CHAPTER 3

ACTIVATION OF MMP-2 BY *Porphyromonas gingivalis* SUPERNATANT IN HUMAN PERIODONTAL LIGAMENT CELLS AND GINGIVAL FIBROBLASTS

3.1 Introduction

Periodontitis is a chronic inflammatory disease characterized by infiltration of leukocytes, loss of connective tissue, resorption of alveolar bone, and formation periodontal pockets. The inflammatory conditions of periodontal disease gradually lead to impairment and destruction of tooth supportive tissue. The extracellular matrix components, including collagen, fibronectin, and proteoglycan, are the major tissue proteins responsible for the structural integrity of tooth anchoring apparatus. Destruction of the supportive apparatus is characterized by a degradation of extracellular matrix components, lead to irreversible loss of periodontal soft connective tissue and alveolar bone. Bacterial pathogens and their products are the primary etiologic agent that directly initiate periodontal disease (Haffajee and Socransky, 2000). Such variability can be attributed to differences in composition of the microflora in subgingival plaque but clinical data from previous study has been shown the composing of subgingival microflora, which most of the suspected pathogens are gram-negative anaerobes (Liljenberg et al., 1994). Suspected pathogens associated in periodontal disease are *Actinobacillus actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis)* and *Bacteroides forsythus (B. forsythus)* (Zambon, 1996). The gingival sulcus, which is

0.5-mm deep crevice (in healthy gingiva) between the tooth and gingiva, is the portal of entry and niche for a unique bacterial ecology system, composed of highly adapted microorganism. A large body of evidence suggests that *P. gingivalis* is one of the most putative pathogen, which have been associated chronic adult periodontitis (Schwartz et al., 1997; Lamont and Jenkinson, 1998).

The gram-negative obligate anaerobe *P. gingivalis* is one of the organisms most strongly associated with chronic adult periodontitis. A multitude of virulence such as fimbriae, hemagglutinin, lipopolysaccharide, and various bacterial enzymes and proteases which are capable of hydrolyzing collagen, immunoglobulins, iron-binding protein, and complement factors. Eventhough periodontal destruction is partly caused by proteinases secreted from this group of bacteria; it is now accepted that host response to such bacterial products is the major cause of the pathogenesis at the site of infection. (Kilian, 1981; Carlsson et al., 1984a; Sundqvist et al.,1988a; Uitto et al., 1989; Yoneda et al., 1990; Imatani et al., 2001; Takahashi and Sato, 2002). Products of *P. gingivalis*, such as lipopolysaccharide, membrane proteins and bacterial proteinases, are known to be able to induce the response of local cells to secrete high level of several cytokines and proteinases that lead to periodontal tissue destruction (Kornman et al., 2000). Many of these virulence factors are not only present on membrane but these virulence factors could be secreted as bacterial products such as proteinase, membrane blebs or vesicle (Mayrand and Grenier, 1989). Increasing literature indicates that member of periodontopathogenic bacteria (including *P. gingivalis)* are not only capable of a direct attack and destruction of host tissue, but they could communicated with host cells to stimulate the production of host mediators, cytokines and also host-derived enzymes (Yamazaki et al., 1997; Suchett-

Kaye et al., 1998). Previously, a number of studies have implicated matrix metalloproteinases (MMPs), playing a central role in a pathogenesis of the disease (Birkedal-Hansen et al., 1993; Makela et al., 1994; Korostoff et al., 2000).

Matrix metalloproteinase (MMP) are a family of proteolytic enzymes that mediate the degradation of extracellular matrix macromolecules, including interstitial and basement membrane collagens, fibronectin, laminin and proteoglycan core protein. The enzymes secreted in latent form and become activated in pericellular environment by disruption of a Zn^{++}-cysteine bond which block the reactivity of the active site (Birkedal-Hansen et al., 1993). It has been shown that MMP which involve in periodontal tissue destruction including MMP-1, MMP-8, MMP-9 and recently MMP-2 (Birkedal-Hansen, 1993b; Makela et al., 1994; Komatsu et al., 2001). MMP-2 was found in the crevicular fluid of periodontitis patients in low amount (Ingman et al., 1994), on the contrary, high amount of MMP-2 was found in the gingival tissue of the patients. In addition, increase of active MMP-2 was recently shown in chronic adult periodontitis (Korostoff et al., 2000). These results suggest the involvement of MMP-2 in periodontal destruction.

Activated form of MMP-2 is thought to play a crucial role in periodontal destruction. Most cells of mesenchymal origin have the ability to synthesize and secrete MMP-2 in latent form (pro-enzyme) but must be further stimulated to enable activation of MMP-2 for proteolytic function (Pulyaeva et al., 1997). Therefore MMP-2 activation is an important regulatory process before its function.

The correlation between *P. gingivalis* products and MMP secretion has been described (Uitto et al., 1989; Sorsa et al., 1992; Fravalo et al., 1996; DeCarlo et al., 1997; Firth et al., 1997). The products that have been shown to contain MMP induction ability include the outer membrane extract and the bacterial phospholipase C (Ding et al., 1995). Furthermore, proteinases secreted from *P. gingivalis* contain the inductive ability as well as the activation ability, other than its enzymatic function. Therefore, it is interesting to examine the correlation between these *P. gingivalis* products and the activation of MMP-2. Specifically, the biological significance of MMP-2 activation in human adult periodontitis by bacterial supernatant of *P. gingivalis* remains to be clarified.

Periodontium is composed of numerous cell types, including fibroblast, epithelial cells and inflammatory infiltrate of neutrophils and mononuclear cells, all of which may contribute to these inflammatory processes. Previously, a variety of bacterial virulence factors induces cellular response has been investigated extensively in macrophage and monocytes. Fibroblasts and epithelial cell were previously considered as cells primarily concerned with providing physical barriers and structural components in periodontium. Importantly, it is now recognized that these local cells may play a crucial role in periodontal destruction. In periodontitis, human periodontal ligament (HPDL) cells and gingival fibroblasts (HGF) are a major group of cells highly affected by the *P. gingivalis* products. To date, there is no study focusing on the effect of *P. gingivalis* on HGF and HPDL cells. The objective of this study was to investigate the correlation between *P. gingivalis* and periodontal ligament cells in the aspect of MMP-2 activation. The human primary cell culture, HPDL and HGF, was

established and used to direct demonstrate the response of these cells to *P. gingivalis* supernatant.

3.2 Materials and Methods

Cell culture

HPDL and HGF cells were cultured from explants obtained from freshly extracted third molars for orthodontic reason. The detail was mention in materials and methods part of Chapter 2.

Clinically isolated *Porphyromonas gingivalis*

Subjects who had been referred to Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, were selected to participate in this study and gave informed consent to our experimental procedure. The patients were clinically diagnosed as adult periodontitis, free of systemic disease and did not receive any periodontal or antibiotic therapy for 6 months prior to this experimental procedure. Gingival crevicular fluid (GCF) were collected using sterile fine paper points (Johnson and Johnson, East Windsor, United Kingdom) according to the method mentioned in the previous study (Condorelli et al., 1998). Briefly, the area of the collection site was isolated with cotton roll and dried with air. After supragingival plaque was carefully removed, the sterile paper point was insert to periodontal pocket until mild resistance was felt and held for 10 seconds. Collected GCF samples in reduced transport fluid (RTF) and stored at –80 °C until using.

Selective agar were prepared on the basis of drug resistance. Kanamycin sulfate was added into the agar in order to permit the growing of black pigmented gram-negative anaerobic bacteria. The GCF samples were inoculated and spreaded on Tryptic soy agar (TSA; BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% blood, 5 μg/ml hemin, 1 μg/ml menadione and 100 μg/L Kanamycin sulfate (Sigma Chemical Co., St. Louis, MO). *Porphyromonas gingivalis*, which resist to Kanamycin sulfate could grow on these selective agar and were selected on the basis of size, color, shape, and gram-staining. The gram-stained technique was conducted to distinguish the structural differences between bacteria. *Porphyromonas gingivalis* is gram-negative rod or short rod bacteria (Slots and Reynolds, 1982).

Since *Prevotella intermedia* (*P. intermedia),* another species which posses black pigmented gram-negative anaerobic bacteria, could also be detected on these selective agar. A fluorescence test by longwave UV light was used to exclude *P. gingivalis* from another black-pigmented, anaerobic, gram-negative rod (Slots and Reynolds, 1982).

Biochemical profile

The bacteria were further identified by the different of biochemical profile including of the carbohydrate fermentation abilities and the presence of bacterial enzymes. Biochemical profile was performed to further confirm the identity of *P. gingivalis.* The Rapid ID 32 A strip (Biomerieux a la Balme, Les Grottes, France) contained dehydrated-test substrate. The assay was done according to the instruction

of manufacturer. After 4 hours of incubation in aerobic condition, reading can be performed. Identification is obtained by using the identification software.

After selection of the colonies by subculture one well-isolated colony on a freshly prepared blood agar plate (TSA with 10% blood, 5μg/ml hemin and 1μg/ml menadione) which is necessary for black pigmented gram-negative anaerobe. Incubate at 37 ^{o}C in anaerobic jars and generate the appropriated anaerobic condition by using Gas Pak® (BBL Microbiology Systems, Cockeysville, Md.). Harvest the growing colonies on the blood agar plate. Make the bacterial suspension with turbidity equivalent to 4 McFarland (Biomerieux). The number of each McFarland standard suspension represent an average value valid for bacteria. For the No. 4 McFarland standard equivalence to bacterial concentration 1200x10 ^{6} cells/ml in sample and this value correspond to optical densities of bacterial suspension (OD 1.000). Pipette 55 μl bacterial suspension into each cupule (55 μl/ cupule). The strips were incubated in aerobic condition at 37 ^{o}C for 4 hours. Reading the strip by visual reading. Refer to the reading table (as described in Appendix C). Finally, identification was obtained by using the identification software according to manufacturer's instruction.

P. gingivalis W50 (ATCC 53978) was used as reference strain in this study. Bacteria were inoculated on the blood agar plates maintained at 37°C in anaerobic conditions as described above.

A well-isolated colony of bacteria was subcultured for 3-4 passages, until obtaining the constant colony morphotype and verified the purity of bacterial colonies by gram-stain. After the obtaining of a pure culture, other tests can be used in order to

identify the species of bacteria. The growing of *P. gingivalis* W50 and clinical isolated strains have to further identify bacterial species by polymerase chain reaction (PCR) method.

Genetic identification by Polymerase Chain Reaction

Polymerase chain reaction (PCR) was use to genetically identified the isolated *P. gingivalis*. Bacterial DNA was extracted by modified the previous methods (Marmur, 1961; Sambrook, 1989). In brief, all strains of *P. gingivalis* were culture in TSB supplemented with 5 μg/ml and 1 μg/ml menadione for 5 days. After harvesting, bacteria were sedimented in microcentrifuge. After the bacterial pellet was resuspended in lysis buffer (SET buffer; 20% sucrose, 50 mM Tris-HCl, 10 mM EDTA pH 8.0). 10 mg/ml lysozyme was added and incubated at 37 ° C for 1 hour to assure that all cells were lyzed. Membrane was dissolved in 0.5% (w/v) SDS. Then 100 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) was added and incubates at 37 ° C for 2-5 hours. Add 1/10 volume of 3 M sodium acetate pH 5.2. Protein was precipitated by phenol: chloroform: isoamyl alcohol (Sigma Chemical Co., St. Louis, MO), repeated this step 3-4 times or until there were no precipitated protein at the inter-phase layer. Pipette the upper phase into the new tube, DNA was precipitated by adding 2 volume of isoamyl alcohol, resuspend DNA pellet in TE-buffer. Stored the extracted DNA at -20 ° C before using. DNA of bacteria was used as the template for amplification of specific sequences in PCR.

Primers were designed from the sequences of 16S r-RNA subunits for *P. gingivalis*, obtained from GenBank, National Center for Biotechnology Information.

The specific sequences of *P. gingivalis* 16S rRNA gene (GenBank L12345) were aligned. Two pair of organism-specific oligonucleotide primers including the forward primer; 5'...AGG CAG CTT GCC ATA CTG CG...3' corresponding to the residues 692-711 and the reverse primer; 5'...ACT GTT AGC AAC TAC CGA TGT 3' corresponding to the residues 1076-1096. PCR products were approximately 490 base pairs. PCR was performed in a 25 μl volume. The reaction mixtures contained 1 units of Taq DNA polymerase, 2.5 μl of (10X) PCR buffer (Promega, Madison, Wis), 1.5 mM $MgCl_2$, 0.4 μM of forward and reverse primer, 0.2 mM dNTP and 1 ng of bacterial DNA. Cycling condition were started at 95 ° C for 2 min. Then the PCR working condition was set for 38 cycles; DNA dissociation at 94 ° C for 1 min, primer annealing at 60 ° C for 1 min, chain elongation at 72 ° C for 1.45 min, and in the last cycle extension process was continued at 72 ° C for 10 min. The amplified products were analyzed using 2% agarose gel electrophoresis. The gel was stained by ethidium bromide and visualized by UV illuminator (BioRad Laboratories, Richmon, California).

Preparation of *P. gingivalis* supernatant

A single colony from the agar plate of each strain (*P. gingivalis* W50 and clinical isolated strains) was grown in a test tube containing 10 ml of Tryptic soy broth (BBL Microbiology System, Cockeysville, Md.) supplemented with 1.5% yeast extract (Difco Laboratories, Detroit, Mich.), 5 μg/ml hemin (Sigma) and 0.2 μg/ml menadione (Boehringer Mannheim, Indianapolis, Ind). After the bacterial growth yield OD_{660} at 1.000, the supernatant was harvested by centrifugation at 10000x g for

15 minutes at 4°C. The collected supernatant was filtered twice through 0.2 μm membrane (Pall Gelman Science, Ann Arbor, Michican) and kept at –80°C until used.

Activation of HGF and HPDL cells with *P. gingivalis* supernatant

The toxicity of supernatant was analyzed by MTT [3-(4,5-Dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide) assay after 24 hours of treatment. Briefly, cells were seeded in 24-well plates (Nunc, Roskilde, Denmark) at a density of 50,000 cells/ml/well (25,000 cells/cm^2) and allowed to attach for 16 hours. Cells were silenced overnight in serum-free medium containing 0.02% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO) before the treatment. Cells were treated with various concentrations of *P. gingivalis* supernatant in serum-free condition. The equal amount of TSB was also added to the control. For MTT assay, medium was replaced by fresh medium containing 5 mg/ml of MTT at the last 4 hours. The blue formazan product in each well was then dissolved by dimethyl sulfoxide (DMSO) and quantitated by measuring at wavelength 570 nm using spectrophotometer model Ultraspec 3000 (Pharmacia Biotech, Piscataway, NJ.). Cell numbers were determined by comparing to the standard curve established from the known cell number (standard curve of cell number was shown in Appendix E).

Various concentrations of *P. gingivalis* supernatant that do not show any toxicity were added into the culture medium. After 48 hours, the medium was collected and kept at -20°C prior to the MMP-2 analysis. For the heat treatment, the supernatant was boiled for 15 minutes before adding to the culture.

In the inhibitory experiment, cells were treated with either proteinase inhibitors or NF-kB inhibitor for 30 minutes before *P. gingivalis* supernatant was added. Proteinase inhibitors used in the experiment included 10 μM phenanthroline, 2 mM Ethylene diamine tetra-acetic acid (EDTA), 55 ng/ml (0.25 Trypsin inhibitor unit; TIU) aprotinin, 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 50 μg/ml leupeptin. Cells were preincubated with 50 μg/ml NF-kB inhibitor, APDC, for 30 minutes before adding *P. gingivalis* supernatant. All inhibitors were obtained from Sigma.

Gelatin zymography

MMP-2 activity was evaluated by gelatin zymography. As describe in Chapter 2.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Cells were seeded in 6-well plates at a density of 25,000 cells/cm^2 and treated with 10 μl/ml of *P. gingivalis* supernatant. After 48 hours, total cellular RNA was extracted with Trizol (GibcoBRL, Grand Island, NY) according to manufacturer's instructions. One μg of each RNA sample was converted to cDNA by reverse transcription using Avian myeloblastosis virus (Promega) for 1.5 hours at 42°C. Subsequent to the reverse transcription, polymerase-chain reaction was performed for detection of MMP-2 DNA. The primers specific to MMP-2, glutaldehyde 3 phosphate dehydrogenase (GAPDH) were prepared by using reported sequences from GenBank

(MMP-2; GI: 180670, GAPDH; GI: 4503912) The oligonucleotide sequences of MMP-2 and GAPDH primers were:

MMP-2	sense	5'...CAA GAA GTA TGG CTT CTG CC...3'
	antisense	5'...GCA CCC TTG AAG AAG TAG CT..3'
GAPDH	sense	5' TGAAGGTCGGAGTCAACGGAT 3'
	antisense	5' TCACACCCATGACGAACATGG 3'

The PCR reaction was performed using Tag polymerase (Qiagen, Dusseldorf, Germany) with PCR volume 25 μl. The reaction mixtures contained 25 pmol of primers and 1 μl of RT reaction. The PCR working condition was set at denaturation for 1 min at 94 °C, primer annealing for 1 min at 60 °C, and chain elongation for 1.45 min at 72 °C on DNA thermal cycler (Omn-E Thermal cycler, Hybaid, UK). The amplified DNA was then electrophoresed on a 2% agarose gel and visualized by ethidium bromide fluorostaining. The PCR product of MMP-2 and GAPDH amplified cDNA is 926 bp and 395 bp, respectively.

Western analysis of MMP-2

Cells were seeded in 6-well plates (Nunc, Roskilde, Denmark) and treated with 10 μl/ml *P. gingivalis* supernatant as described above. For MMP-2 analysis, the condition medium was collected and centrifuged to remove cell debris and then concentrated 10 fold by using 10K Nanosep™ (Pall Gelman Science). The amount of protein was determined by BCA™ protein assay (Pierce, Rockford, IL). All samples,

25 μg of total protein per lane, were subjected to electrophoresis under reducing condition on a 10% polyacrylamide gel and then transferred onto nitrocellulose membrane. The membrane was stained for MMP-2 (affinity-isolated antibody against MMP-2 N-terminal) using rabbit anti-human antibody at the dilution of 1:1,000 in 5% non-fat milk for 1 hour. The membrane was subsequently incubated with secondary mouse anti-rabbit IgG_1 (all antibodies were from Sigma Chemical) for 30 minutes, followed by another 30 minutes staining with peroxidase-conjugated streptavidin (Zymed, San Francisco, CA).

After extensive washes with PBS, the membrane was coated with the Chemiluminescence detection for 1 minutes and the signal was captured with CL-Xposture film (Pierce). The amount of protein was quantitated using densitometer model GS-700 and Molecular Analyst Software (BioRad).

Statistical analysis

The data were expressed as percent mean ± standard deviation of triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Scheffe test. A *P*-value of < 0.05 was considered statistically significant.

3.3 Results

Identification of clinical isolated *Porphyromonas gingivalis*

In this study *P. gingivalis* were isolated from 5 patients. The black-pigment colonies which possess kanamycin resistance could grow on these selective agar as shown in **Figure 3.1.** In order to distinguish *P. gingivalis* from *P. intermedia,* another black pigmented gram-negative bacteria, the colonies were subjected to fluorescence test by longwave UV light the absence of fluorescence was considered a rapid taxonomic test to distinguish between *P. gingivalis* and other black-pigmented, anaerobic, gram-negative rods (Slots and Reynolds, 1982).

Next, the bacteria were further identified by the different of biochemical profile including of the carbohydrate fermentation abilities and the presence of bacterial enzymes. The Rapid ID 32 A strip **(Figure 3.2 A)** was used to determine the various kinds of enzyme production and fermentation ability. The results were read by visual reading and were recorded in the result sheets. The sum of each reaction grouping was subjected to computerized process in order to identify bacterial species as shown in **Figure 3.2 B.** Characterization by enzyme-substrate specificity and fermentation ability suggested the biochemical profile of each bacterial species. *P. gingivalis* identification was define as following; αGAL, βGAL, βNAG, ADH, PAL, ArgA, LGA, AlaA, GGA were positive results.

The genetically identification of pure culture obtained from clinical isolated *P. gingivalis* were identified by PCR method. Bacterial DNA was extracted, target DNA

sequence of 16S rRNA gene were amplified in PCR. The reaction mixture of PCR utilizes a pair of primer that is selected from opposing strand of target gene, which is specific to particular bacterial species. In this study the primer were designed and specific complementary to 16S rRNA of *P. gingivalis*. cDNA of 16S rRNA of *P. gingivalis* were synthesized and amplified in reaction mixtures. The amplicons migrated in 2% agarose gel. The presence of band at 495 bp is indicated for *P. gingivalis* as shown in **Figure 3.3.** *P. gingivalis* W50, and clinical isolated strains from patient1-5 was identified for bacteria species of *P. gingivalis* by using specific primer of 16s rRNA. It was found that 4 of 5 isolated strains were identified as *P. gingivalis*. Therefore, we selected the clinical isolated strains from patient 1 and 3 to participate in this study.

Supernatant of *P. gingivalis* activated MMP-2 in HGF and HPDL cells.

HGF and HPDL cells were cultured in the presence of various concentrations (0, 5, 10, 15, 20 and 50 µl/ml) of *P. gingivalis* supernatant with for 24 hours. Toxicity of the supernatant was determined by MTT assay **(Figure 3.4A)**. No different cell numbers was found when cells were treated with 5, 10 and 15 µl/ml of supernatant ($p > 0.05$). Whereas, the number of cells decreased significantly ($p < 0.05$) at 20 and 50 µl/ml, indicating the toxicity of supernatant.

Next, HPDL cells were cultured in the presence of 0, 5, 10 and 15 µl/ml of *P. gingivalis* supernatant for 48 hours and the culture medium was collected for MMP-2 analysis using gelatin zymography. As shown in **Figure 3.4B**, the latent MMP-2 (72 kDa) was detected in all conditions, while the active MMP-2 (62 kDa) was found in

the culture that contained 10 and 15 µl/ml of supernatant. The results indicated that supernatant from *P. gingivalis* at 10 and 15 µl/ml contained the activity that could induce MMP-2 activation in HPDL cells. In order to test whether *P. gingivalis* supernatant could activate MMP-2 in other cell types, HGF were cultured in the presence of *P. gingivalis* supernatant for 48 hours. The results in **Figure 3.5** showed that *P. gingivalis* supernatant could activate MMP-2 in both of cell types, the results obtained from HGF was similar to HPDL cells.

Since 10 µl/ml of supernatant was the first dosage that demonstrated this activity, we continued to use this dosage of supernatant throughout the rest of the experiments. The same rationale was also applied to the amount of TSB used in the control. All strain of *P. gingivalis* (W50 and clinical isolated strain 1 and 3) could induce MMP-2 activation in the same manner as shown in **Figure 3.6.**

Effect of *P. gingivalis* supernatant on MMP-2 expression in HPDL cells

To further identify the bands of MMP-2, cells were activated with 10 µl/ml of *P. gingivalis* supernatant, and analyzed the activation of MMP-2 with Western analysis. In addition, to investigate the effect of *P. gingivalis* supernatant on MMP-2 expression, RT-PCR and Western analysis were performed.

RT-PCR analysis revealed that *P. gingivalis* supernatant has no effect on the expression of MMP-2 as shown in **Figure 3.7A.** Similar result was also obtained from the Western analysis (**Figure 3.7B**). Western analysis showed the 62 kDa and 72 kDa bands confirming that these 2 bands were the latent (72 kDa) and active (62 kDa)

MMP-2. The nature of molecules in supernatant that involved in the activation of MMP-2 was investigated by boiling the supernatant for 15 minutes before adding to the culture. It appeared that the activation of MMP-2 was diminished in the culture treated with heat-treatment supernatant (**Figure 3.8).**

The effect of Protease inhibitors and NF-kB inhibitors on MMP-2 activation by *P. gingivalis* supernatant

In order to find out the mechanism of MMP-2 activation by *P. gingivalis* supernatant, we added various proteinase inhibitors into the culture medium for 30 minutes prior to the application of *P. gingivalis* supernatant. The results in **Figure 3.9** indicated that both MMP inhibitors, phenanthroline and EDTA, could block the activation of MMP-2. However, serine proteinase inhibitors (aprotinin and PMSF) and cysteine proteinase inhibitor (leupeptin) had no inhibitory effect on the MMP-2 activation.

In addition, cells were preincubated with NF-kB inhibitor (APDC) for 30 mimutes prior to the stimulation of cells with *P. gingivalis* supernatant in order to determine the mechanism of MMP-2 activation by *P. gingivalis* supernatant. The results in **Figure 3.10** indicated that APDC completely inhibited the activation of MMP-2.

3.4 Discussion

P. gingivalis is one of the putative periodontopathogen which cause destructive periodontitis in human. The colonization of *P. gingivalis* associated with the progressive form of adult-onset periodontitis. Moreover, it is elevated with high proportions in destructive lesion (Haffajee and Socransky, 2000). The presence of *P. gingivalis* is not sufficient to cause the periodontal disease. Since, the implantation of this organism in the oral cavity could not develop periodontal disease (Holt and Bramanti, 1991). It has been suggested that degradation of periodontal tissue can be cause by either bacterial enzymes or host-derived matrix (MMPs), the later is the main pathway of connective tissue destruction. As a large body of evidence strongly suggested that principal host enzyme that plays a crucial role in extracellular matrix destruction is MMP family. (Birkedal-Hansen, 1993a; Komatsu et al., 2001; Korostoff et al., 2000; Makela et al., 1994). These results indicated the involvement of MMP-2 in periodontal destruction. Therefore, our study is focusing on the influence of bacterial products which secreting from *P. gingivalis* on MMP-2 activation.

This study directly demonstrates the MMP-2 activation processed by *P. gingivalis* supernatant of reference and clinical isolated strain in both HGF and HPDL cells. *P. gingivalis* was isolated from periodontal pockets of periodontitis lesion which identified by gold standard methods such as culturing the bacteria and verified the bacteria by gram-staining and examine by light microscope, biochemical test and also PCR. This study used *P. gingivalis* (W50), reference strain, to compare the capability of bacterial supernatant in MMP-2 activation. Although using selective agar with the presence of kanamycin sulfate is a basic method to isolate *P. gingivalis* but other

species which possess kanamycin resistance could be detected such as *P. intermedia*. However the colony morphology of *P. gingivalis* is mucoid brown to black and glistening character whereas *P. Intermedia* typically dry and also has various colors from tan, gray, reddish brown and black (van Dalen et al., 1998). Light microscopy with gram staining can primarily distinguish between gram-positive and gram-negative organism and can define bacteria by shape and size. *P. gingivalis* is about 0.5 μm x 1-3 μm in size, the cell shape is pleomorphic which is ranging from medium-length rod (5 μm) to cocci (Curtis et al., 2001; Slots and Reynolds, 1982; Tanner, 1992). The gram-negative rod-shape bacteria are most common found in periodontitis whereas gram-positive cocci-shape are associated with health or gingivitis (Listgarten, 1987). To differentiate these two types of bacteria, the colonies were subjected to fluorescence test by longwave UV light (366 nm) and the colony that revealed the absence of fluorescence, was considered to *P. gingivalis*. The bacteria were further identified by the biochemical tests including the carbohydrate fermentation abilities and the presence of bacterial enzymes, using Rapid ID 32 A strip. Although this method is easy to manipulate in aerobic condition and take a few hours to identify bacterial species but there have some difficulties about the judgment of visual reading. Because of the color of some reactions have little changes. The possible outcomes are equally positive to negative results. In addition, there are such wide ranges of color intensities, which make some difficulties for reader judgment. So, the last method for genetically identification is PCR. This method is worldwide accepted and preferable used because of its high sensitivity, rapid and effective detection method for specific periodontal pathogen (Ahmet et al., 1995; Garcia et al., 1997). However, PCR could not reveal the purity of cultured bacteria. Thus, the purity of bacteria was verified by using several methods such as a gram-staining and

examine by light microscope. Moreover, we verified the colonies on both selective and non-selective blood agars and also use the di-primers (primers for specific gene for 16S rRNA of *P. gingivalis and* 16S rRNA of *P. intermedia*) in each PCR reaction parallel with the reaction mixture, which have a single pair of primers. The results show that there was no bacterial specie contamination (data not shown).

Supernatant of *P. gingivalis* (Clinical isolated) and also reference strain (W50) could induce MMP-2 activation in both HPDL and HGF cells. These results are similar to the previous results (in Chapter 2) that mixed-bacterial supernatant also induce MMP-2 activation. The result indicated the capability of bacterial products of *P. gingivalis,* one of the putative periodontopathogens in periodontal disease, could induce host enzymes to enable substrate digestion. Since MMP-2 is one of the major enzymes that involves in degradation process of soft tissue both physiological and pathological way (Shapiro, 1994). MMP-2, like other members of MMP family, is secreted in a latent form and requires activation process to function (Nagase, 1998). However *P. gingivalis* supernatant has no effect on MMP-2 expression, but the most important step for enzyme-function is the process of enzyme-activation. In physiologic condition, regulation of host enzymes were tight regulated in transcription translation and activation. Certainly most of proenzymes were formed as enzyme complex with intracellular inhibitors, TIMPs molecules. After activation TIMPs molecules were dissociated and led enzyme to function. Therefore, if MMP-activation is occurred due to an inbalanced of regulatory molecules such as TIMPs, it may lead to accelerated connective tissue destruction. Our result here also demonstrates that the supernatant from *P. gingivalis* can induce the MMP-2 activation. This result is in agreement with previous study that found the increase of

active MMP-2 in gingival tissue of periodontitis patients (Korostoff et al., 2000). The activation of MMP-2 activation in these patients might reflect the higher degree of tissue degradation that lead to the chronic conditions of the disease.

Mechanism of MMP-2 activation is different from other MMPs, since MMP-2 cannot be activated by serine proteinase like other members of MMP family. It has been proposed the model for MMP-2 activation that involved the function of MT1-MMP, a membrane type MMP (Strongin et al., 1995). According to the model, latent MMP-2 binds to the MT1-MMP by using TIMP-2 (tissue inhibitor of metalloproteinase-2) as bridging molecule to form a tri-molecular complex. Subsequently, another MT1-MMP molecule approaches to the complex and activates MMP-2 by cleaving its propeptide domain. From these results *P. gingivalis* supernatant can induce the activation of MMP-2, this process was partially inhibited by NF-kB inhibitor. Although the previous report suggested that in the presence of metal chelating agent MMP-2 activation was completely inhibited (Azzam and Thompson, 1992; Toth et al., 2000) . Our observation, which revealed that *P. gingivalis* supernatant induced was correspond to these results that MMP-2 require MT1-MMP function. Since phenanthroline and EDTA serve as a zinc and metal ion chelator, respectively, which is requirement for the function of MMPs. Thus, inhibition of MMP-2 activation by these two inhibitors suggests the possibility that MT1-MMP might involve in *P. gingivalis* supernatant induced MMP-2 activation.

Molecules in the supernatant that activate MMP-2 are still unknown. However heat treatment *P. gingivalis*-supernatant could abolish the ability of supernatant on MMP-2 activation. These results suggested that the molecules involving in this

process might be protein(s) in nature. In addition, the ability of *P. gingivalis*-supernatant on MMP-2 activation is not just specific to HPDL cells since the similar response was also found in HGF.

It has been shown that *P. gingivalis* could secrete large amount of trypsin-like activity proteinase (Potempa et al., 1995). In fact, these proteinase not only participated in tissue degradation but also involved in the process of host induction (Sorsa et al., 1992; DeCarlo et al., 1997). Proteinase from *P. gingivalis* could induce host cell to increase secretion of collagenase and plasminogen activator. The increased secretion may be results from the damage of cell surface protein (Uitto et al., 1989). It is possible that the cell surface protein damage lead to the changing of cytoskeleton. As demonstrate (Tomasek et al., 1997), the alteration of actin could induce the activation of MMP-2 in fibroblasts. Thus, the response of HPDL and HGF could be the consequence of the changes in cytoskeleton due to the effects of *P. gingivalis* proteinase on cell surface protein. Understanding in the mechanism of MMP-2 activation may be useful for the effectiveness of periodontal therapy.

Future studies will be necessary to determine the virulence factors in supernatant of *P. gingivalis*, which molecules could induce MMP-2 activation. It is of interest to know how to regulate these molecules in order to control the destruction of connective tissue in periodontal disease.

Conclusion

Supernatant of *P. gingivalis* reference strain (W50) and also clinical isolated could induce MMP-2 activation in both HPDL and HGF cells. These results are similar to the previous results that mixed-bacterial supernatant also induce MMP-2 activation. The activation is mediated through NF-kB dependent pathway and this process requires another MMPs.

Figure 3.1 Black-pigmented colonies on blood agar supplemented with vitamin K1 and hemin. Colony morphotype with 1-2 mm diameter, convex, circular , mucoid texture, smooth border colony. These characterization possibly *Porphyromonas gingivalis* colony.

Figure 3.1 Black-pigmented colonies

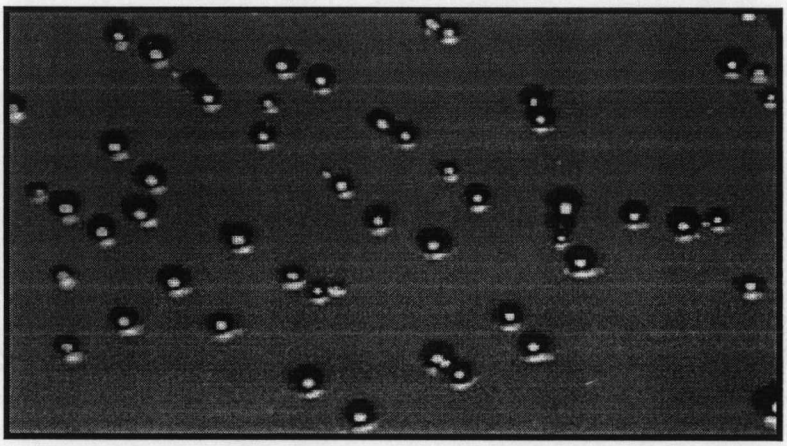


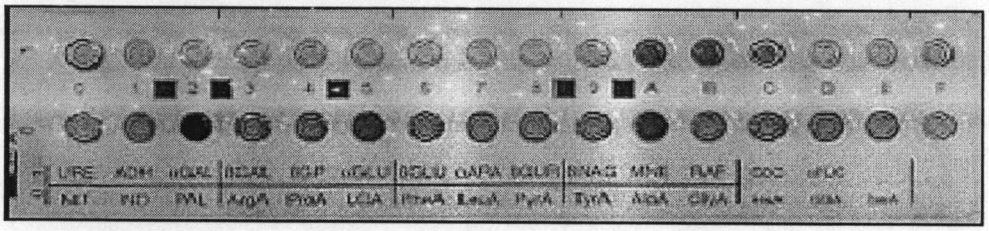
Figure 3.2 Rapid ID 32 A strip

A. The Rapid ID 32 A contains dehydrated-test substrate in each capule. To determine the enzyme production of bacteria, adding bacterial suspension into each capule and incubate in aerobic condition at 37 °C for 4 hours. Visual reading the color of each. Base on enzyme substrate specificity reaction, the appearance of color changing reveal enzyme capability produced by viable bacteria.

B. Record sheet of Rapid ID 32 A, All results obtained by visual reading were recorded +/- and each of result were scored as suggested in record sheet. Collective result were further identify by computerized program.

Figure 3.2 Rapid ID 32 A strip

A. Dehydrated substrate



B. Record sheet

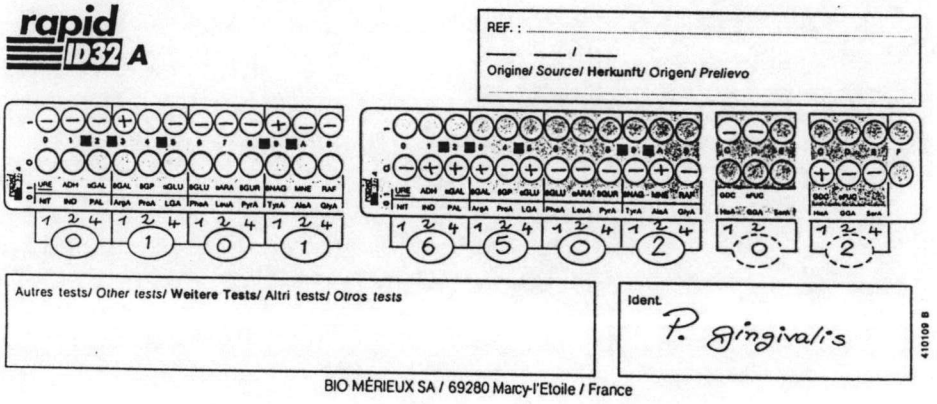


Figure 3.3 Genetic identification by Polymerase Chain Reaction. Using a pair of primer amplified a specific sequence of 16S rRNA subunits for *P. gingivalis.* The PCR working condition was set for 38 cycles; DNA dissociation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, chain elongation at 72 °C for 1.45 min, and in the last cycle extension process was continued at 72 °C for 10 min. The amplified products were analyzed using 2% agarose gel electrophoresis. The gel was stained by ethidium bromide and visualized by UV illuminator. The presence of PCR products 495 bp indicated for *P. gingivalis.* Lane 1and 8: DNA ladder; lane 2: *P. gingivalis* W50 (reference); Lane 3-7: Clinically isolated strain of *P. gingivalis* from periodontal pockets of patient1-5, respectively.

Figure 3.3 Genetic identification by Polymerase Chain Reaction

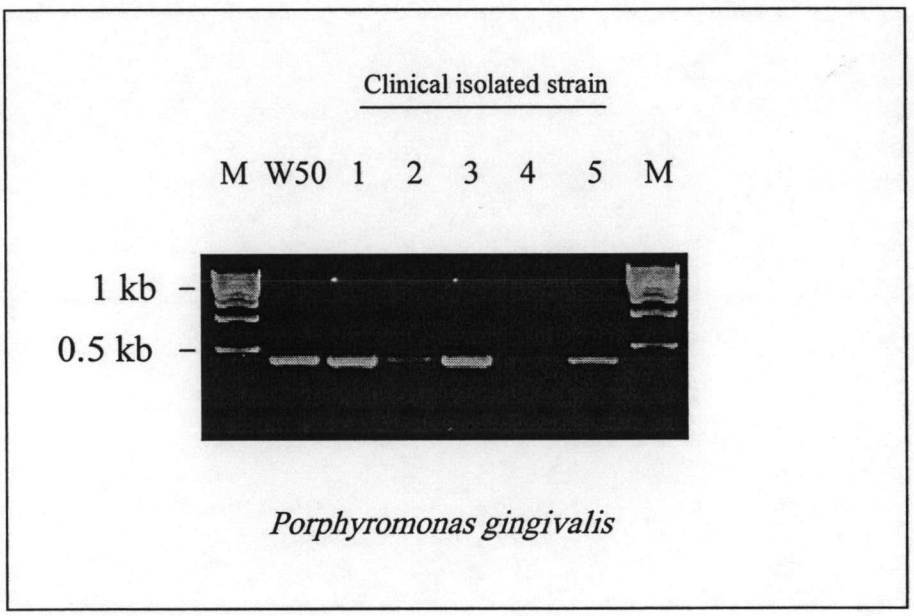


Figure 3.4 Effect of *P. gingivalis* supernatant

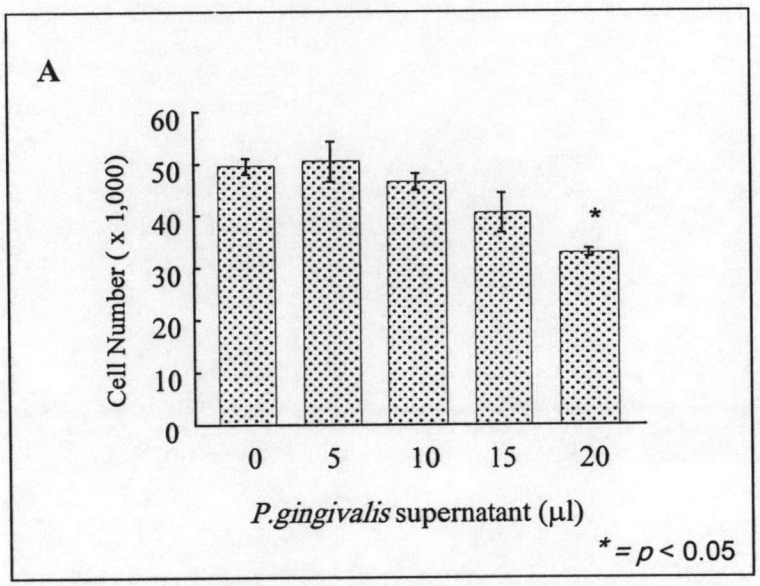
A. Cytotoxicity of *P. gingivalis* supernatant

Toxicity of *P. gingivalis* supernatant determined by MTT assay cell were treated with 0, 5, 10, 15, 20 µl/ ml of supernatant for 24 hours. Data were shown in mean ± SD from three independent experiments. Toxicity of supernatant was observed at 20 µl/ ml. Statistical analysis was performed by one way analysis of variance (ANOVA) using Scheffe test. A p-value of < 0.05 was considered statistically significant.(*).

B. Gelatin zymography of MMP-2 activation by *P. gingivalis* supernatant

Cultured cells were treated with supernatant 0, 5, 10, 15 µl/ml for 48 hours. The activation of MMP-2 was analyzed by gelatin zymography. Equal amount of protein was loaded in each lane. The position of latent and active MMP-2 (72 and 62 kDa) were indicated on the right. Activation of MMP-2 appeared in the culture exposed to 10 and 15 µl/ ml of supernatant.

Figure 3.4 Cytotoxicity of *P. gingivalis* supernatant and MMP-2 activation



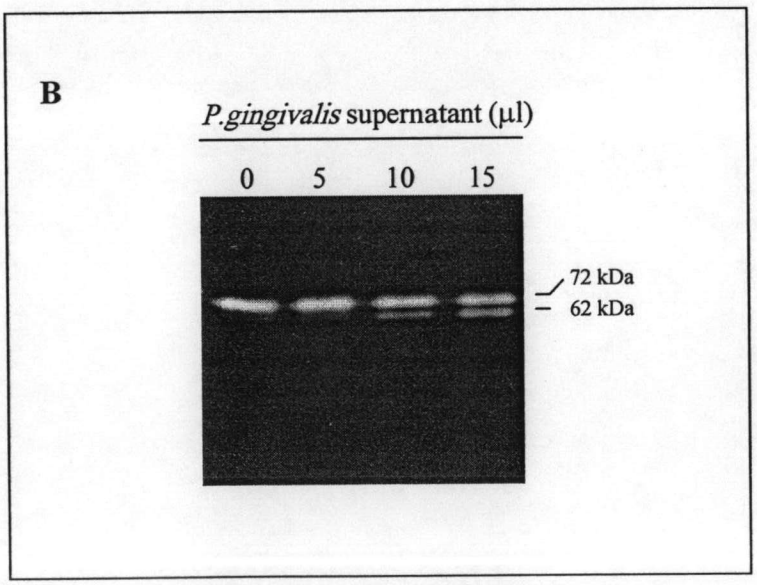


Figure 3.5 Gelatin zymography of MMP-2 activation by *P. gingivalis* supernatant in HGF and HPDL cells
Gingival fibroblast and periodontal ligament cells were grown in the absence or presence of various concentration of *P. gingivalis* supernatant (0, 1, 5, 10 µl/ml) for 48 hours. The media were collected for gelatin zymography. Both of cell types were response in dose dependent relationship. The position of latent and active MMP-2 were indicated on the right.

Figure 3.6 Gelatin zymography of MMP-2 activation by *P. gingivalis* supernatant of reference and clinical isolated strains.
P. gingivalis supernatant of both reference and clinical isolated strains (1 and 3) could induce MMP-2 activation in HPDL cells. Cultured cells were grown in the presence of *P. gingivalis* supernatant 10 µl/ml for 48 hours, then the culture medium were collected to determine the MMP-2 activation.

Figure 3.5 MMP-2 activation in HGF and HPDL cells

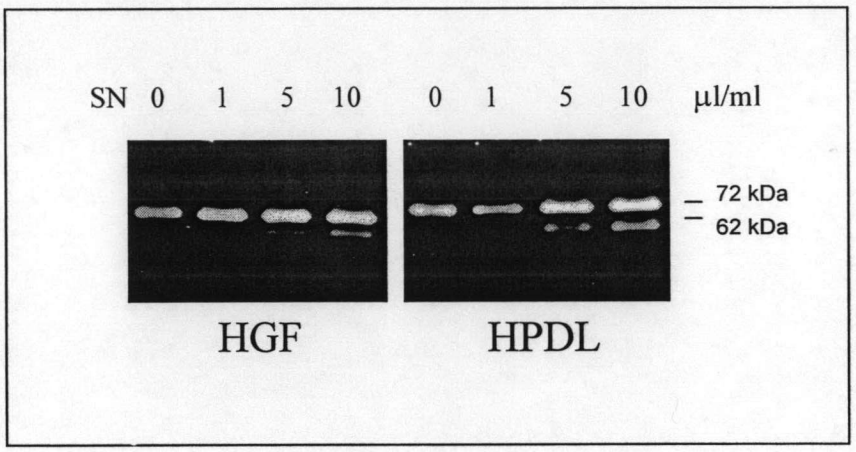


Figure 3.6 *P. gingivalis* (W50 and clinical isolated) supernatant

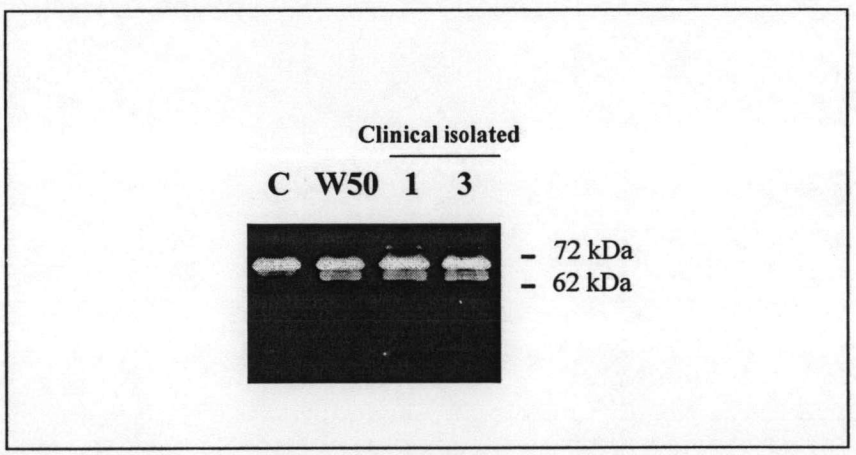


Figure 3.7 Effect of *P. gingivalis* supernatant on MMP-2 expression HPDL cells were grown in the absence (control;C) or presence of *P. gingivalis* supernatant (10 μl/ml) for 48 hours.

A. RT-PCR

Total cellular RNA were extracted and mRNA of MMP-2 were convert to cDNA by reverse transcription (RT). cDNA was subsequently amplified by Polymerase Chain Reaction (PCR). GAPDH was used as internal control. PCR products of MMP-2 and GAPDH were 926 bp and 395 bp, respectively.

B. Western analysis.

For MMP-2 analysis condition medium was collected, removed cell debris, and concentrated by ultrafiltration (Molecular weight cut off 10 kDa). Equal amount of total proteins were electrophoreses. Proteins were blotted to nitrocellulose membrane. MMP-2 was detected by monoclonal antibody (rabbit anti-human antibody), subsequently incubated with mouse anti-rabbit antibody, follow by staining with kit the amount of protein was quantitated by using densitometer.

Figure 3.7 Effect of *P. gingivalis* supernatant on MMP-2 expression

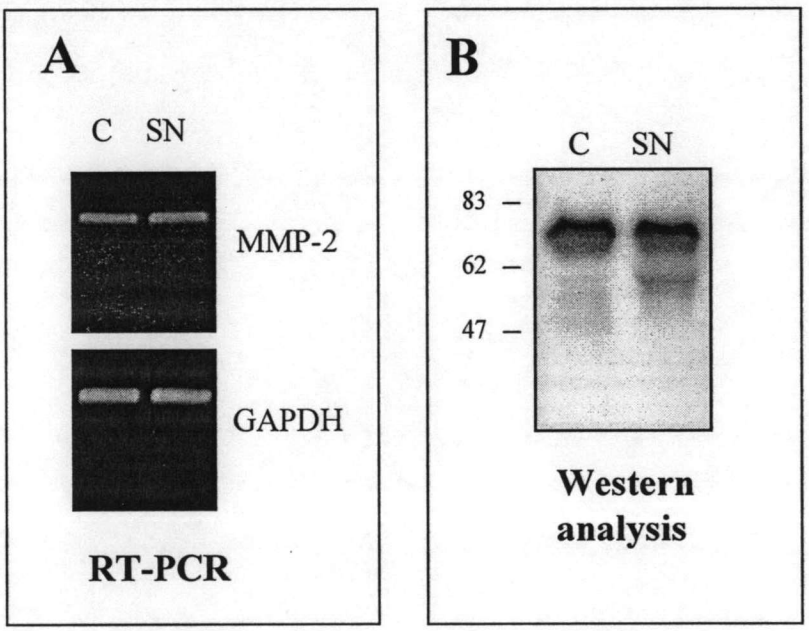


Figure 3.8 Effect of heat treatment on *P. gingivalis* supernatant
Gelatin zymography of conditioned medium obtained from HPDL cells incubated in the absence (control; C) , or presence of *P. gingivalis* supernatant with heat treatment (+H) and native supernatant (-H) for 48 hours. The position of latent and active MMP-2 (72 kDa and t 62 kDa) was indicated on the right, respectively.

Figure 3.8 Effect of heat treatment on *P. gingivalis* supernatant

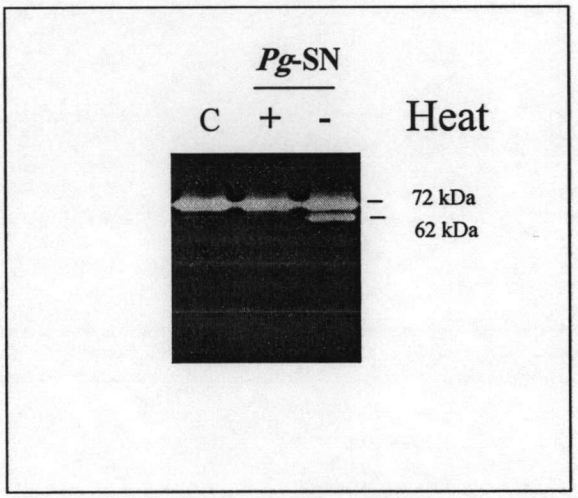


Figure 3.9 Effect of protease inhibitors on MMP-2 activation by *P. gingivalis* supernatant

In the inhibitory experiments, cells were treated with proteinase inhibitors for 30 minutes before *P. gingivalis* supernatant was added. Three groups of proteinase inhibitors used in the experiment included **metal chelating agents**; 10 μM phenanthroline, 2 mM Ethylene diamine tetra-acetic acid (EDTA), **serine proteinase inhibitors**; 55 ng/ml aprotinin, 0.2 mM phenylmethylsulfonylfluoride (PMSF) and **cysteine proteinase inhibitor;** 50 μg/ml leupeptin. Conditioned medium of each was collected and determined MMP-2 activation by gelatin zymography.

Figure 3.9 Effect of protease inhibitor on MMP-2 activation

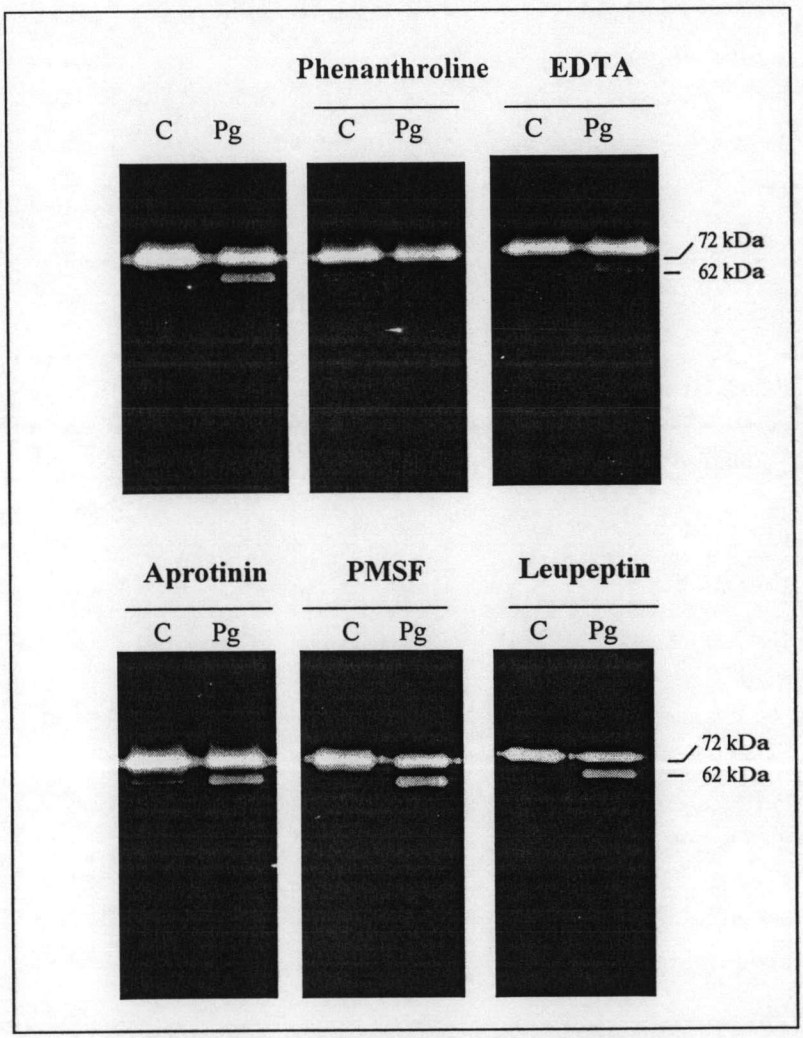


Figure 3.10 Effect of NF-kB inhibitor on MMP-2 activation by *P. gingivalis* supernatant

In the next inhibitory experiments, cells were preincubated with NF-kB inhibitors for 30 minutes before *P. gingivalis* supernatant was added. NF-kB inhibitor was used to investigate the MMP-2 activation is cell-dependence pathway, cells were preincubated with 50 μg/ml NF-kB inhibitors (APDC) for 30 minutes before adding *P. gingivalis* supernatant. **SN** represent *P. gingivalis* supernatant, **APDC**: NF-kB inhibitor, **C**: control, ***Pg* W50**: Pg reference strain, ***Pg*-1** and ***Pg*-3**: *P.gingivalis* clinical isolated strain.

Figure 3.10 Effect of NF-kB inhibitor on MMP-2 activation