KININOGENASE ACTIVITY OF ENDODONTIC ORGANISMS – DEGRADATION OF KININOGEN AND FIBRINOGEN BY *PORPHYROMONAS ENDODONTALIS*

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by
Hoonji Jang, D.D.S., M.S
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Thesis Approvals:

Dr. Eugene Whitaker, DMD, PhD, Thesis Advisor, Department of Restorative Dentistry
Dr. Cemil Yesilsoy, DMD, MSD, Department of Endodontology
Dr. Roni Nissan, DMD, Department of Endodontology

ABSTRACT

Introduction*:* Localized infection to pulp tissue results in a vascular response. The endothelial cells of the microvasculature contract and become leaky, so that high molecular weight plasma proteins accumulate in the affected site. This inflammation is initiated and controlled by chemical mediators known as the kinins. The kininogens are multifunctional plasma proteins that participate in various phases of the inflammatory process. Both high molecular weight kininogen (HK) and low molecular weight kininogen (LK) contain the vasodilatory nonapeptide bradykinin (Bk). The interaction of kininogen with bacteria frequently found in infections of the pulp has not been investigated. Hence, we propose to study the in vitro effect of three endodontic pathogens on kininogen. A related aim is to characterize the kininogenase activity of *Porphyromonas endodontalis*.

Purpose: This study aims to determine the significant in vitro interaction between *P. endodontalis*, an endodontic pathogen, and the plasma proteins kininogen, a modulator of the inflammatory response, and fibrinogen, a major protein substrate of the coagulation cascade.

Materials and Methods: Whole cells (10^5 cells/µl) or sonic extracts (1mg/ml) of the endodontic pathogens *P. endodontalis*, *Enterococcus faecalis*, and *Fusobacterium nucleatum* were incubated with low molecular weight kininogen (LMWK) (0.1mg/ml) and fibrinogen (0.1mg/ml) for 1 hour, 4 hours and 24 hours at 37°C. Degradation of these proteins was detected by polyacrylamide gel electrophoresis (PAGE). Kininogenase activity was assayed by incubating *P. endodontalis* sonic extracts (PESE, 30µl, 1mg/ml) or purified fractions of PESE with either chromogenic amide substrate N-benzoyl-pro-

phe-arg-pNA (70µl, 7µM) or LMWK (70µl, 0.1mg/ml, 10^{-3}µM). Release of pNA was determined spectrophotometrically and release of bradykinin from kininogen was measured by a competitive enzyme-linked immunosorbent assay (ELISA). Kinogenase activity was partially purified from PESE by gel chromatography and characterized by assaying in the presence of protease inhibitors.

Results: *P. endodontalis* sonic extract (PESE) degraded both kininogen and fibrinogen as evidenced by PAGE. Sonic extracts of *E. faecalis* and *F. nucleatum* did not exhibit this activity. PESE also cleaved the protease substrate at the arginine residue releasing pNA linearly. Bradykinin (100pg/ml) was released from LMWK when incubated with PESE. Kininogenase activity of *P. endodontali*s was purified 17 fold and characterized as a kallikrein-like serine protease.

Conclusion: PGSE degrades both kininogen and fibrinogen in vitro. These data suggest that *P. endodontalis* may contribute to the pathogenesis of pulpitis and periodontitis by modulating the inflammatory response via its effect on LMWK as well as hemostasis by its ability to degrade fibrinogen.

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CHAPTER 1
INTRODUCTION

Endodontic treatment is required mainly due to the localized inflammation or infection to pulp tissue by bacteria mostly from decay. Molecular based studies have revealed a complex microbiota in cases of endodontic infection including the frequently found bacteria *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Porphyromonas endodontalis* (1-3). *E. faecalis* is frequently cultured from endodontically treated teeth with persistent apical periodontitis (4). *F. nucleatum* is more abundant in primary infection and flare-up (5).

P. endodontalis is a gram-negative microorganism that is a black-pigmented, obligate anaerobic, and asaccharolytic (6-8). It is commonly found in root canal infections and periapical abscesses of dental origin (9). Additionally, *P. endodontalis* has been identified in radicular cysts, oral submucous abscesses, and subgingival plaque (8, 9). These bacteria play a role in recruiting white blood cells, triggering the production of cytokines, and causing cytotoxic effects in inflamed and necrotic dental pulp. *P. endodontalis* has been shown to stimulate the production of IL-8 and monocyte chemo-attractant protein in mononuclear cells, inhibit the growth and proliferation of periapical fibroblasts, and induce plasmin activity in gingival fibroblasts (10-12). Furthermore, the lipopolysaccharide of *P. endodontalis* stimulates the production of IL-6 and IL-1b in periodontal ligament cells and human dental pulp cells (13, 14) These findings indicate that *P. endodontalis* is closely associated with the development of oral infections and inflammation.

These endodontic pathogens contribute to localized infection to pulp tissue resulting in a vascular response (15). The endothelial cells of the microvasculature contract and become leaky, so that high molecular weight plasma proteins accumulate in the affected site. Bacteria, in turn, can enter the circulation system (16). The inflammatory response results from an integrated series of biochemical reactions involving the production of humoral mediators. This inflammation is initiated and controlled by chemical mediators known as the kinins (17). The kininogens are multifunctional plasma proteins that participate in various phases of the inflammatory process (18). Both high molecular weight kininogen (HK) and low molecular weight kininogen (LK) contain the vasodilatory nonapeptide bradykinin(Bk) (19).

The human contact system is named for its initiation mechanism, i.e., contact with artificial or biological negatively charged surfaces trigger its activation. This system, otherwise known as the plasma kallikrein-kinin system, is made up of coagulation Factor XII, prekallikrein and kininogen (20). It is regarded as an inflammatory response because it ends up generating the kinins. Many species of bacteria are known to activate contact factors on their surface and hence indirectly generate bradykinin. (21). However, there is greater interest in pathogens that have developed additional mechanisms to activate the contact system directly, via. bacterial proteases to release bradykinin. Whereas activation by surface contact may serve to protect the host, activation by the pathogen vectors tends to lead to detrimental effects on the host (22). Some examples of bacterial proteinases that activate the contact system by proteolysis are as follows: *Porphyromonas gingivalis* gingipains (23); microbial cysteine proteases from group A streptococci (24), staphopain from *Staphylococcus aureus* (25); cruzipain from *Treponema* (26); and serine protease

from the gastroenteritis associated pathogen *Aeromonas sobria* (27). These proteases can be secreted, cell-bound, or membrane-bound and are able to amplify the cascade of bradykinin production resulting in vascular leaking activity.

Bradykinin plays a significant role in inflammation and is associated with various symptoms in the pulp, e.g., edema and pain (28). The cleavage of kininogen in blood plasma generates bradykinin, and when it is released during the inflammatory processes, bradykinin acts as a mediator of vasodilation, increasing blood flow (29). This vasodilation helps deliver immune cells and substances to the inflamed area, assisting the elimination of pathogens and the initiation of the repair. Also, the enhanced capillary permeability allows immune cells and fluids to move more freely from the blood vessels to the affected tissue (17). Emigration of polymorphonuclear leukocytes through dilated vessels, with release of lysosomal enzyme from neutrophils, could lead to further tissue breakdown (28, 29). However, bradykinin can be broken down rapidly and can be a short-lived mediator, unless it is produced in large volumes in an infected and contained space like the infected pulp (29).

The investigation of the interaction between kininogen and bacteria commonly found in pulp infections has been largely unexplored. Therefore, verifying the kininogenase activity in endodontic pathogens can significantly contribute to the pain mechanism and clinical impact on pain management. Our working hypothesis is that bacterial proteases cleave kininogen, leading to the release of bradykinin within the pulp. Consequently, bradykinin induces increased capillary permeability, edema, and pain. This study specifically aims to examine the in vitro effect of three endodontic pathogens, *E. faecalis*, *F. nucleatum*, and *P. endodontalis* on potential proteolysis of kininogen and

fibrinogen. Additionally, we seek to assess if bradykinin is release due to proteolysis of kininogen and to characterize the kininogenase activity.

CHAPTER 2
MATERIALS AND METHODS

Materials

The following items were purchased from Sigma Chemical Co., St. Louis, MO: the chromogenic substrate N-Benzoyl-Pro-Phe-Arg-p-nitroanilide (S2302); human low molecular weight kininogen (LMWK) and high molecular weight kininogen from plasma (HMWK); aprotinin; leupeptin; DL-dithiothreitol; L-cysteine; Triton X-100; $MgCl_2$; gel filtration markers bovine serum albumin and human haptoglobin; human fibrinogen, hemoglobin, albumin, and immunoglobulin G and electrophoresis markers low range (6-65,000). Sepharose 2B was purchased from Amersham Pharmacia Biotech Inc, Uppsala, Sweden. Mini-Protean Precast Gels (7.5%), and a Colorimetric Alkaline Phosphatase Western Blot Kit were purchased from Bio-Rad Corp. Hercules, CA. DCL Dual Vue Western blotting molecular weight markers were purchased from Thermo Fisher Scientific, Waltham, Mass. Rabbit anti-human kininogen polyclonal antibody was purchased from My Biosource Inc. San Diego CA. A Bradykinin ELISA kit was purchased from Enzo life Sciences, Farmingdale, NY.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed on 7.5% gels according to the method of Laemmli (30). The running buffer was 25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3. The gels were stained with Coomassie Blue R-250 (0.1% Coomassie Blue R-250, 40% methanol, 10% acetic acid), and destained with a solution of 10% acetic acid, 40% methanol.

Bacterial Cultivation and Preparation of Cells

Enterococcus faecalis (19433) cells, *Fusobacterium nucleatum* (25586) cells, and *Porphyromonas endodontalis* (35406) and were purchased from the American Type Culture Collection (Manassas, VA). *E. faecalis* was inoculated in brain heart infusion (BHI) broth and allowed to grow for 24 hours at 37°C. *F. nucleatum* was inoculated in brain heart infusion broth and allowed to grow for 24 hours at 37°C in anaerobic jars using a Gas Pak system (Mitsubishi Gas Chemical Co., Tokyo, Japan). *P. endodontalis* was grown in BHI supplemented with hemin (5mg/l), menadione (1mg/l), and resazurin (0.01%) for 48 hours at 37°C in anaerobic jars using a Gas Pak system (Mitsubishi Gas Chemical Co., Tokyo, Japan). Cells were removed by centrifugation (6000 x g) and washed twice in isotonic saline. The pelleted cells were suspended in 10mM phosphate buffer (pH = 7.4) and adjusted to an OD = 0.5 (600 nm) equal to that of a half standard McFarland solution (31). For *P. endodontalis*, the numbers of cells that correspond to the McFarland turbidity values were determined by quantitative polymerase chain reaction (qPCR). Working solutions were made by diluting McFarland standards by ½ and ¼ in 0.1 M phosphate buffer, pH 7.4.

Determination of Bacterial Cell Numbers

For qPCR, *P. endodontalis* DNA (5µg/ml ATCC 35406) was subjected to two-fold dilutions from ½ to 1/256 and used to generate a standard curve and used to compare against DNA extracted from whole cells. DNA was extracted from whole cells using Instagene Matrix solution (BioRad Laboatores). Each dilution of cells (1ml) was added to

1ml Instagene Matrix, boiled for 5 minutes, and pelleted by centrifugation. The supernatant containing extracted DNA was used in qPCR assays.

The complete sequence of the dipeptidylpeptidase 11 (DPP 11) gene of *P. endodontalis* was downloaded from GenBank (AB610284) and forward (5'-ATCGCTCCCTTGATC-3') and backward (5'-CAGCTTTTGGTGC-3') primers were synthesized and used to augment a segment of the gene by qPCR (32). To 10µl of each primer (2µM) was added 20µl Chai Green Master Mix and 10µl quantitative whole genomic DNA from *P. endodontalis.* Amplifications were performed in a single channel Chai Open (Qpcr) thermocycler (Chai Biotech, Santa Clara, Ca) with cycling as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and at 72°C for 30 seconds. The terminal denaturation was performed at 72°C for 5 minutes. PCR products were detected by monitoring the increase in fluorescence.

Cleavage of LMWK by Bacterial Cells

Aliquots (100µl) of *E. faecalis, F. nucleatum and P. endodontalis* cell working solutions were incubated with LMWK (100µl, 0.1mg/ml) for 24 hours at 37°C. Cells were removed by centrifugation (6000 x g) and the supernatant was analyzed for cleavage of LMWK by disappearance the LMWK protein band on electrophoretic gels.

Cleavage of Both Artificial Plasma HMWK and LMWK by *P. endodontalis* cells

Artificial plasma, created by mixing 100µl of HMWK, LMWK, human serum albumin, haptoglobin, human IgG (each 1mg/ml, 150µl) was incubated with *P. endodontalis* cells

(OD 600nm = 0.5, one half McFarland, 300µl) for 24 hours at 37°C. Aliquots (10µl) were withdrawn every 4 hours and applied to electrophoretic gels (SDS-PAGE). Cleavage of plasma kininogen was detected by Western blot procedure as follows: after electrophoresis on 7.5% gels, proteins on the gel were transferred to Immuno-blot PVDF membranes in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Corp). The blotting buffer was 20mM Tris, 150mM glycine, 20% methanol, and transfer was done in an ice-cooled chamber, for 1 hour at 200 volts. Membranes were incubated in blocking buffer (1% non-fat dry milk in Tris buffered saline [TBS; 20mM Tris-HCl, 500mM NaCl, pH 7.4]) and then kininogen bands were detected by incubating membranes in primary antibody solution containing polyclonal rabbit anti-human kininogen IgG (1µg/ml in TTBS [20mM Tris-HCl, 500mM NaCl,] .05% Tween 20, pH 7.4)); washing; and then incubating in secondary antibody solution containing goat anti-rabbit IgG (1µg/ml, in TTBS) conjugated to alkaline phosphatase (AP). AP, now conjugated to kininogen, was assayed by an AP conjugate substrate kit (Bio-Rad Corp).

Preparation of Sonic Extract of *P. endodontalis*

Sonication of *P. endodontalis* cells was performed at 1500 cycles/sec in phosphate buffer for 5 minutes at 4 °C in the presence of the detergent TritonX-100 (1%). Cell debris was removed by centrifugation (6000 x g) for 10 minutes and then the supernatant was dialyzed exhaustively at 4°C in phosphate buffer to eliminate Triton. Protein concentration of sonic extract was measured by the BCSA protein assay reagent (Peirce Biochemicals, Rockford III).

Cleavage of LMWK by *P. endodontalis* Sonic Extract

Sonic extract (0.75mg/ml) was incubated with LMWK (1.0mg/ml) at 37°C for 10, 20 and 30 minutes and cleavage of LMWK was visualized on PAGE. Release of bradykinin from LMWK resulting from cleavage with sonic extract was measured by a Bradykinin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Enzo Life Sciences). Aliquots of the reaction mixtures were added to compete with standard bradykinin in the kit. Bradykinin standards were conjugated to biotin. Both standards and standards plus samples were added to wells that were coated with goat-antirabbit antibody. Rabbit polyclonal antibody to bradykinin was added to the mixture and allowed to incubate 2 hours. During this incubation, the rabbit antibody binds, in a competitive manner, to bradykinin in the sample or conjugate. Rabbit antibody stays on the coated plate. After washing, a solution of streptavidin, which binds biotin, conjugated to horseradish peroxidase is added to each well. After incubation for 1 hour, the wells were washed and a HRP catalyzed reaction generates a color reaction that can be read on a spectrophotometer at 420nm. Since generated bradykinin in samples competes with standard bradykinin, the amount of signal is inversely proportional to the level of bradykinin in the sample. The amount of bradykinin release per hour was calculated from a standard curve and reported as picogram bradykinin/0.1 mg sonic extract/hr.

A parallel, secondary assay, was employed to measure kallikrein-like kininogenase activity by incubating sonic extract (0.75mg/ml) with N-Benzoyl-Pro-Phe-Arg-p-nitroanilide (S2302, chromogenic substrate). Hydrolysis at the arginine residue results in release of the chromophore para-nitroanilide which turns yellow and can be read on a spectrophotometer at 410nm. The reaction mixture consisted of 0.1ml S2302

(0.15mM), 0.8ml of Hank's balanced salt solution, and 0.1ml of sonic extract (0.75mg/ml). The reaction was run for 1-10 minutes, and stopped by the addition of 0.01ml of 1M acetic acid. One unit of activity was defined as the liberation of 1 nmol of p-nitoaniline per min per mg.

Gel Filtration (Sepharose 2B) Chromatography

Sonic extract (1ml, 10mg/ml) was applied to a Sepharose 2B column (1.8 x 30cm, total volume 50ml) equilibrated with 10mM phosphate buffer, pH 7.4. The column was calibrated for molecular weight using bovine serum albumin (mw 66,0000, 1mg/ml) and human haptoglobin (mw150,000, 1mg/ml). The flow rate was controlled at 1ml/min and 1ml fractions were collected. Fractions were assayed for protein by absorbance at 280nm and for kallikrein-like amidase activity at 410nm by incubating with the chromogenic substrate S2302. Typically, 0.1ml of each fraction was incubated with 0.1ml S2302 for 10 minutes.

BLAST Nucleotide Query

Since *P. endodontalis* is phylogenetically closely related to *P. gingivalis*, a BLAST search (Basic Alignment Search Tool, National Library of Medicine) was done to query if *P. endodontalis* encodes the gingipains. *P. gingivalis* lys-gingipain (Kgp) and arg-gingipain (Rgp) are cysteine proteases that cleave kininogen and fibrinogen. Thus, the genomes of Kgp and Rgp were downloaded from NLM and imported into a nucleotide BLAST search of the complete genome of *P. endodontalis* ATCC to look for similar nucleotide sequences.

Characterization of *P. endodontalis* Kallikrein-like Kininogenase activity

The pooled active fractions from Sepharose 2B column were incubated with various enzyme inhibitors. The reaction mixture, incubated at 37° C for 1 hour, consisted of partially purified kininogenase from Sepharose 2B (0.1ml, 0.1 mg/ml), either 0.1ml S2302 (0.15mM) or 0.1ml LMWK (0.1mg/ml), and 0.1 ml inhibitor as follows: 0.5mM aprotinin; 0.1mM leupeptin; 1mM cysteine; 10mM $MgCl_2$). Aliquots were assayed for either release of bradykinin from LMWK by ELISA or for release of pNA from S2302 spectrophotometrically.

Cleavage of Kininogen and Fibrinogen by Partially Purified Kininogenase

The pooled active fractions from Sepharose 2B column (0.1ml, 1mg/ml), were incubated at 37°C with either LMWK (0/1ml, 0.1mg/ml) or fibrinogen (0/1ml, 0.1mg/ml) for 10–30 minutes. Aliquots from selected time points were subjected to SDS-PAGE and cleavage of kininogen and fibrinogen was observed on the gels.

Statistics

Experiments were repeated three times. For electrophoresis gels, only one gel for each experiment is presented. The author observed 8 images of protein gel electrophoreses on two different occasions to become familiar and self-calibrate the appearance and disappearance of protein bands indicating protein degradation. The calibration result was were reported as % repeatability index (33).

Enzyme activity experiments using either bradykinin release or cleavage of chromogenic substrate were likewise repeated three times, including those in the presence of inhibitors. The values for each experiment are reported along with the average (34).

CHAPTER 3
RESULTS

Effect of *E. faecalis, F. nucleatum,* and *P. endodontalis* Cells on LMWK

After growing bacterial cells to stationary phase, the cells were washed, pelleted and suspended in 0.01 phosphate buffer (pH 7.4) at a concentration equal to one half McFarland.

Dilutions of this suspension (working solutions) were incubated with pure LMWK for 24 hours. As seen in Figure 1, only *P. endodontalis* digested kininogen. Dilutions of the McFarland standard of *P. endodontalis* cells by 1/2 through 1/10 dilutions also hydrolyzed kininogen (data not shown). Intraoperator repeatability for observation of gels was determined to be 100%.

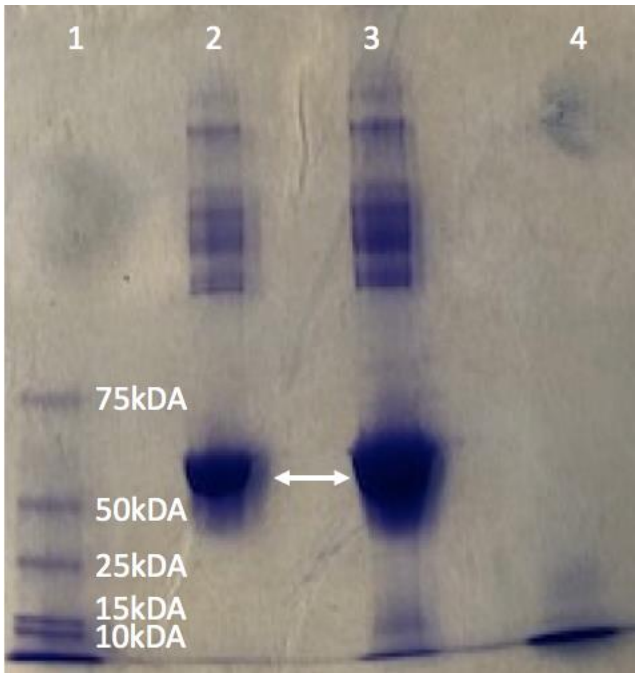

Figure 1. Degradation of LMWK by Whole Cells of *P. endodontalis*. This gel, showing all stained proteins, indicates that LMWK (arrow) is digested overnight by *P. endodontalis* in lane 4, but not by *E. faecalis* cells in lane 2, or *F. nucleatum* cells in lane 3. Lane 1 are molecular weight markers.

Quantitation of *P. endodontalis* Cells

As compared to a standard curve generated by *P. endodontalis* DNA, we found that turbidity of half McFarland standard corresponded to 10^5 cells/μl as determined by qPCR. We found that activity was confined to the cells, as cell supernatant did not degrade kininogen (data not shown).

Effect of *P. endodontalis* Cells on Artificial Plasma Kininogen

To investigate the effect of *P. endodontalis* cells on kininogen in a plasma-like environment, we solubilized plasma proteins and incubated the mixture with *E. faecalis* cells (10^5 cells /μl) for 4-24 hours. Aliquots were withdrawn and analyzed by Western blotting with anti-kininogen antiserum (Figure 2). Both parent molecules, the 120 kDa HMWK and the 60 kDa LMWK, showed progressive destruction from 4-24 hours with the appearance of more and thicker low molecular weight breakdown peptides (Figure 2).

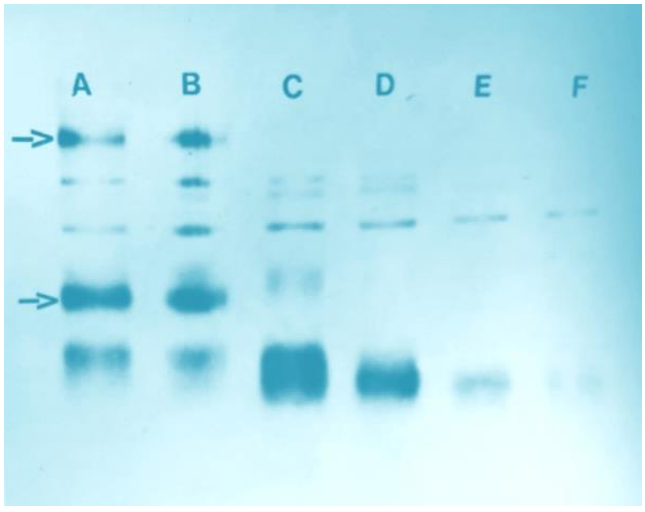

Figure 2. Western Blot assaying effect of *P. endodontalis* cells on artificial plasma. Western blot procedure detects only kininogen by using specific antibody to kininogen. We can see progressive breakdown of both HMWK (upper arrow) and LMWK (lower arrow) over 24 hours.

Extraction of *P. endodontalis* Kininogenase

Since the kininogenase activity was associated with whole cells, it was presumed to be membrane bound. Cells were disrupted by sonication in the presence of a weak non-ionic detergent, Triton X-100. This fraction (PESE) was dialyzed exhaustively and then incubated with LMWK for 10-30 minutes. As seen in Figure 3, the Triton sonic extract (PESE) contained kininogenase and degraded LMWK in as soon as 10 minutes.

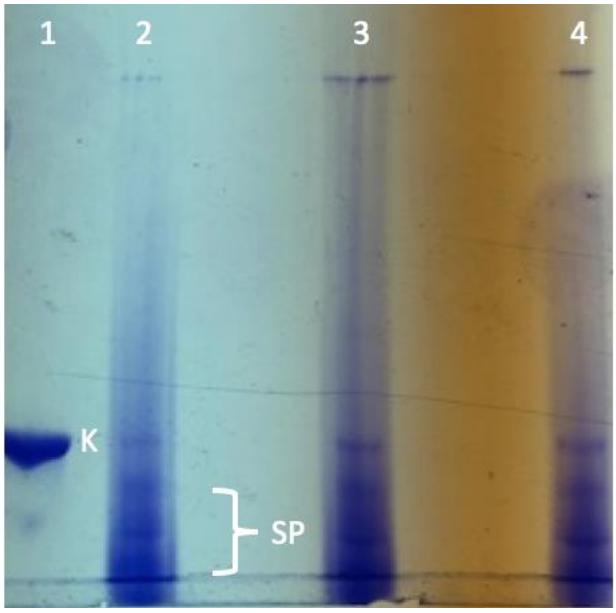

Figure 3. Time Course of Sonic Extract Cleavage of LMWK. PAGE of assay for PESE with LMWK. PESE was incubated with LMWK (K) for 10, 20 and 30 minutes and aliquots were applied to the gel (Lanes 2,3,4 respectively). Small peptides are seen (SP) indicating breakdown of LMWK.

Release of Bradykinin from LMWK and Correlation with Hydrolysis of S2302

Just as PESE degraded LMWK, it also hydrolyzed the chromogenic substrate S2302 linearly during a 10 minutes assay (Figure 4). Data from three sonic extracts yielded specific activities 520, 450 and 315 nmoles released per min per mg, with an average of 418 nmoles/mg/min. The data in the figure is from one assay.

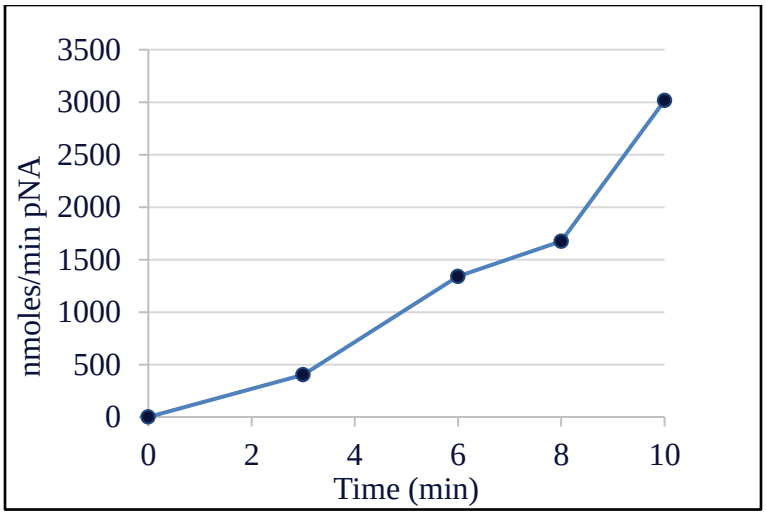

Figure 4. Cleavage of Chromogenic Substrate by Sonic Extract. Release of the chromophore pNa from S2302 by 0.1mg PESE as measured spectrophotometrically.

The degradation of LMWK, as seen on electrophoretic gel, was correlated to release of bradykinin. The same mixtures were found to release bradykinin as determined by an ELISA, using a rabbit antibody to bradykinin (Figure 5). Data were generated from three different sonic extracts yielding specific activities of 1311, 1200 and 1115 (pg bradykinin released per hour, for and average or 1208 pg/mg/hr. The data in the figure is from one assay.

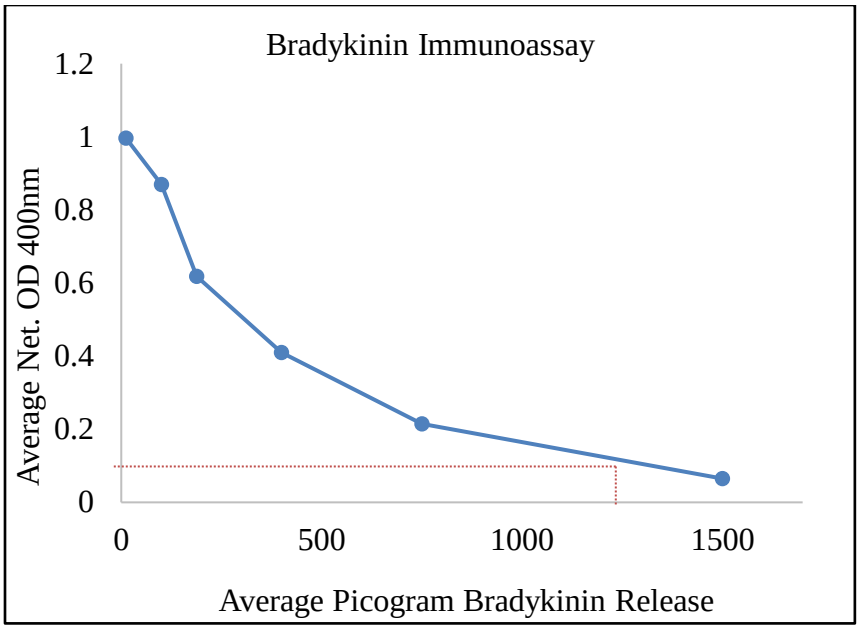

Figure 5. Release of Bradykinin from LMWK. Measured by competitive ELISA, values are reported as pg bradykinin released per hr per 0.1mg sonic extract.

Purification of *P. endodontalis* Kininogenase

PESE (10mg/ml) was applied to a Sepharose 2B column. Fractions were analyzed for protein at 280nm and for hydrolysis of the chromogenic substrate. The fractions with activity against S2302 eluted at apparent molecular weight of 100,000 daltons (Figure 6), and was purified 17 fold from sonic extract (Table 1).

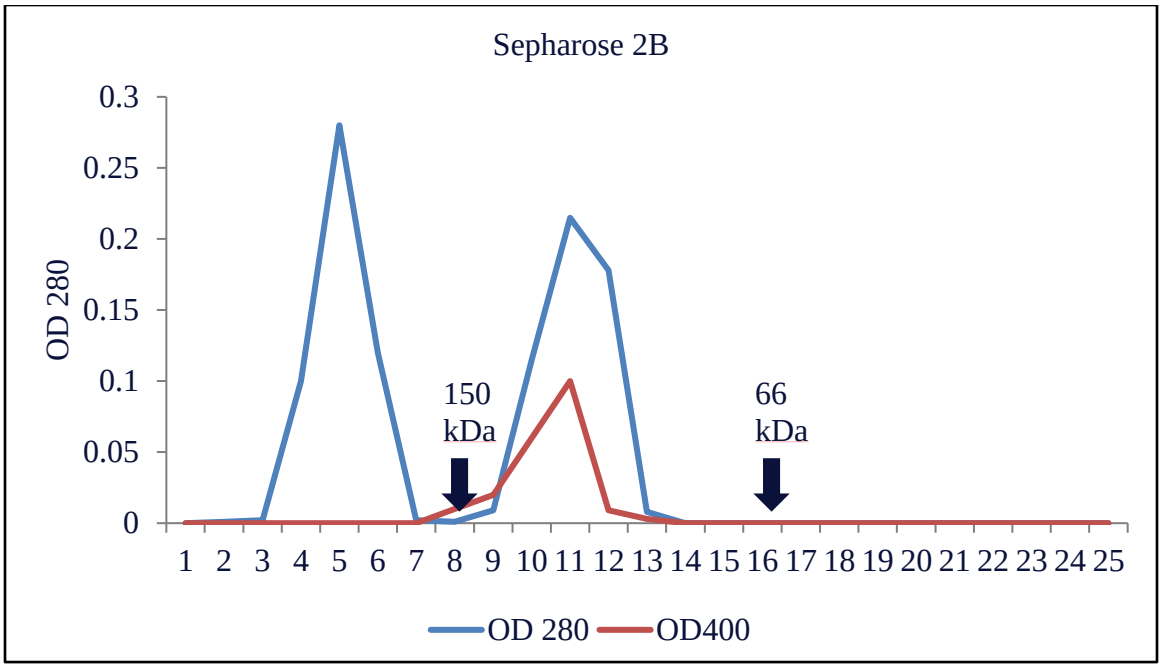

Figure 6. Sepharose 2B Chromatography of Sonic Extract. Active fractions were eluted with 100mM phosphate buffer. The blue line is protein measurement and the red line is amidase activity.

Fraction	**Concentration Mg/ml**	**S2302 Release Nmole/min**	**Specific Activity Nmole/mim/mg**
Sonic Extract	10	450	45
Active Fractions	1.5	1200	800
Purification(fold)			**17**

Table 1. Purification of *P. endodontalis* Kininogenase. Specific activity is expressed as release of pNA from S2302 or release of bradykinin from kininogen.

Comparison of Kgp and Rgp Genes with *P. endodontalis* Genome

A BLAST search comparing the complete genome of *P. endodontalis* ATCC to the gene sequences of kgp and rgp genes revealed that these cysteine proteases, native to *P. gingivalis*, do not exist in *P. endodontalis*.

Characterization of *P. endodontalis* Kininogenase

A series of inhibitors was used to characterize the activity isolated from the Sepaharose 2B column. Both the amidase activity against S2302, and the release of bradykinin from LMWK, were inhibited by boiling for 1 minute and by the serine protease inhibitor aprotinin. On the other hand, the thiol protease inhibitors, leupeptin and cysteine, and the metalloprotease inhibitor or activator Mg++, had no effect on the activity (Figure 7).

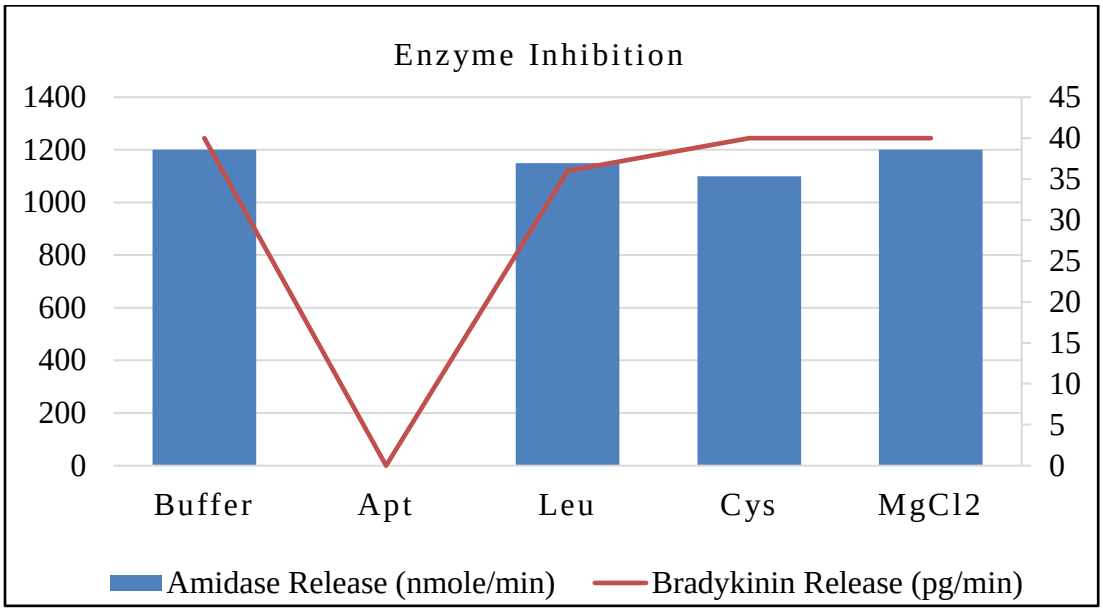

Figure 7. Characterization of *P. endodontalis* Kininogenase. PESE was incubated with S2302 or LMWK, in the presence of inhibitors, and assayed for release of pNA or bradykinin. The data are expressed as averages for three experiments.

Cleavage of Fibrinogen by *P. endodontalis* Kininogenase

Because of a previous report of a 88kDA protease form *P. endodontalis* that cleaves fibrinogen (35), we tested whether our serine protease also had fibrinolytic activity. When kininogenase from the active fractions was incubated with fibrinogen for 10-30

minutes, cleavage products were observed on PAGE, indicating some breakdown of fibrinogen (Figure 8).

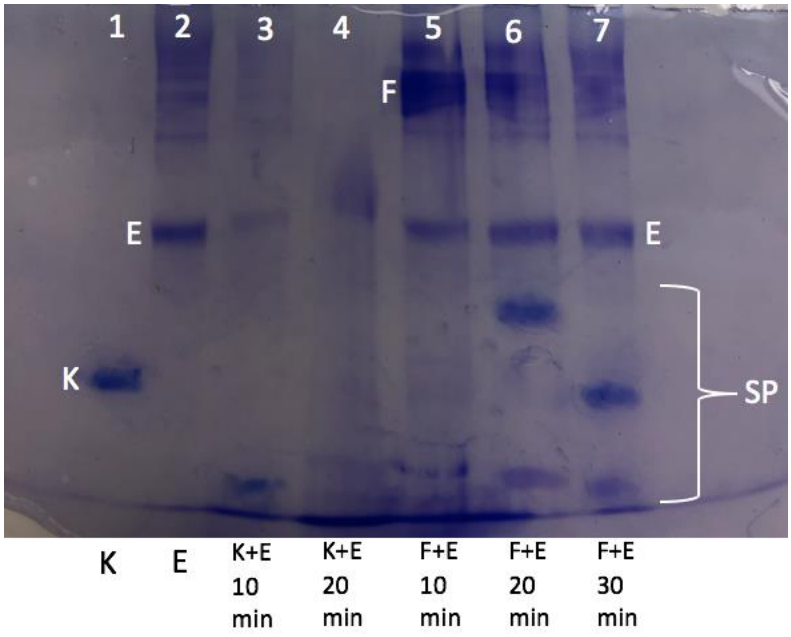

Figure 8. Cleavage of Fibrinogen by *P. endodontalis* Kininogenase. PAGE of samples of active enzyme from Sepharose 2B column incubated with low molecular weight kininogen (K) or fibrinogen (F) for 10, 20 and 30 minutes. Lane 1 kininogen; Lane 2 enzyme (E); Lanes 3, 4 kininogen + enzyme incubated for 10 and 20 minutes; Lane 5, 6, 7 fibrinogen (F) incubated with enzyme for 10, 20, 30 minutes. Breakdown of kininogen and fibrinogen can be seen on the gels as small peptides (SP).

CHAPTER 4
DISCUSSION

In cases of irreversible pulpitis, bacteria invade the pulp and come into direct contact with pulp tissue. Such localized infection to tissue results in a vascular response in the affected site, whereby the endothelial cells of the pulp microvasculature contract and become leaky, so that high molecular weight plasma proteins and fluid accumulate in the surrounding area (36). Histologic studies of pulps with irreversible pulpitis reveal endothelial gaps opened on vascular walls (37). Although early colonizers may initiate an inflammatory response, molecular based studies have shown that certain bacteria persist in infected pulps, namely *Porphyromons endodontalis, Enterococcus faecalis* and *F. nucleatum* (3).

As a consequence of infection, the pulp has the capacity to harbor bacteria and their products. Depending on the degree of infection, as well as the duration of infection, the host responds in various ways. Non-specific inflammatory reactions as well as specific immunological interactions result in pathological changes (38). Inflammatory mediators known as the kinins, along with the vasoactive amines and the complement system, produce many of the characteristic signs of inflammation (39). The biosynthetic precursors of the vasoactive kinins are the plasma proteins known as the kininogens. Human plasma contains two distinct kininogens, high molecular weight kininogen (HMWK, mw 120,000) and low molecular weight kininogen (LMWK, mw 65,000) (18). The kininogens can be cleaved to generate several types of kinins: the nonapeptide bradykinin (arg-pro-pro-gly-phe-ser-pro-phe-arg), the decdapeptide kallidin (lys-bradykinin), or the undecapeptide met-lys-bradykinin (40).

The plasma kallikrein-kinin system is made up of coagulation Factor XII, prekallikrein and kininogen (20). This system is initiated when Factor XII binds to a negatively charged surface, whether artificial or biological, which activates Factor XII to hydrolyze prekallikrein to kallikrein, which in turn cleaves kininogen to release bradykinin. In theory, any bacteria that enters the pulp could trigger the kallikrein-kinin system, by providing a negatively charged surface. The surfaces of bacteria, i.e., the lipopolysaccharide (LPS) of gram-negative bacteria and teichoic acid of gram-positive bacteria, are negatively charged, and thus may provide a surface to activate this system. However, in the absence of the contact system and in the presence of only pure LMWK, our results indicate that *P. endodontalis* whole cells, but not *E. faecalis* or *F. nucleatum*, hydrolyze LMWK. Although *F. nucleatum* is associated with various systemic diseases, its mechanism is one of attachment to eukaryotic cells (41). Likewise, its interaction with *E. faecalis* invaders in endodontic infections has been studied extensively, but proteolytic activity has not been identified as a pathogenic vector in these organisms (42).

P. endodontalis whole cells not only cleave pure kininogen proteolytically, but also both HMWK and LMWK kininogen in artificial plasma. This is an important finding since it mimics the clinical scenario and shows that *P. endodontalis* contains a kininogenase that can function in a plasma-like environment. We found that 10^7 cells incubated overnight in artificial plasma cleaved plasma kininogen. Kininogen usually circulates in plasma at a concentration of 70μg/ml (43). Since 1mg of dental plaque contains 10^8 to 10^9 bacteria, 1mg of plaque harboring as little as 0.01% *P. endodontalis* would in theory cleave kininogen. As opposed to whole cells, we could not identify activity in the cell broth or supernatant, suggesting that the enzyme is cell-bound.

Bacterial proteases are important for the survival of organisms because they contribute to the nutrition of organisms as they provide essential amino acids. However, these proteases may also be involved in the pathogenesis of the pulpitis (44).

As a result of improved methods for the identification, isolation and classification of oral bacteria, the role of specific microorganisms in the etiology and pathogenesis of irreversible pulpitis has been appreciated. The black-pigmented *P. endodontalis* can be isolated almost exclusively from infected root canals and it has proteolytic activity comparable to *P. gingivalis* (6). It is intriguing that *P. endodontalis* has enzymes that degrade fibrinogen and activate plasminogen (35, 45). The relationship between the presence of *P. endodontalis* in the infected pulp and extracellular proteolysis of these proteins of the coagulation system may figure into the pathogenesis pulpitis. Our finding that *P. endodontalis* degrades kininogen may be equally important since kininogens are multifunctional plasma proteins, that connect the kinin producing pathway with the blood coagulation cascade, the acute phase response and the inhibitor defense system.

An important discovery in this research is that the proteolysis of kininogen is linked to the release of bradykinin. Vasoactive bradykinin can be released from kininogen in plasma by the action of kallikrein, or kallikrein-like enzymes. Although kallikrein is considered the dominant producer of bradykinin in vivo, a number of tissue kallikriens also release bradykinin. These kallikriens are all serine proteases, i.e., they have serine at their active site, and hydrolyze peptides at arginine and lysine residues. The human cysteine protease calpain also cleaves kininogen to release bradykinin. Bradykinin, however formed, binds to the bradykinin B1 receptor on endothelial cells, and stimulates endothelial cell nitric oxide (NO) formation, i.e., endothelial cell

relaxation factor, to produce vasodilation (46). Bradykinin in plasma is degraded in seconds by a number of kininases; therefore, to have biologic function, it must be released in sequestered locations, like the dental pulp, and be produced quicker than it is degraded.

Bradykinin, a small nonapeptide, has a history of being difficult to assay (47). This explains why the interaction of oral bacteria with the kallikrein-kinin sytem has not been extensively investigated. However, there is renewed interest in this system, with the advent of new assays and the discovery of bacterial enzymes that cleave kininogen, namely the gingipains of *P. gingivalis*, which may augment the production of bradykinin (23). An immunologic assay was used in this research to identify bradykinin. A variety of immunologic assays are available including immunofluorescent microscopy, latex agglutination, flow cytometry and enzyme linked immunosorbent assay (ELISA). In principle, the ELISA is based on the fact that when an antibody is chemically linked to an enzyme, the resulting conjugate retains a great portion of its immunologic activity. The simplicity of this procedure derives from the use of detection substrates that are initially colorless but yield a colored product after enzymatic degradation.

Increased kinin activity has been shown to be associated with oral inflammation, both periodontal disease and pulpitis (48). There is a positive correlation between levels of bradykinin in gingival crevicular fluid and the degree of chronic gingivitis and periodontitis. Likewise, mean extracellular levels of bradykinin within pulp tissue diagnosed with irreversible pulpitis is significantly higher than that found within normal pulps (29). Although the nonapeptide, bradykinin, is known for its capacity to effect the

dilatation and permeability of blood vessels, some data indicate that it is also a potent stimulator of bone resorption, which may account for the periapical lesions (49, 50).

The association between proteolytic activity of the genus Porphyromonas and resultant inflammation has long been recognized. The proteases of *P. gingivalis* have been extensively studied. In addition to gingipains, *P. gingivalis* produces collagenase, immunoglobulin A and G protease, proteases that digest complement and serum proteinases inhibitors, fibrinogenase, proteases that degrade haptoglobin and hemopexin, and protease that inactivates the prothrombinase complex (51). Although less extensively studied, *P. endodontalis* is equally proteolytic (52). Several highly proteolytic dipeptidyl peptidases have been isolated in addition to an 88kDa protease that degrades fibrinogen (35, 53). Any of these proteases could be important virulence factors by acting directly on host proteins or indirectly by crippling the host defense proteins and cells, and importantly, bacteria proteases are not inhibited by host proteinase inhibitors (54).

Since kininogenase activity was not found in the cell broth, and since recent reports used detergents to extract and stabilize enzymes from *P. endodontalis* and *P. gingivalis*, we sonicated whole cells in the presence of the non-ionic detergent Triton X-100 (23, 35). We found kininogenase in the Triton sonic extract indicating that the enzyme is membrane associated. Triton is a surfactant or surface solubilizing agent that is used to isolate and stabilize proteins that otherwise may denature at the surface of a solution. The extraction procedure is both easy and efficient, providing for the isolation of kininogenase with relatively few contaminating proteins. Proteins vary markedly in their solubilities in detergents and it is obvious from the electrophoretic analysis of the sonic extract that few proteins were extracted along with kininogenase.

Because kininogen-cleaving enzymes frequently prefer arginine peptide bonds, we assayed *P. endodontalis* sonic extract and purified fractions for kallikrein-like activity using a chromogenic substrate containing "P1" arginine. We isolated an enzyme that we call "kallikrein-like" because of its specificity for an artificial, chromogenic substrate that has arginine in the "P1" position, i.e. P3'-P2'-P1'-X, where X is a chromophore that can be released by proteolytic cleavage of the P1'-X bond, and hence, read on a spectrophotometer. Using the chromogenic substrate S2303 (pro-phe-arg-pNA) to assay fractions for activity, we were able to purify the enzyme 17-fold by applying sonic extract to a Sepharose 2B column. The low degree of purification is a consequence of the relative purity of the enzyme after extraction. The enzyme eluted from the column at molecular weight 100,000 daltons, which was confirmed by SDS-PAGE.

Gingipain-K from *P. gingivalis* interferes with the blood-clotting system by cleaving both fibrinogen and kininogen to release the vasoactive peptide, bradykinin (23). This enzyme, along with gingipain-R, is a cysteine protease. Since *P. endodontalis* is phylogenetically related to *P. gingivalis*, we queried whether *P. endodontalis* kininogenase also belongs to the cysteine protease family. Hence, we downloaded the gene sequences of the *P. gingivalis* gingipains, both kgp and rgp, and performed a BLAST search through the National Library of Medicine Center for Biotechnology Information. We found that the genes for the gingipains do not exist in *P. endodontalis.*

Since the *P. endodontalis* genome does not contain the gingipains, a series of inhibitors was used to further classify the partially purified enzyme eluted from Sepharose 2B. Cleavage of both the chromogenic substrate for release of pNA and LMWK for release of bradykinin was measured in the presence of leupeptin, cysteine,

magnesium, and aprotinin. Neither leupeptin, which inhibits most cysteine proteases and some serine proteases, and cysteine, a competitive inhibitor of cysteine proteases, inhibited the activity. Magnesium neither inhibited or activated the activity, indicating that the enzyme is most likely not a metalloprotease. On the other hand, the serine protease aprotinin (Trasylol), which is a strong inhibitor of kallikrein, blocked chromogenic activity and bradykinin release. We concluded that a serine protease was isolated.

Since a previous report identified a 88kDa protein that cleaved fibrinogen, we assayed our kallikrein-like enzyme with fibrinogen and looked for cleavage of fibrinogen over 10, 20, and 30 minutes. We discovered that both kininogen and fibrinogen were cleaved in a time-dependent manner releasing smaller peptides. This is an important finding, so that, just as bradykinin release could account for increased vascular permeability, cleavage of fibrinogen could account for the hemorrhagic like bleeding associated with irreversible pulpitis. In this way, *P. endodontalis* kininogenase is similar to other enzymes that cleave both kininogen and fibrinogen, e.g., tryptase, and neutrophil elastase (55, 56). Thus, the high proteolytic activity allows *P. endodontalis* to participate in the inflammatory response on several levels, and may further explain its implication in irreversible pulpitis as depicted in Figure 9.

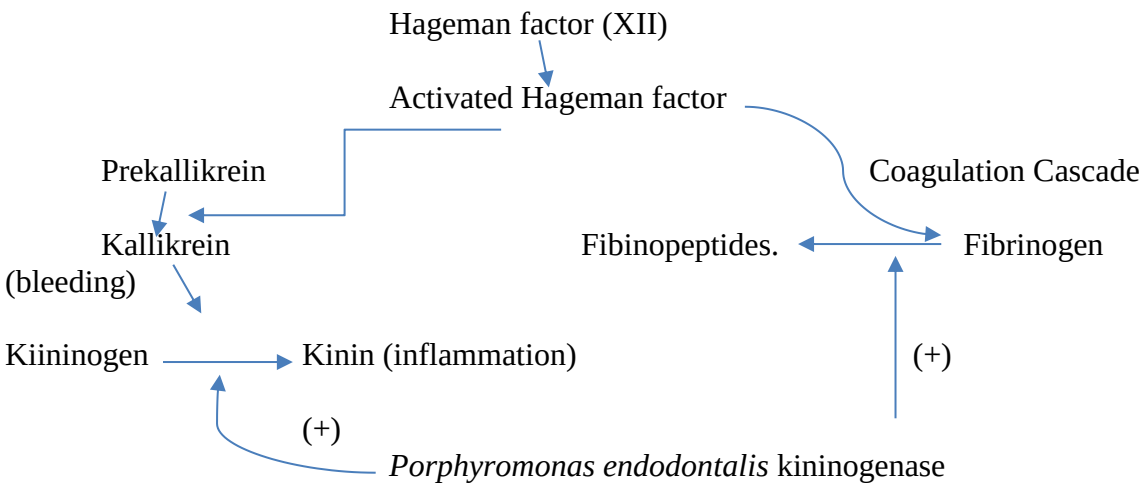

Figure 9. Diagram of Interaction of *P. endodontalis* and Contact System. The role of *P. endodontalis* kallikrein-like kininogenase in dysbiosis of both the inflammatory and coagulation cascades by production of kinin and depletion fibrinogen that normally would be converted to fibrin.

CHAPTER 5
CONCLUSIONS

These in vitro studies support previous studies implicating *Porphyromonas endodontalis* in pulpal disease. This organism harbors a highly proteolytic serine protease that degrades kininogen to release bradykinin, but also degrades fibrinogen. The enzyme is a kininogenase that has kallikrein-like activity, since it releases bradykinin. It also hydrolyzes kininogen in plasma. These data suggest that *P. endodontalis* may contribute to the pathogenesis of pulpitis on several levels, by modulating the inflammatory response via its effect on LMWK as well as hemostasis by its ability to degrade fibrinogen.

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