CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents Used in This Study and Their Sources

All reagents used were of analytical grade or equivalent and obtained from:-

Sigma (Sigma Aldrich, St. Louis, MO, USA)

Biotinamidocaproate-N-hydroxysuccinimide ester, bovine serum albumin, polyoxyethylene sorbitan monolaurate (Tween 20), sodium phosphate, TRIZMA-hydrocholride (Tris[hydroxymethyl]-aminomethane hydrochloride), trypsin type XIII from bovine pancreas, trypsin inhibitor type I-S from soybean, papain from papaya latex, cystatin from white egg, elastase from pancreatic, phenylmethyl sulfonylfluroxide, diisopropyl flurophosphate, α-1 protease inhibitor, α-N-Benzoyl-DL-Arginine-β-Naphthylamide (BANA), N-Succinyl-Ala-Ala-Val-nitroanilide (SAAVNA),4-chloromercuribenzoic acid (CMB), Fast Garnet GBS salt, Brij-35 solution, ethylenediaminetetraacetic acid, acrylamide, aminum persulfate, glycine, sodium dodecylsulfate, standard protein MW 6,500-205,000, N,N,N',N'-tetramethyl ethylenediamine (TEMED), potassium phosphate, azocoll, casein

BDH Laboratory Reagent (Poole, England)

Sodium acetate trihydrate

Carlo Erba reagenti (Rodano (Mi), Italy)

Di-sodium hydrogen phosphate anhydrous

Potassium sulfate

Sodium chloride

Sodium-dihydrogen phosphate

Fluka (Buchs, Swizerland)

Tri-sodium citrate dihydrate

Merck (Darmstadt, F .R. Germany)

Sodium acetate trihydrate

Sodium hydrogen carbonate

Sodium carbonate anhydrous

Citric monohydrate

Potassium chloride

Dimethylsulfoxide (DMSO)

2.2. Group of Subjects

The study was conducted at Department of Odontology and Oral Pathology, and Department of Oral Surgery, Faculty of Dentistry, Chiang Mai University. Research Ethics Committee, Faculty of Medicine, Chiang Mai University, approved this study. A total number of 117 adult human subjects were evaluated by dentist for oral screening examination. The subjects were examined for the presence of periodontal diseases by measuring the community periodontal index of treatment needs (Ainamo et al., 1982). (Turku study) or the presence of periodontal pockets and gingivitis (gingival index of Löe and Silness, 1963) (UBC study).

The scoring procedure used to assess gingivitis was the Löe-Silness Gingival Scoring Index as modified by Talbolt et al (1977). According to the Löe-Silness Index, scoring criteria are as follows:

0 = absence of inflammation

1 = Mild inflammation slight change in color and texture.

There is no bleeding upon probing

2 = Moderate inflammation. Moderate glazing, redness, edema and hypertrophy.

There is bleeding upon probing

3 = Severe inflammation. There is marked redness and hypertrophy,

a tendency to spontaneous bleeding and ulceration

The clinical scoring procedure was done on the facial and lingual surface of the teeth. Each tooth is scored in six areas: 1) disto-facial, 2) mid-focial, 3) mesio-facial, 4) disto-lingual, 5) mid-lingual, 6) mesio-lingual. A Löe-Silness index for each subject is determined by adding all the individual scores and dividing their sum by the total number of areas scored. The clinical measurements were determined on all the fetch by the same dentist. This study was carried out with three groups of subjects. The criteria used to categorize the subject 6 periodontal status was as follow:

Group A Forty healthy gingiva volunteers, 12 male and 28 female and age between 20 to 59 yr., were selected. Each subject had pockets not exceeding 3 mm. and bleeding score smaller than 0.2 after probing to the bottom of the pocket mesially, distally and interproximally in all the teeth.

Group B Thirty-seven gingivitis volunteers, 21 male and 16 female, were selected, age between 21 to 49 yr. Each subject had pockets around 3 mm more or less and bleeding score more than 1.0

Group C Forty periodontitis volunteers, age between 21 to 79 yr., who have pocket depth $\geq$ 4 mm. were selected.

2.3. Collection of Whole Saliva

All saliva samples were randomly taken between 9.00 – 12.00 a.m. priors to the clinical measurements. Whole saliva was collected by spitting without stimulation into the polypropylene tube. The saliva samples were collected over 5-min period and the average saliva flow rate was calculated from the total volume. Whole saliva was measured for pH by using pH meter and transfer into an ice-cooled vessel. Immediately, it was centrifuged at 10,000 rpm at room temperature for 15 min to remove cells and bacteria so as prevent the degradation of salivary proteins. After centrifugation the supernatant was kept at –20°C in aliquots for further analysis.

2.4. Determination of Protein Concentration

2.4.1. Principle

A protein concentration of the human saliva was determined by the Lowry procedure (Lowry et al., 1951). The principle of the method is to let the peptide nitrogen (s) reacts with the copper ion (s) under alkaline condition and to measure the subsequent reduction of the Folin-Ciocalteau phosphomolybdicphospho-tungstic acid to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acids. This

method is sensitive to low concentration of protein. The suggested concentration range is 5-100 μg/ml

BSA standard solution (5-60 μl) or 20 μl of saliva sample was added into the microcentrifuge tube in duplicate. The total volume was adjusted to 100 μl with 0.15 M NaCl. The 1.0 ml of alkaline copper solution (solution C) was added to each sample with mixing. After standing at room temperature for 10 minutes, 100 μl of Folin-Ciocalteu reagent (solution D) was added and the resulting mixture was allowed to stand another 30 minutes at room temperature. The absorbance at 750 nm of standard BSA or saliva sample was determined using the spectrophotometer. Another tube containing distilled water replacing the protein samples was treated as above and served as a blank. A standard curve of total protein content determination is presented in Figure 2.1, from which the protein content in the sample was determined.

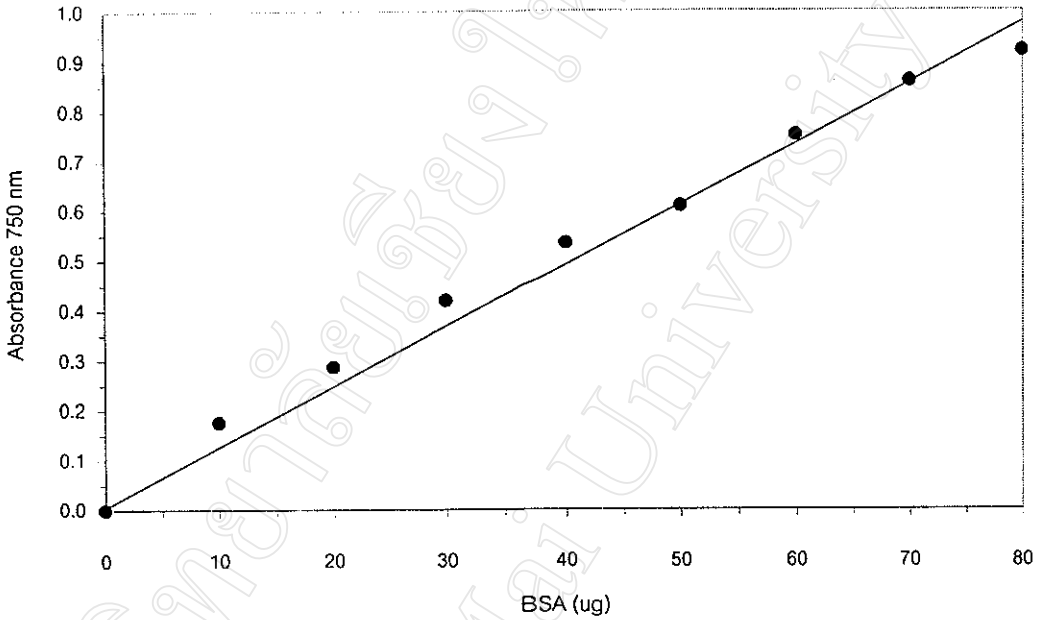

Figure 2.1 The standard curve of protein concentration was shown.

2.5. Determination of Total Proteinase Activity

2.5.1. Principle

Total proteinase activity of salivary sample was masured using an enzyme-linked immunosorbant assay method (Koritsaa and Atkinson, 1995). The principle of method is based on a solid phase assay employing substrate biotinylated gelatin adsorbed onto microtiter plates. The measurements of proteinase activity are based on the loss of biotin resulting from proteolytic action on the gelatin biotin complex prebound to microtiter plate wells (Figure 2.2). Removal of gelatin peptide fragments by proteinase results in fewer biotin moieties for anti-biotin antibody binding (Figure 2.2 A and B). The level of biotin-gelatin complex remaining after proteolysis, which bound to the well, was determined by the addition of peroxidase conjugated anti-biotin antibody, followed by peroxidase substance (Figure 2.2).

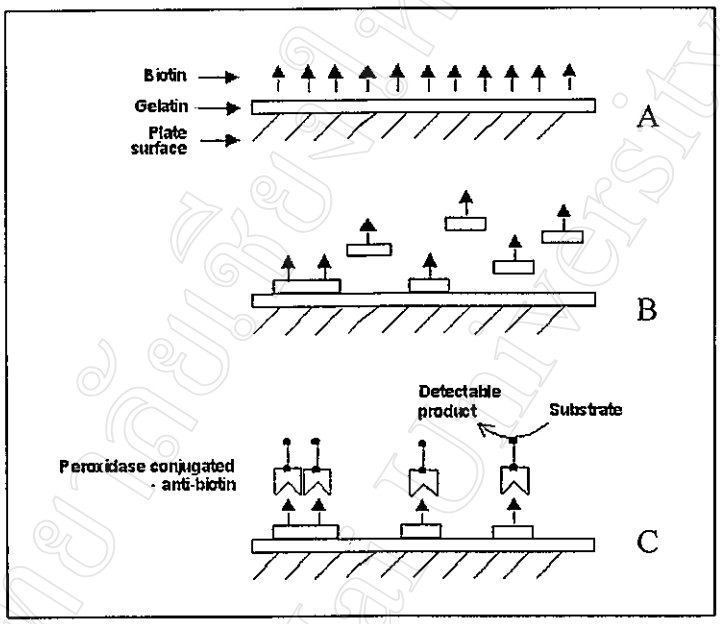

Figure 2.2 Schematic diagram represents the proteinase assay using biotinylated gelatin as substrate, (A); Proteinase is added to the well containing the immobilized substrate, (B); The enzyme hydrolyzes the substrate, releasing peptide–biotin fragment in solution, (C); Cleaved products are washed out and any remaining biotin sites interact with peroxidase conjugated anti-biotin antibody. Peroxidase substrate is then added and the amplified signal is measured photometrically.

2.5.2. Procedure

2.5.2.1.Preparation of Biotinylated Gelatin

To 5 ml of gelatin solution (1 mg per milliliter in 0.2 M Na_2HCO_3, pH 8.8, containing 0.15 M NaCl) 100 µl of biotinyl-N-hydroxysuccinimide ester (4 mg in 1 ml dimethylfromamide) was added and the reaction was allowed to proceed at room temperature with gentle stirring for 15 min. The reaction was stopped by the addition of 75 µl 1 M NH_4Cl (pH 6.0) and the solution was dialyzed exhaustively at 4°C against water. The dialyzed biotinylated protein was lyophilized and aliquots of the lyophilized biotinylated geletin was stored at –20°C.

2.5.2.2.Immobilization of Biotin – Gelatin on Microtiter Plates

Stock biotin-gelatin was diluted to 25 ng/ml in coating buffer and 50 µl was applied to each well of a microtiter plate. The proteinase substrate was left to bind overnight at 4°C and unbound biotinylated protein was removed by washing with PBS. Uncoated area was then blocked with 50 µl/well of 1% (W/V) gelatin in PBS for 30 min at 37°C, washed again with PBS three times, and then three times with water. Plates were ready for use after the water was removed.

2.5.2.3.Proteinase Assay

The biotin-gelatin-coated microtiter plates were incubated in 50 µl of the appropriate buffer for 10 min at 37°C prior to the addition of the proteinase. Buffer was removed and 50 µl/well of proteinase or sample added to the wells and incubated at 37°C 20 min. The reaction was stopped by washing five times with PBS-Tween. 100 µl peroxidase conjugated anti-biotin (1:8000 dilution) in PBS was added to each well and incubated for 60 min at 25°C. The plates were washed three times again with PBS-

Tween. Peroxidase substrate (100 µl/well), OPD was added and incubated at 37°C for 20 min to allow the color to develop. The reaction was stopped by addition of 50 µl/well of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the titertek Multiskan M340 multiplate reader. The 500 µl of each saliva sample was lyophilized and weighed. 0.5 mg of the lyophilized sample was diluted to 50 µl with TNC buffer (0.05 M Tris-HCl buffer pH 7.6, contained with 0.2 M NaCl and 0.005 M $CaCl_2$). The concentration of proteinase was compared with proteinase standard control in a sample. Proteinase activity was calculated using the absorbance value (at 492/690) for well treated only with buffer to represent no digestion.

2.5.2.4.Proteinase Inhibitor Assay

Stock soybean trypsin inhibitor (SBTI) was dissolved in water and added to the proteinase or trypsin solution of a final incubation concentration. The proteinase-inhibitor mixtures were preincubated for 20 min at 37°C before addition to biotin-gelatin-coated wells. After 60 min. at 37°C, the proteinase-inhibitor mixture was washed five times with PBS-Tween and the amount of peroxidase conjugated antibiotin bound was measure by the addition of peroxidase substrate. Values are mean absorbance at 492/690 nm. Parallel controls contained inhibitor solvent only. The lyophilized saliva sample was diluted to 50 µl with TNC buffer and boiled for 5 min at 100°C before preincubation with the trypsin solutions.

2.6. Determination of Cysteine Proteinase Activity

2.6.1. Principle

Quantitation of papain activity (cysteine proteinase) in saliva sample was determined by spectrophotometric assay using α-N-Benzoyl-DL-Arginine-β-Naphthylamide (BANA) as a synthetic substrate according to Barrett (1972). The enzyme (activated by cysteine and EDTA) hydrolyses BANA at pH 6.0, releasing free β-

naphthylamine. The reaction is terminated by the addition of the coupling reagent. 4-chloromercuribenzoic acid (CMB) in the coupling reagent halts the enzyme activity by blocking the free thiol groups of the cysteine at the active site of this enzyme. Fast Garnet GBC couples with free naphthylamine, producing a crimson azo dye; the reaction is accelerated in the presence of a neutral detergent, which also maintains the colored product in a soluble form.

2.6.2. Procedure

2.6.2.1.Enzyme Assay

The reactions were adapted for use in a microtiter-plate assay as follows: a 50 µl saliva was diluted in duplicate in 125 µl of incubated buffer containing 0.2 M potassium phosphate buffer pH 6.0, 4 mM EDTA, 0.2 mM cysteine and pre-incubated at 37°C for 5 min. Subsequently, 50 µl BANA (3 mg/ml in 10% DMSO) was added. After 10 min of incubation at 37°C the enzymatic reaction was stopped with 60 µl of 5 mM 4-chloromercuribenzoic acid and the cleavage product, 2-napthylamide, was coupled with 60 µl of fast Garnett GBC (0.15 mg/ml in 4% W/V brij-35), producing a red color. The absorbance at 490 nm was measured with a microtiter-plate reader.

A standard reference assay for papain activity in saliva measures the ability of the enzyme to generate a substrate. Eight concentration of papain ranging from 40.0 µ g/ml to 0.1 µg/ml was plotted against absorbance at 490 nm. The linear regression was used to determine the point of best fit standard curve plots which were linear over for entire concentration range ($r = 0.999$). The lowest concentration of papain giving and absorbance are reading of 0.002-0.003.

2.6.3. Inhibitory Assay for Total cystatin

Total cystatin (cysteine proteinase inhibitor) activity of the salivary sample was determined by measuring the inhibition of papain by salivary cystatin. In this assay the activity of free (unbound) salivary cystatin was measured. The saliva was heated at 100°C for 30 min. 50 µl of the diluted saliva sample was pre-incubated at 37°C for 5 min with 75 µl of papain solution (20 µg/ml). Cystatin from chicken egg – white was used as a standard and was diluted in the same way as the sample (starting concentration 1.0 µ g/ml). Subsequently, 50 µl BANA was added and performed similarly. After measurement, the inhibition curves were constructed by plotting the percentage of residual activity against the amount of saliva added. Apparent cystatin concentration of the saliva samples was calculated by comparison with the inhibition curve obtained with chicken egg-white cystatin.

2.7. Determination of Elastase Activity

2.7.1. Principle

Elastase activity has been found in the pancreas, PMNs, platelets, alveolar macrophage, saliva and several microorganism (Stein et al., 1989; Uitto et al., 1996). With a spectrophotometric assay system was using a synthetic substrate, N-Succinyl-Ala-Ala-Val-nitroanilide (SAAVNA), which has been widely used for assaying elastase activity. This substrate is cleaved by the enzyme to release the chromophore (Bieth et al., 1974). In this study, the effectively quantitation of elastase activity in saliva sample can be accomplished by measuring the breakdown of SAAVNA, and determining the amount of product, *p*-nitroaniline (NA), which has been released. NA concentrations were assayed using a modified HPLC method of Kuwada and Katayama (1984).

2.7.2. Procedure

2.7.2.1.Enzyme Assays

SAAVNA was dissolved in a solution presenting the following final concentration: 1 mM substrate, 0.1% DMSO, 0.2 M NaCl and 0.05 M Tris-HCl buffer, pH 7.5. Elastase standard has been diluted from a stocking solution with water to achieve concentration of 0.69-200 μg/ml. A 100 μl aliquot of saliva was preincubated with 125 μl of 0.2 M NaCl 0.05 M Tris-HCl buffer, pH 7.5 at 37°C for 10 minutes in microtiter-plate. A 25 μl of substrate solution was added to rich a 250 μl final volume. For the elastase activity, 100 μl of elastase dilutions were incubated with 125 μl of buffer solution and 25 μl of substrate solution for 2 hours at 37°C. Afterwards, the mixture was used for measurement of elastase activity by the spectrophotometric method, and a 75 μl was used for measurement by the HPLC method.

2.7.2.2.Inhibitory Assay for Saliva Elastase Inhibitor

The elastase inhibitors, phenylmethyl sulfonylfluroxide (PMSF), at various dilution in buffer solution were tested for the activity. To assay the inhibitor in saliva, saliva samples were boiled at 100°C for 30 minutes to kill the enzyme activity. 50-μl aliquot of inhibitor samples with 50 μl of elastase (100 μg/ml) was preincubated at 37°C for 1 hour. Remaining activities of incubation mixture were performed as described for elastase in a final volume of 250 μl.

2.7.2.3.Spectrophotometric Method

Elastase activity was measured by using a spectrophotometric technique as decribed by Uitto and co-worker (1996). Formation of yellow color was read with a spectrophotometric ELISA reader at 405 nm. All samples were assayed in duplicate.

2.7.2.4.HPLC Apparatus and Chromotographic System

The HPLC arrangement is composed of a Consta Metric 3500 programmable isocratic pump, Consta Metric 3200 solvent delivery system, and a Spectromonitor 3200 detector (LDC Analytical Co., USA). The chromatographic separation was performed on a Spherisorb ODS-2 (4.6 x 250 mm, 5 μm) analytical column capped with a Spherisorb ODS-2 (4.6 x 10 mm, 5 μm) guard column instead of μ-Bondapack C18 column. Mobile phase consisted of a mixture of 0.01 M Sodium phosphate buffer, pH 3.0, /methanol (1:1, v/v), which was filtered though membrane (0.45 μm, 47 mm in diameter) and simultaneously degassed with a Millipore Filtration equipment. 75 μl of readily filtered sample was injected into the Rheodyne 7125 25-μl loop connected to HPLC system, then eluted with a mobile-phase solvent at the flow rate of 0.6 ml/min (back pressure 2000-3000 psi). The column effluent was continuously monitored at 405 nm with the detector. Peak area was integrated using a BDS program.

2.7.2.5.Standard Curves for HPLC Assay

Under the chromatographic condition described previously, the peak that resulted from *p*-nitroaniline (NA) had a retention time of 4.8 min, (Figure 3.7). Six different concentration ranges of elastase were prepared for the HPLC analysis of elastase and inhibitory activity in saliva sample. Standard curve for all samples varied between 3 and 100 μg/ml of elastase (Figure 3.8). The concentrations of inhibitor were obtained from OD values that compared with the OD of standard elastase activity (Figure 3.8).

2.8. Determination of Plant Inhibitory Activity

2.8.1. Gelatin Digestion Method

2.8.1.1.Principle

The detection of proteinase activity was modified using unprocessed X-ray films by Cheung and co-worker (1991). Proteinase has been detected by incubation on the surface of unprocessed X-ray films. This procedure utilized the gelatin coating of the unprocessed films as a substrate. Proteolytic degradation of gelatin on the surface results in a clear area against a dark background. The utility of this simple assay has been extended to study the inhibitory activity from plant extracts after pre-incubation with the enzyme in a clear area and then decreases.

2.8.1.2.Procedures

Preparation of Plant Extract

Plant samples were purchased from a local market in Chiang Mai, Thailand. Each plant sample was cleaned, and dried at 60°C for overnight in an incubator. They were blended to a fine powder. A small amount of the dry powder was extracted with distilled water by a ratio of 1:5 (w/v). One tube stirred at room temperature and another tube at 100°C for 2 hours, were filtered by cotton gauze. The aqueous extract of plant material was decanted, clarified by centrifugation of 24,000 g for 30 min, and the supernatant fraction was stored at –20°C prior to use. Plant extracts were prepared from each of the thirty-two plants and are shown in Table 3.5.

Assay for Total Proteinase

The gelatin coating or unprocessed X-ray film (20.3 x 25.4 cm) of Kodak chemical Corp. was used as a substrate for a variety of proteolytic enzymes. For the assay of the standard trypsin activity, 50 µl of a various trypsin solution diluted with 0.1

M Tris-HCl buffer, pH 7.5 was applied onto the film which was then incubated in an incubator for 1 hour at 42°C. The film was gently washed with running tap water for 1 minute. The appearance of the plastic below the gelatin film as indicated by a clear zone at the site of application was considered positive. The film was dried and visually determined in the detection of clear zones. The resulting clear zones at different trypsin concentrations were showing Figure 3.14.

Assay for Total Proteinase inhibitor

Each inhibitor extract from many kinds of plants was diluted in tube with 0.1 M Tris-HCl buffer, pH 7.5. Various dilution of inhibitor was pre incubated with equal volume (25 µl) of the required trypsin (0.08 mg/ml) at 37°C for 10 minutes. The mixtures were applied on the film, which was performed as described for standard trypsin. The percent of inhibition was calculated from the calibration curves of proteolytic degradation of gelatin, which were made with amount of the standard trypsin inhibitor as shown in Figure 3.15.

2.8.2. Specific Substrate Method

2.8.2.1.Principle

Trypsin inhibitor activity was assayed by using benzoyl arginin p-nitroanilide hydrochloride (BApNA) as a synthetic substrate (Erlanger, 1961). This synthetic substrate is hydrolyzed by trypsin. The hydrolysis product is a yellow *p*-nitroaniline compound, which can be measured by a spectrophotometry.

2.8.2.2.Procedure

Assay for Trypsin Standard

75 µl of 1 mM BApNA solution was placed in a microtiter-plate that contained 205 µl of 0.1 M Tris HCl buffer, pH 7.5. This mixture was preincubated at 37°C for 10 minutes. Finally, each 20-µl of eight concentrations of trypsin running from 3 to 1000 µg/ml was added to the mixture and incubated for 20 minutes at 37°C. A 300 µl of total volume mixture was measured the absorbance at 405 nm for p-nitroaliline. The trypsin activity was calculated from the values of p-nitroaniline formation.

Assay for Total Proteinase Inhibitor

A trypsin-inhibitor extract from ten kinds of Thai herbs that showed higher than the fifty- percent inhibitory activity (Table 3.6). It was diluted in microtiterplate and preincubated with an equal volume (20 µl) of trypsin (1000 µg/ml) in 185 µl of 0.1 M Tris HCl buffer (pH 7.5) at 37°C for 10 minutes. Trypsin inhibitor was used as a standard and was diluted in the same condition. The reaction was started by adding 20 µl 1 mM BApNA and carried out 37°C for 20 min. At the end of incubation, the reaction was measured at 405 nm. The inhibitory activity was calculated from standard curve. The residual enzyme activities were plotted for various concentration of the inhibitor and the IC_{50} value was determined

Table 2.1 The amount of substrate and enzyme use in the assay of trypsin inhibitory activity.

Well	Substrate BApNA (µl)	Inhibitor Solution (µl)	Tris buffer (µl)	Enzyme solution (µl)	Total volume (µl)
Blank	75	20	205	-	300
Control	75	20	185	20	300
Assay	75	20	185	20	300

Calculation

$$\% \text{ Inhibition} = \frac{T - T^*}{T} \times 100$$

When T = optical density of p-nitroaniline in well (control – blank)

T* = optical density of p-nitroaniline in well (assay-blank)

Assay for Cysteine Proteinase and Cystatin

See section 2.6

Assay for Elastase and Elastase Inhibitor

See section 2.7

2.9. Determination of Enzyme Inhibition of Periodontitis Saliva Adding with the Most Inhibitory Effect of Thai herbs

The saliva samples collected from periodontitis subjects were tested with extract of Thai-herbs, which had the highest inhibition of enzymes. See section 2.5, 2.6 and 2.7 for more detail.

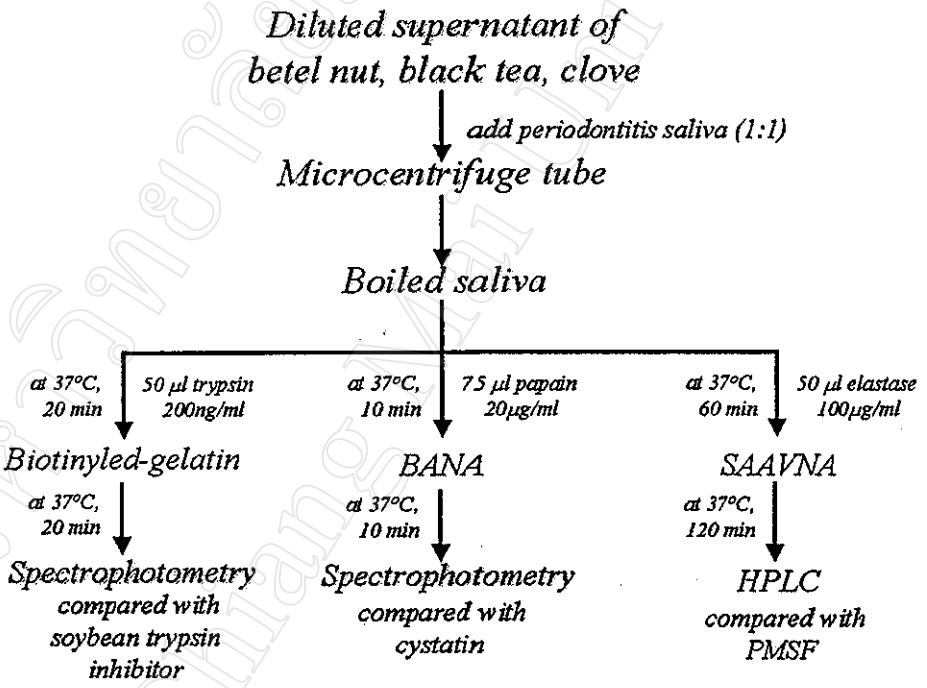

Figure 2.3 Diagram of determination of enzymes inhibition of periodontitis saliva with thai herbs

Normal saliva samples (n=40)

Gingivitis saliva samples (n=37)

Periodontitis saliva samples (n=40)

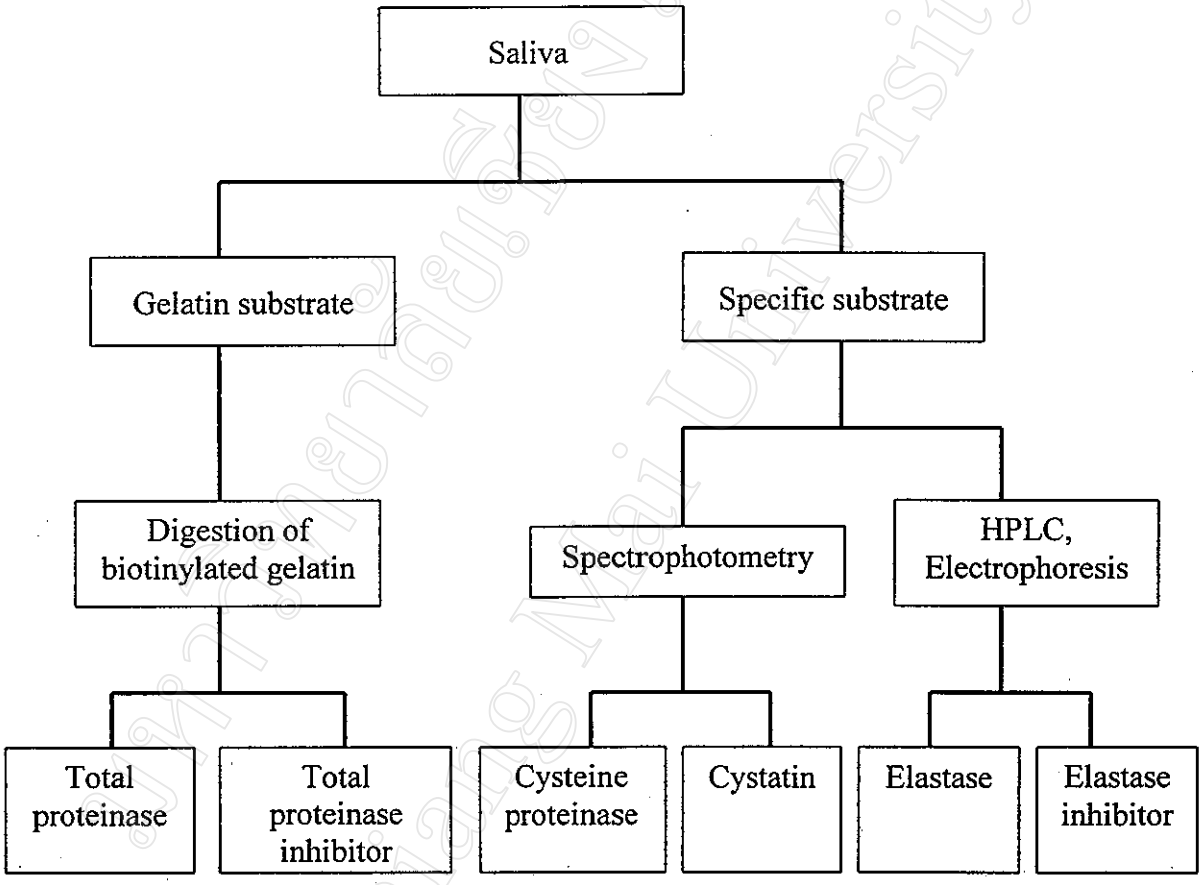

Figure 2.4 Diagram of determination for saliva samples in this study.

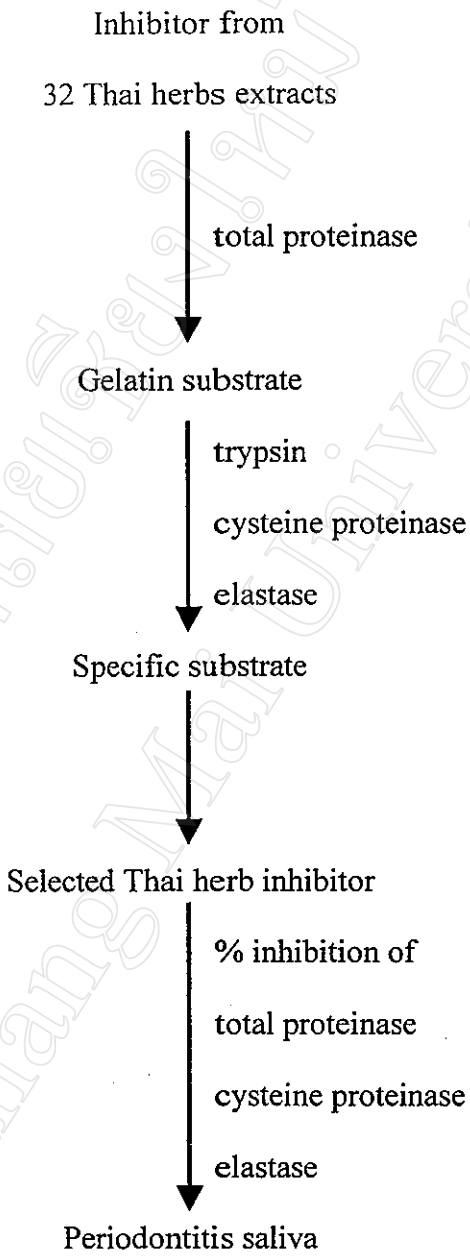

Figure 2.5 Diagram for determination of inhibitor from Thai herbs

2.9.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970)

2.9.1.1.Principle

This technique was used to identify proteins and determine their molecular weights. The protein was denatured by sodium dodecylsulfate (SDS). When applied to an electrical field, the negatively charged SDS-polypeptides were moved towards the anode at rates dependent upon their molecular weight. A small protein molecule can thread its away through the gel easily and hence migrates faster than a larger molecule.

2.9.1.2.Apparatus

Mini-PROTEAN 3® system components and power supply

2.9.1.3.Procedure

The saliva samples were taken from section 2.4, 2.7 and 2.9. Protein contents were determined according to Lowry method (see section 2.4) SDS-PAGE was performed according to Laemmli's method.

Electrophoresis was run as described below, all glass plates were first cleaned in detergent, washed with water and ethanol, and allowed to dry. The glass-plate sandwich using a spacer plate of 0.75-mm gel thickness was assembled with a short plate on top of it. The sandwich was locked to the casting stand and placed a comb completely into the assembled gel cassette, and mark the glass plate 1 cm below the comb teeth. This is the level to which the separating gel is poured and removes the comb. The separating gel (12% sodium dodecyl sulfate polyacrolamind gel) solution was poured into the glass plate by a Pasteur pipette. The gel was overlaid with deionized water to ensure a flat surface and to exclude air and gel was allowed to polymerize for 30 minutes. Then, the water was removed and the stacking gel (4% gel) was poured on the top of the set

separating gel and a comb was inserted into the stacking gel. After the gel polymerized (approximately 30-45 minutes) the comb was removed and the wells were rinsed with electrophoresis buffer to remove unpolymerized monomer. The gel sandwich was attached to the buffer chamber using manufacture's instruction. An aliquot of protein sample was analyzed at least 1:1 (v/v) with sample buffer and heat at 100°C in a sealed screw-cap microcentrifuge tube. A 20 µl protein samples was loaded into the well under electrode buffer with a pipette using gel-loading tip. After protein application, the electrophoresis was carried out using 120 volts of electricity until the bromphenol blue tracking dye had migrated approximately 0.5 cm from the bottom (~90 minutes). Then electrophoresis is complete, turn off the power supply and discard electrolyte buffer. The gels were carefully removed from the gel cassette and can be stained overnight with commassie blue at room temperature. The stained gel was destained in frequently changes of the destaining buffer until the protein bands were clear from the background (usually 1 to 3 hours).

2.10. Statistic Analysis

The statistical differences in the levels of enzyme and inhibitory activity were calculated using One Way Analysis of Variance (ANOVA). Comparisons between the percent of inhibition in periodontitis saliva with and without adding Thai herbs were evaluated with repeated measure ANOVA on Ranks. The statistical calculations were performed on a computer using Sigmastat2.01 software. Values of $p<0.05$ were accepted as statistically significant.