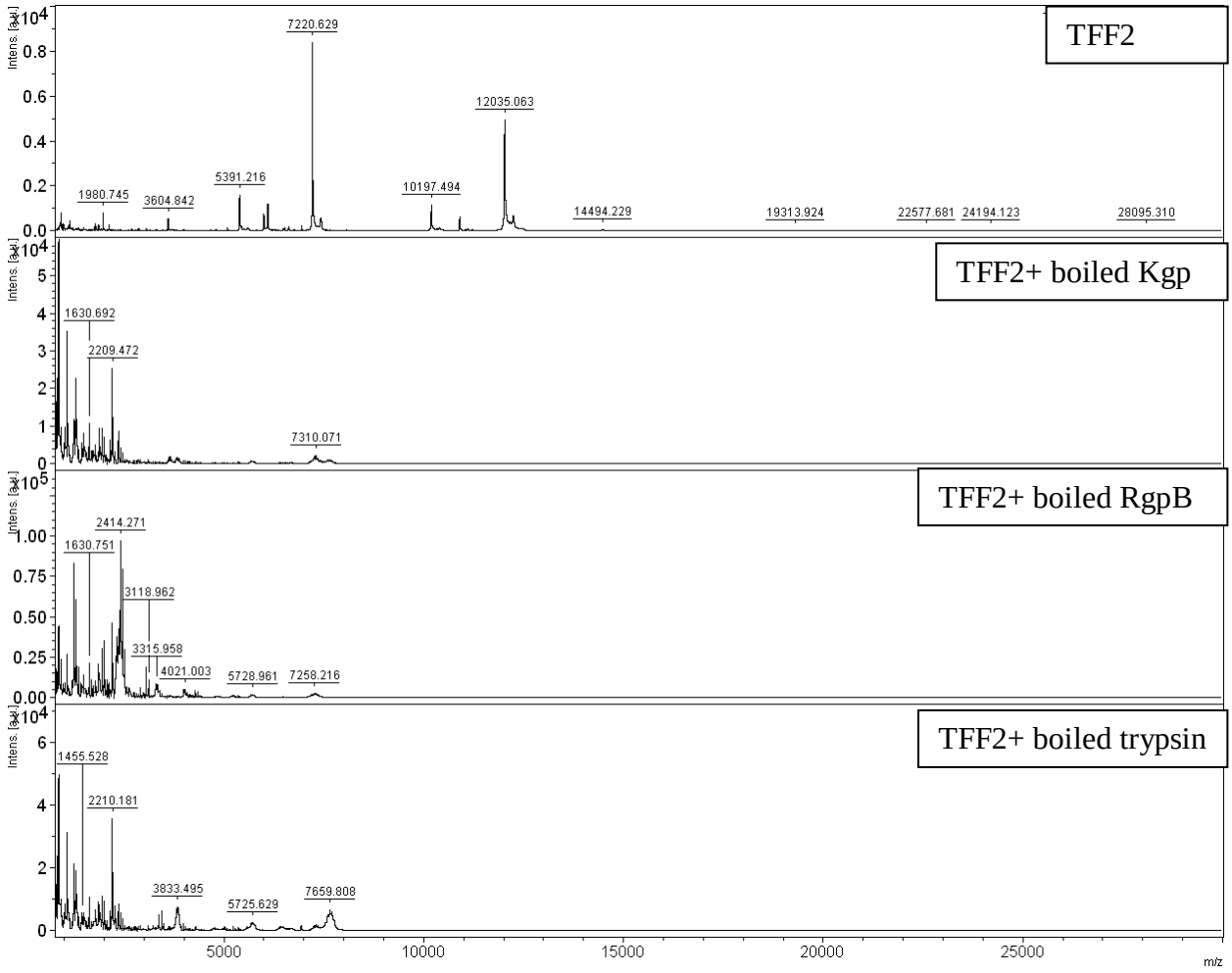


Figure 3A Pattern of TFF3 mass signals when TFF3 peptides were digested by Kgp, RgpB, and trypsin, respectively.

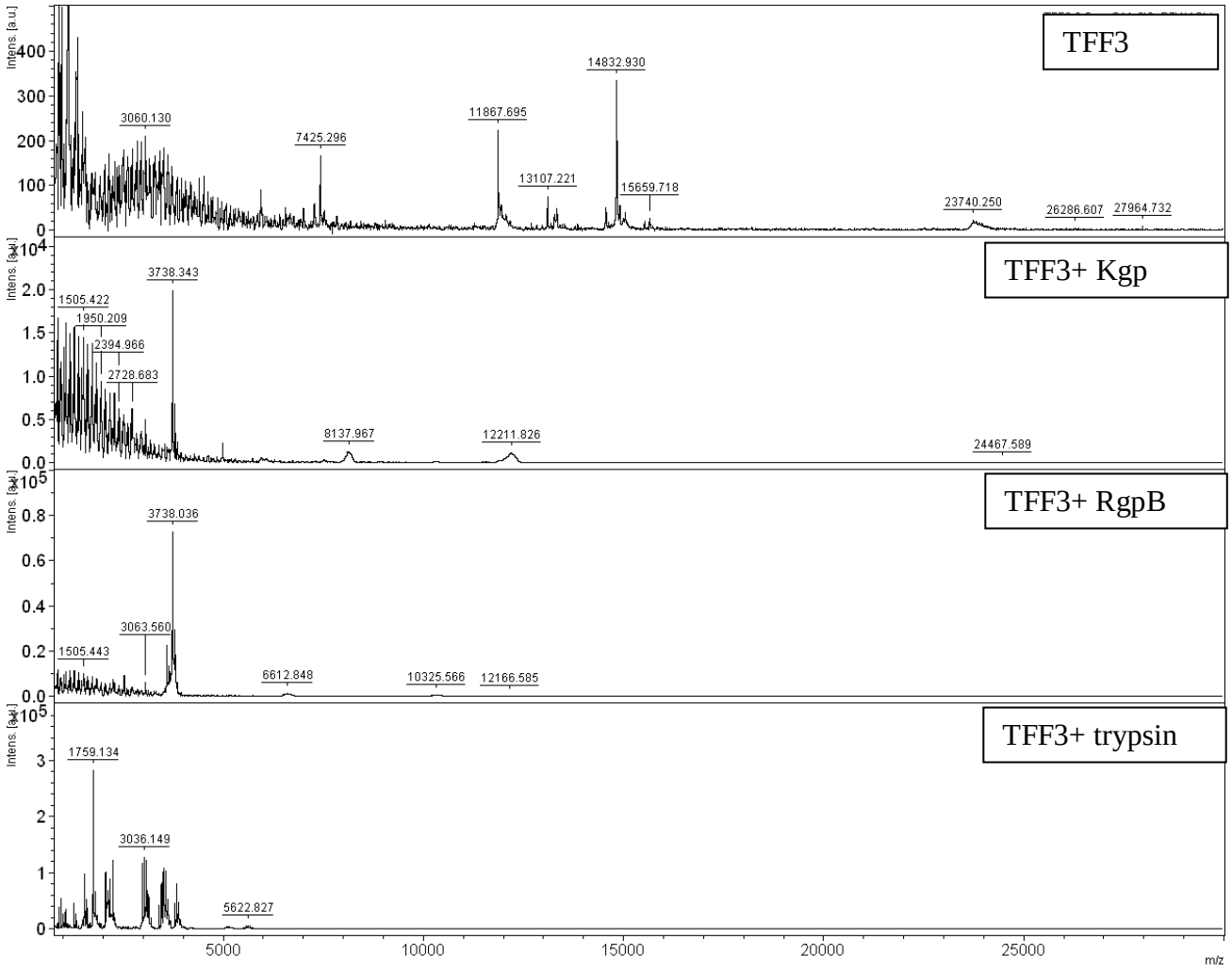
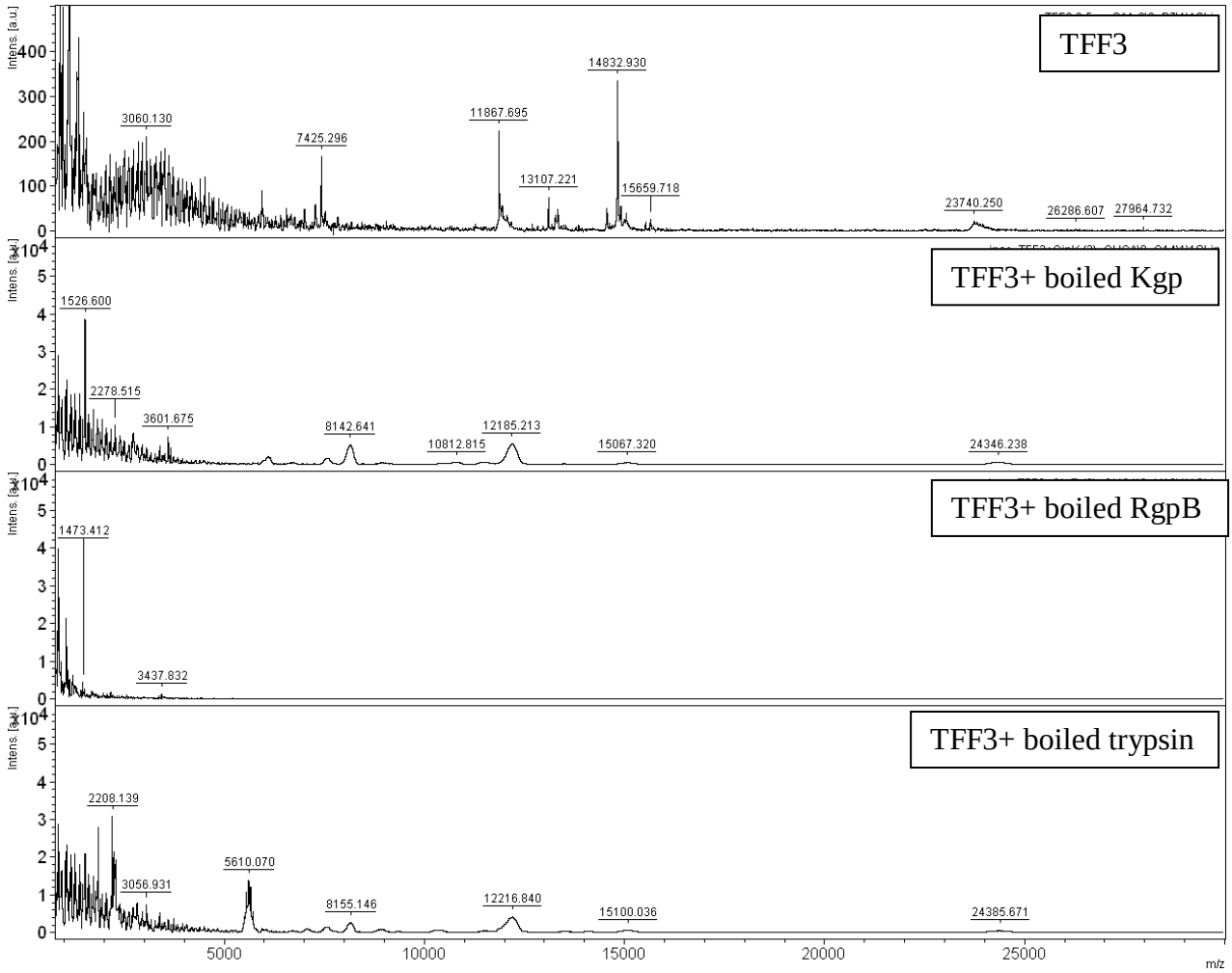


Figure 3B Pattern of TFF3 mass signals when TFF3 peptides were digested by boiled Kgp, RgpB, and trypsin, respectively.



[2] การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์

-

2.2 เชิงนโยบาย

-

2.3 เชิงสาธารณะ: การสร้างเครือข่ายความร่วมมือเกี่ยวกับการวิจัยทรีฟอยล์แฟคเตอร์

With international TFF researchers at The 7th International Conference on Trefoil Factor Family Peptides, Friedrich-Alexsander University, Erlangen, Germany

2.4 เชิงวิชาการ: มีการนำข้อมูลความรู้เกี่ยวกับการวิจัยจินจิเพนไปพัฒนาการเรียนการสอนในระดับบัณฑิตศึกษา

Evaluation of salivary arginine gingipain-B (RgpB) in patients with periodontal diseases

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Abstract

Gingipains are proteolytic enzymes that account for 85 percent of total proteolytic enzymes of *P. gingivalis*, which is one of the bacteria involving in chronic periodontal diseases. Gingipains have been detected significantly in gingival crevicular fluid (GCF) of patients with chronic periodontitis. The objective of this study was to evaluate the levels of salivary gingipains, especially arginine gingipain B (RgpB) and number of *P. gingivalis* in subgingival plaque from patients with periodontal diseases. Whole saliva samples and sub-gingival plaque were collected from 10 gingivitis patients and 10 chronic periodontitis (CP) patients. Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the level of salivary RgpB. Number of *P. gingivalis* was quantified by real-time polymerase chain reaction. Levels of salivary RgpB were not significantly different between gingivitis and periodontitis patients. Level salivary RgpB was negatively correlated with mean pocket depth (PD). Number of *P. gingivalis* was positively correlated with mean bleeding on probing (BOP). In conclusion, our study provided additional information that there were correlations between levels of salivary RgpB and a clinical parameter for periodontal diseases including PD. These findings would help us to understand the pathological role of RgpB in periodontal diseases. However, further investigations of gingipains are still required to clarify the exact role of salivary RgpB in association with periodontal diseases.

Keywords: Arginine gingipain B (RgpB), Whole saliva, *Porphyromonas gingivalis*

2.5 อื่นๆ

- ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ: วิทยาสารทันตแพทยศาสตร์

วันที่ 25 พฤษภาคม พ.ศ. 2559

เลขที่ JDAT2016-18

เรื่อง แจ้งผลการพิจารณาบทความ

เรียน รศ.ดร. พลธรรม ไชยฤทธิ์

วิทยาสารทันตแพทยศาสตร์ขอขอบคุณที่ท่านให้ความสนใจในการส่งบทความและแก้ไขบทความเพื่อตีพิมพ์ในวิทยาสารทันตแพทยศาสตร์

กองบรรณาธิการมีความยินดีเป็นอย่างยิ่งที่จะเรียนให้ท่านทราบว่าบทความของท่านเรื่อง "Current Update in Human Saliva and Its Role in Oral and Systemic Health and Diseases" ซึ่งเป็นบทความปริทัศน์ จะได้รับการตีพิมพ์ในวิทยาสารทันตแพทยศาสตร์ ปีที่ 66 ฉบับที่ 4 ตุลาคม – ธันวาคม พ.ศ. 2559

ขอแสดงความนับถือ

เอกมน มหาโกคา

สาราณียกร

- การเสนอผลงานในที่ประชุมวิชาการ:

Oral Presentation: Challenges in exploration of trefoil factors in oral compartments

The 7th International Conference on Trefoil Factor Family Peptides, Erlangen, Germany

(May 20-22, 2015)

FRIEDRICH-ALEXANDER UNIVERSITÄT ERLANGEN-NÜRNBERG
MEDIZINISCHE FAKULTÄT

7th International Conference on Trefoil Factor Family Peptides Erlangen Germany

20.-22.05. 2015

Department of Anatomy II
Universitätsstraße 19
D-91054 Erlangen
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May, 20th

10.00 - 2.00
Registration

2.00 - 2.05
Welcome

2.05 - 3.30
Session 1 - Physiopathology

2.05 - 2.50
Keynote - TFF peptides: from biosynthesis to function
Werner Hoffmann, Magdeburg, Germany

2.50 - 3.10
CXCR4 and CXCR7 mediate TFF3 signaling induced cell migration independently from the ERK1/2 signaling pathway
Julia Dieckow, Wolfgang Brandt, Stefan Schob, Ute Schulze, Philipp Ackermann, Saadettin Sel, Friedrich Paulsen; Halle (Saale), Leipzig, Düsseldorf, Heidelberg and Erlangen, Germany

3.10 - 3.30
Challenges in exploration of trefoil factors in oral compartments
Ponlatham Chaiyarit, Kon Kaen, Thailand

3.30 - 4.00
Coffee break

4.00 - 5.45
Session 2 - Metabolism

4.00 - 4.45
Keynote – Function of TFFs in obesity
Mirela Baus-Lončar, Zagreb, Croatia

4.45 - 5.05
Fatty acid and protein profiling in the liver of Tff2 and Tff3 deficient mice
Maro Bujak, Martina Mihalj, Ivana Tartaro Bujak, Srđan Vučinić, Anita Horvatić, Sanja Novak, Katarina Misković , Vjekoslav Kopačin, Branka Mihaljević, Ines Drenjančević, Mirela Baus Lončar; Zagreb and Osijek, Croatia

May, 20th

5.05 - 5.25
The effect of trefoil factor family peptide 3 (TFF3) on the formation of cancellous bone in the secondary centers of ossification
Nikola Bijelić, Željka Perić Kačarević, Tatjana Belovari; Osijek, Croatia

5.25 - 5.45
Role of TFF3 in bone and cartilage
Patricia Klinger, Friedrich Paulsen, Sebastian Schmidt, Bernd Swoboda, Arif Ekici, Kolja Gelse; Erlangen, Germany

5.45 - 6.25
Session 3 - Poster presentation

5.45 - 6.25
Poster demonstration (each responsible poster author will briefly present its poster to the audience)

6.25 - 8.30
Get together (Schlosspark)

Posters (May, 20th)

1. An expression of Trefoil Factor 3 in elderly major salivary glands: A cadaveric study
Parichat Prachaney, Rarinthorn Sumrid, Kowit Chaisiwamongkol, Ponlatham Chaiyarit; Khon Kaen, Thailand

2. Expression and localization of trefoil factor family peptides in the different laryngeal compartments
Ronny Fischer, Martin Schicht, Fabian Garreis, Lars Bräuer, Friedrich Paulsen; Erlangen, Germany

3. Localization of trefoil factor family peptide 1 and 3 in epithelial tissues originating from all three primary germ layers of developing mouse embryo
Nikola Bijelić, Tatjana Belovari, Mirela Baus Lončar; Zagreb and Osijek, Croatia

4. TFF peptides in synovial fluid of patients suffering from osteoarthritis and rheumatoid arthritis
Judith Bechmann, Patricia Klinger, Kolja Gelse, Stefan Sesselmann, Horst Claassen, Michael Tsokos, Saskia Guddat, Friedrich Paulsen, Martin Schicht; Erlangen, Halle (Saale) and Berlin, Germany

5. Close anatomical relationship of chemically characterized nerve fibers to putative Ki67 and TFF positive stem cells in mouse gastric mucosa"
Lukas Roßner, Vanessa Strauß, Winfried Neuhuber; Erlangen, Germany