CHAPTER III

RESULT

3.1. Clinical Measurements

All 117 subjects participating in the study were clinically characterized by measurement of various clinical parameters. The median levels of measured parameter are given in table 3.1 periodontitis group had higher the median plaque and Löe-Silness Gingivitis Index than other group. The distribution of the percentage of sites in class of probing pocket depth (PPD) for each periodontitis subjects. Which showed a large heterogeneity within the periodontitis group: 40% of the sites had a PPD between 1 and 3 mm, 50% of the sites had a PPD between 4 and 5 mm and 10% of the sites had a PPD of 6 mm or more. For all periodontitis subjects, mean PPD was 4.1. An analysis of variance (ANOVA ; Kruskal-Wallis) indicated that there was a statistically significant difference in the median values of age, Löe-Silness Gingivitis and Plaque Index ($P < 0.05$).

3.2. Biochemical Analysis of Saliva

The flow rates in salivary sample of all subjects were measured, and subsequently a number of biochemical parameters showed in Table 3.2. Significant differences were in the median values of salivary pH ($p = 0.016$) and flow rates ($p = 0.057$) in the three groups. The salivary protein concentrations among the three groups were statistically significant differences ($p = 0.001$). The mean protein concentration in saliva of periodontitis group (2.0 mg/ml) was significantly higher ($p < 0.05$) than in the normal (1.6 mg/ml) and gingivitis groups (1.6 mg/ml).

Table 3.1 Descriptive data of normal and patient groups

Group	Total	Sex		Median (Mean ± SD)			
		M	F	Age (year)	Probing (mm)	Gingival Index	Plaque Index
Normal	40	12	28	29.0 (30.8±8.4)	1.83 (1.8±0.3)	0.46 (0.8±0.4)	0.75 (0.8±0.4)
Gingivitis	37	21	16	26.0 (28.7±7.1)	2.25 (2.3±0.4)	1.17 (1.4±0.4)	1.75 (1.8±0.7)
Periodontitis	40	24	16	45.0 (43.7±2.0)	2.67 (2.4±0.8)	1.50 (1.5±0.7)	2.67 (2.4±0.8)

Table 3.2 Salivary parameter of normal and patient groups

Salivary Parameter	Median (Mean ± SD)		
	Normal	Gingivitis	Periodontitis
pH	7.06 (7.1±0.3)	7.00 (7.0±0.2)	6.98 (6.9±0.3)
Flow rate (ml/min)	0.60 (0.7±0.4)	0.60 (0.5±0.3)	0.40 (0.4±0.4)
Total protein (mg/ml)	1.66 (1.7±0.4)	1.60 (1.6±0.6)	2.02 (2.0±0.6)

3.3. Quantitation of Total proteinase and its inhibitor

Measurements of total proteinase activity are based on the loss of biotin resulting from proteolytic action on the gelatin – biotin complex prebound to microtiter-plate well (Figure 2.2). Trypsin was selected as a total proteinase to represent their respective catalytic class in salivary enzyme. The hydrolysis of gelatin – biotin after 30 min was proportional to the amount of trypsin, and a standard curve trypsin was shown in Figure 3.1. and the range of detectable concentration was 10-1000 ng/ml.

The effect of standard soybean trypsin inhibitor (SBTI) for trypsin was shown in Figure 3.2. The appropriate amount of trypsin 100 μg/ml (50μl/well) and the required trypsin inhibitor concentrations were preincubated for 20 min at 37°C before addition to biotinylated -gelatin coated wells. After 60 min at 37°C, the trypsin-inhibitor mixture was washed five times with PBS-Tween and the amount of antibiotin-peroxidase conjugate bound was measured by the addition of peroxidase substrate at absorbance 492/690 nm.

An assay for determining total proteinase and its inhibitor in salivary samples was developed using biotinylated – gelatin. Analysis of total proteinase specific activity showed that there was a statistically significant difference among the three groups ($p = 0.001$). The median values of periodontitis group (346.3 μg/mg protein) was significantly higher than in gingivitis (60.0 μg/mg protein) and normal group (156.0 μ g/mg protein).

In addition the total proteinase inhibitor was analyzed, it was shown that there was a statistically significant difference of median values among the three groups (Table 3.4) ($P = 0.050$). The median values of periodontitis group (4.9 μg/mg dry saliva) and gingivitis (5.4 μg/mg dry saliva) were significantly lower than in the normal group (29.6 μg/mg dry saliva).

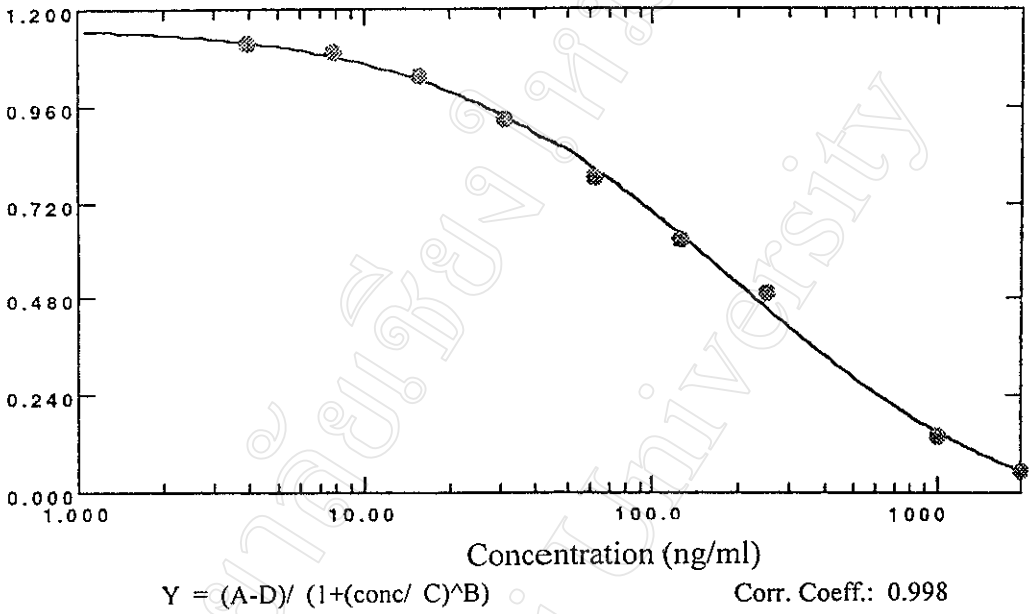


Figure 3.1 The standard curve of hydrolysis of biotin-gelatin at various concentration of the trypsin (range 3-2000 ng/ml) was shown. Fifty microtiters of the proteinase in buffers was added to the well and incubated for 20 min at 37°C.

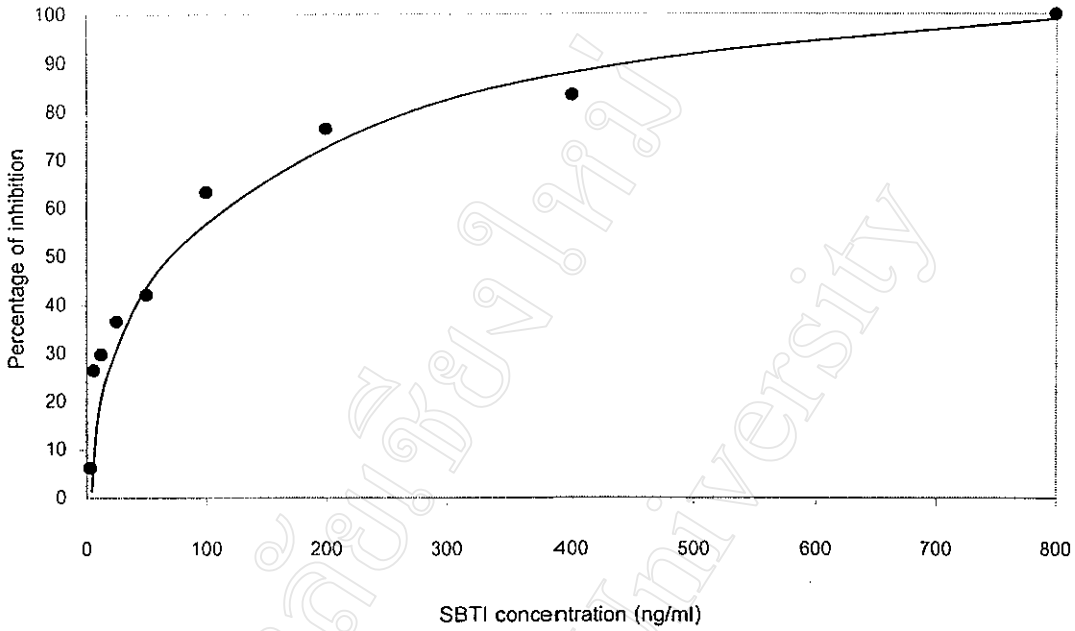


Figure 3.2 Percent inhibition of trypsin at different concentrations of standard soybean trypsin inhibitor by gelatinolysis method.

3.4. Quantitation of Cysteine Proteinase and Total Cystatin

Papain (cystein proteinase) activity was determined with α-N-Benzoyl-DL-Arginine-β-Naphthylamide (BANA) as a substrate. Enzyme catalysed release of β-napthylamine can also be determined by using a stable diazonium salt, Fast Garnet GBC, which detected the absorbance at 490 nm. Measuring BANA-hydrolysis checked papain activity of the saliva sample. The standard curve of papain activity was show in Figure 3.5. After 10 min at 37°C, the amount of papain was measured by the addition of its substrate (150 μg) at absorbance 490 nm. From the standard curve it was found that the assay was in the range 0-25 μg/ml of papain concentration. Analysis of papain specific activity showed that there was statistically significant difference among the three groups ($p = 0.039$). The median values of the gingivitis group (3.5 μg/mg protein) and periodontitis group (2.9 μg/mg protein) were significantly higher than the normal group (2.0 μg/mg protein) (Table 3.4).

Next, studies were done to determine the total cystatin in saliva under our assay condition, the appropriate amount of papain (1.5 μg) and the required standard cystatin concentration were preincubated for 10 min at 37°C before addition to BANA (150 μg). After incubation, the remaining papain activity was detected the absorbance at 490 nm. The standard curve of inhibition of papain activity was shown in Figure 3.6. The cystatin concentration of the saliva sample of the subject was analyzed. Statistical analyses of the data (Table 3.4) indicated that there was statistically significant difference among the three groups ($P < 0.001$). The median total cystatin of the gingivitis (0.7 μg/mg dry saliva) and periodontitis group (1.0 μg/mg dry saliva) were significantly lower than in normal group (8.3 μg/mg dry saliva).

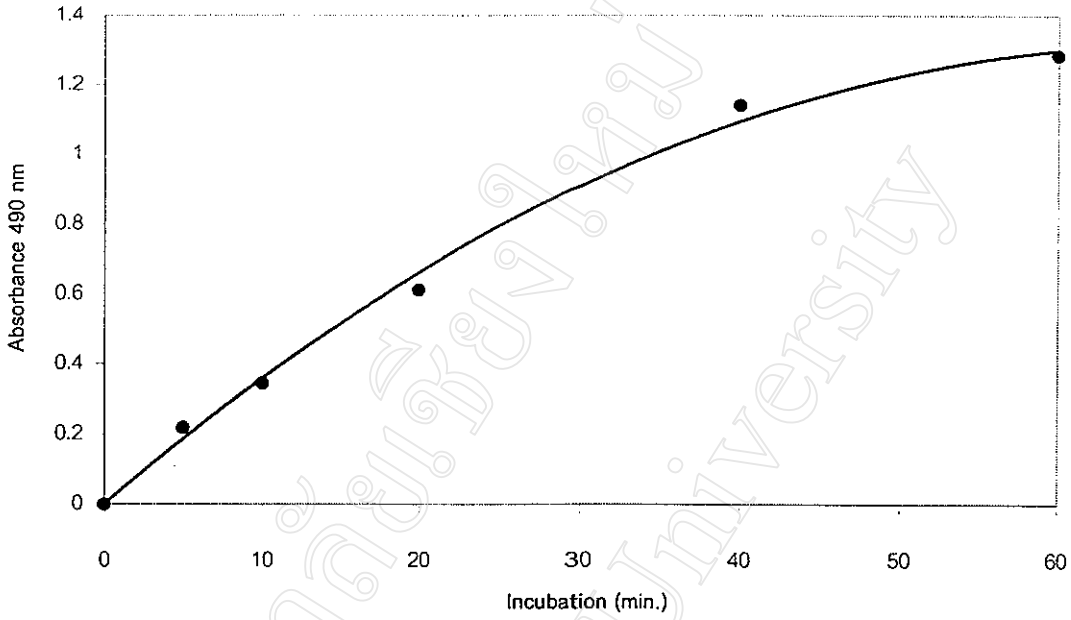


Figure 3.3 The optimum incubation time of papain.

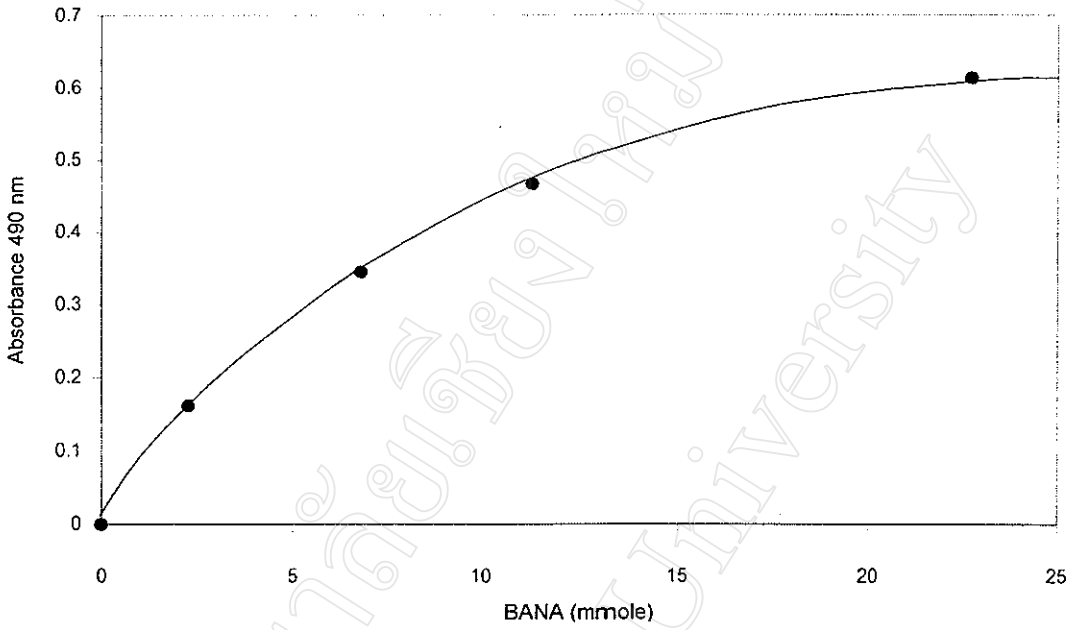


Figure 3.4 Standard curve for papain assay by using specific substrate (BANA).

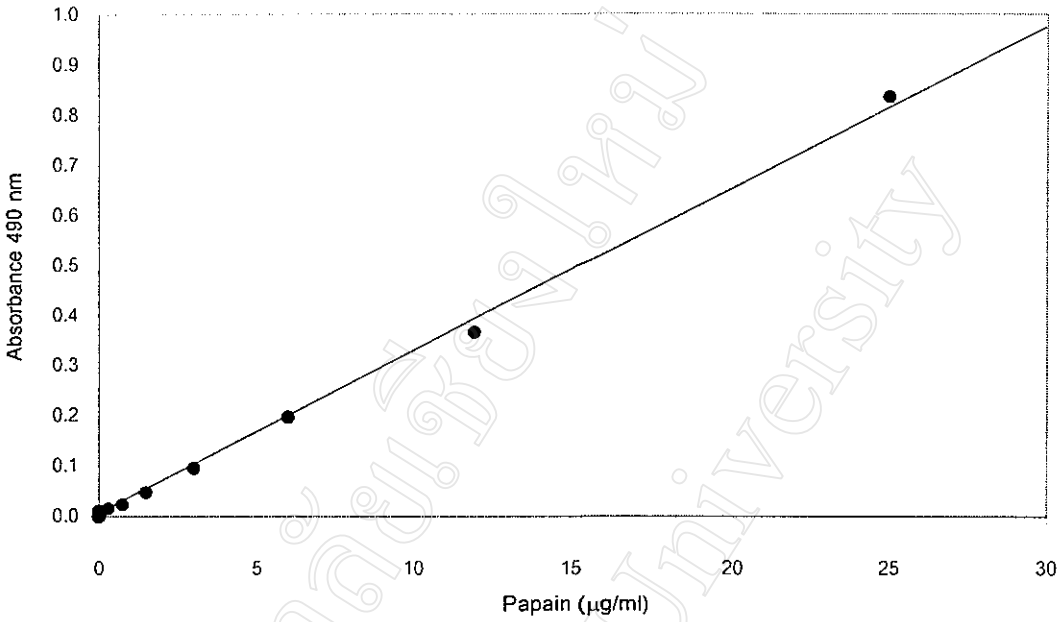


Figure 3.5 This graph demonstrated the standard curve of papain concentration.

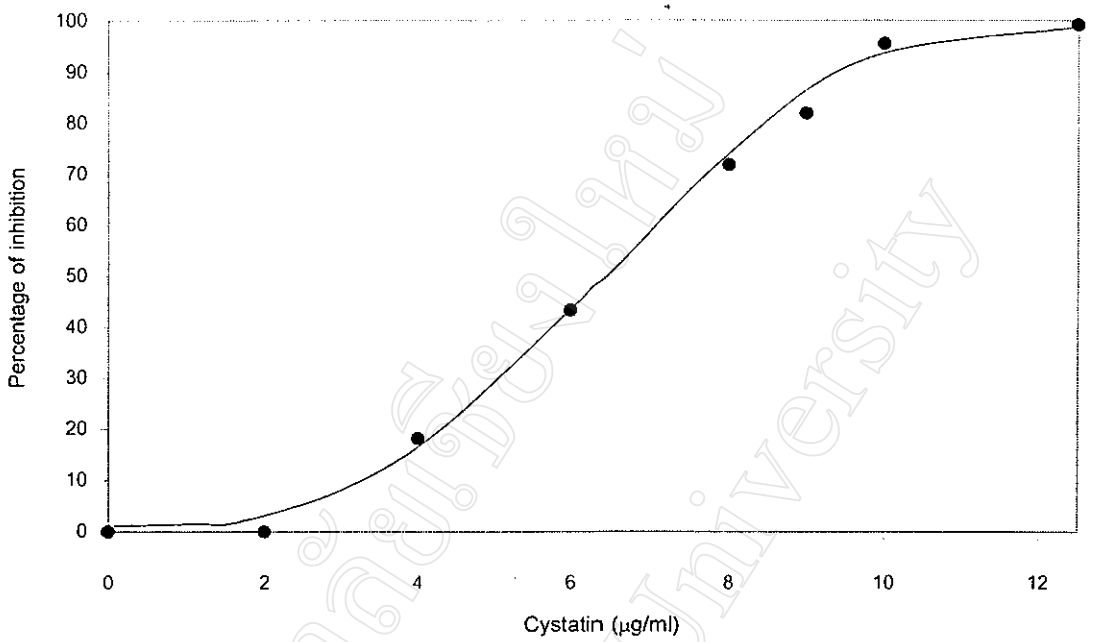


Figure 3.6 Percent inhibition of papain (150 µg) at different concentration of cystatin were illustrated obtained by spectrophotometric method.

3.5. Quantitation of Elastase and Its Inhibitor

HPLC was used to detect the activity of elastase and its inhibitor on the basis of substrate specificity (N-Succinyl-Ala-Ala-Val-nitroanilide; SAAVNA) by separating the product p-nitroaniline (NA) of substrate cleavage. Figure 3.7 showed the separation of NA on a single reverse phase column under our condition, and NA was separated within 12 min. The retention time of NA peak was averaged 4.83 minute. The standard curve of elastase activity was shown in Figure 3.8 while NA concentration was detected. The linearity of curve has been achieved in the range of 0-100 μg/ml. Analysis of elastase specific activity in saliva samples showed that there was a statistically significant difference ($P < 0.001$) among three groups (Table 3.4). The median of periodontitis group was higher (3.6 μg/mg protein) than the normal (0.6 μg/mg protein) and gingivitis group (0.4 μg/mg protein).

On the other hand, when PMSF was added to the incubation, NA decreased. The standard curve of inhibition of elastase activity was shown in Figure 3.9. From the percentages of inhibition in saliva were analyzed, the elastase inhibitor showed that there was not statistically significant difference among the three groups ($P = 0.056$).

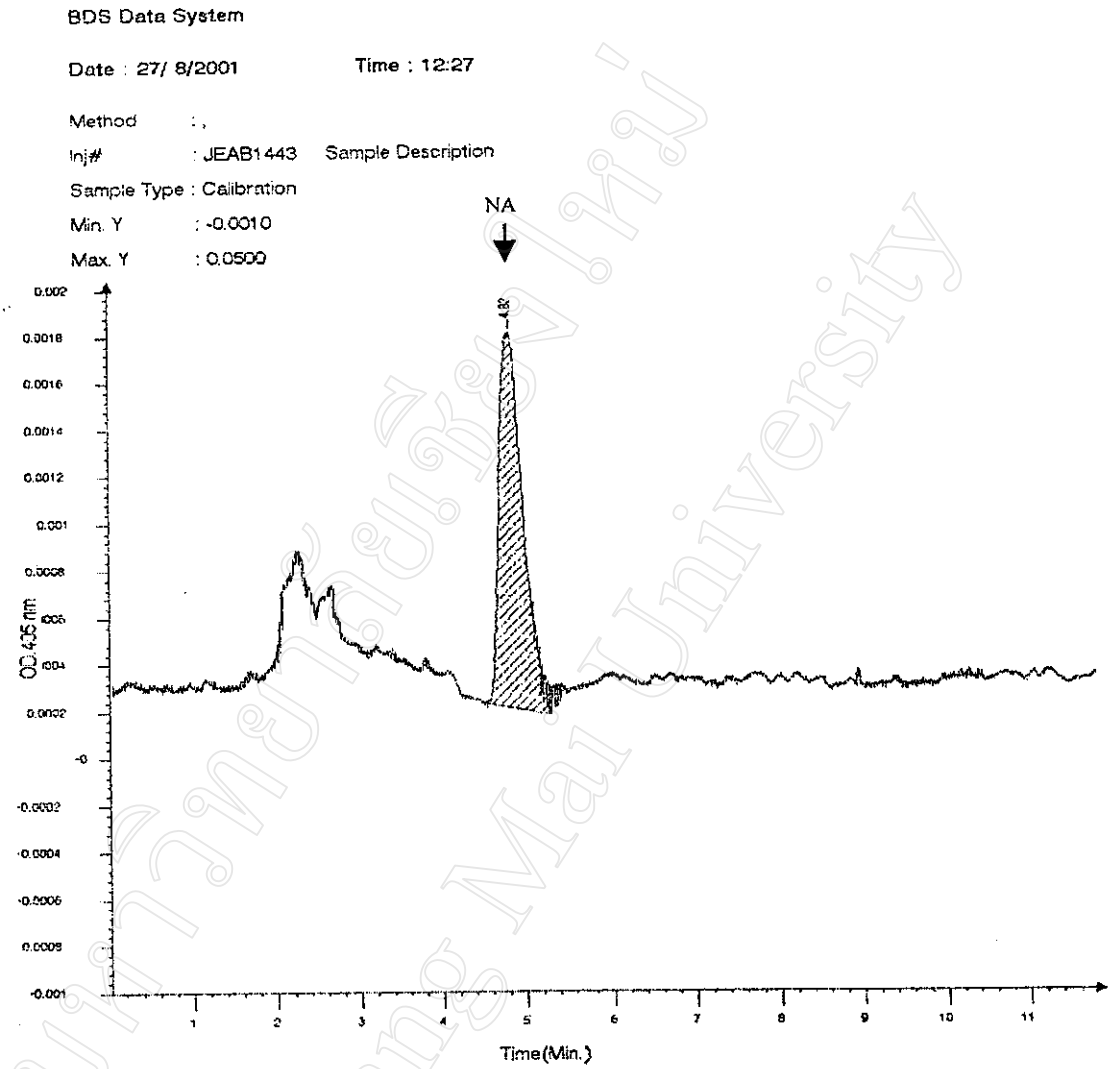


Figure 3.7 Chromatograms of enzymatic digests of N-Succinyl-Ala-Ala-Val-nitroanilide by elastase. 25 µl of 1mM SAAVNA was incubated with 50 µl of elastase at incubation and the solution was analyzed by HPLC (NA = *p*–nitroaniline).

Figure 3.8 Standard curve of elastase activity at different concentrations by HPLC method.

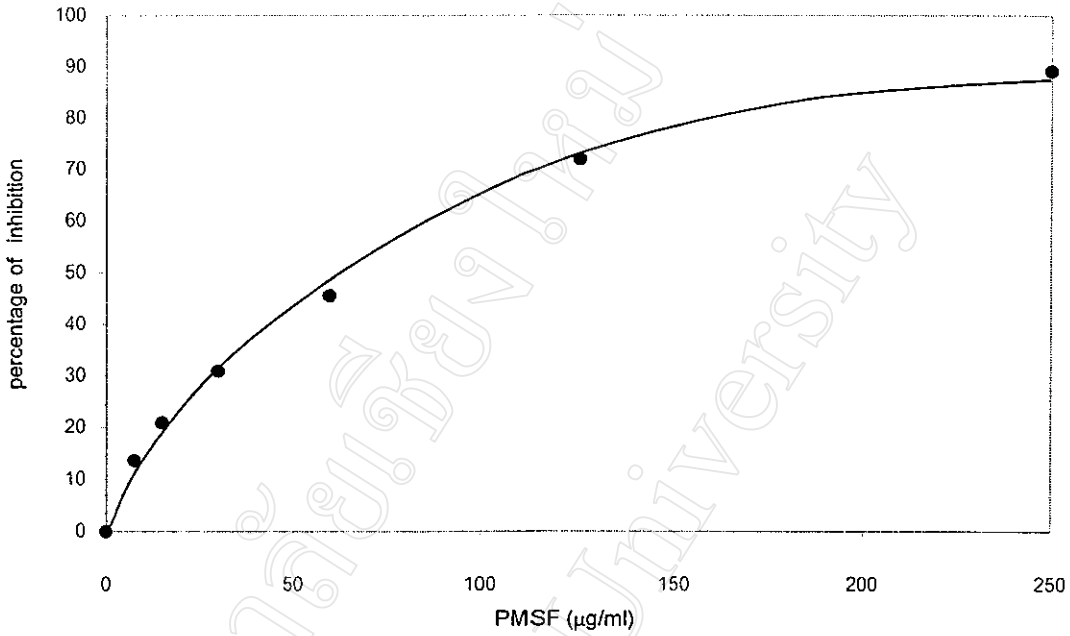


Figure 3.9 Percent inhibition of elastase activity at different concentration of PMSF by HPLC method.

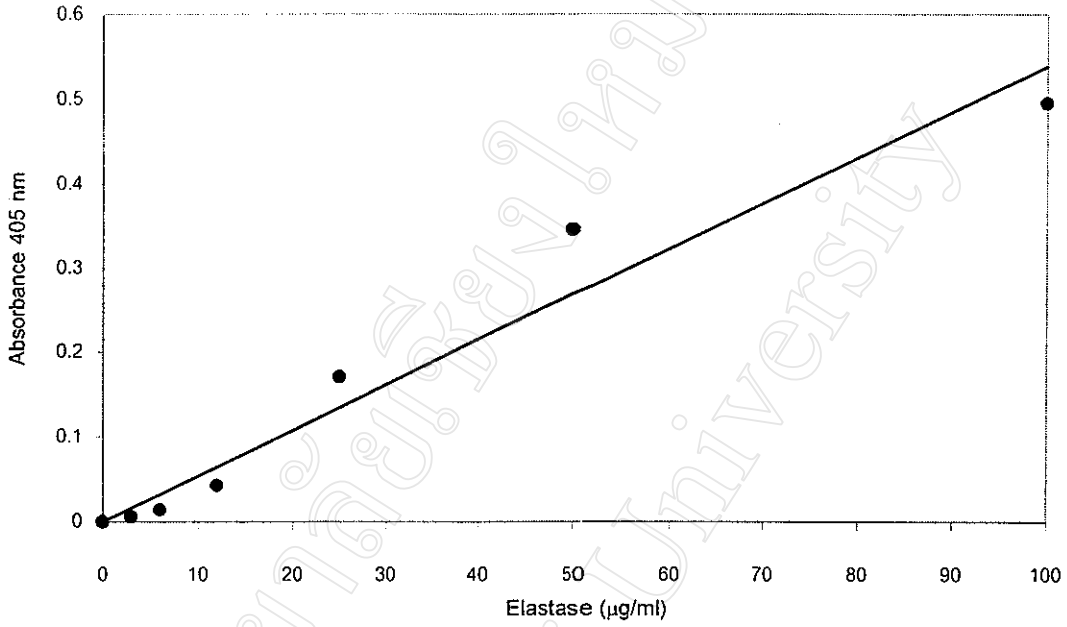


Figure 3.10 Standard curve of elastase activity at different concentration by spectrophotometric method.

3.6. Comparison of HPLC and Spectrophotometric Determination of Elastase Activity

For saliva, the spectrophotometric method was used to determine concentration of elastase. There was a statistically significant difference ($P < 0.001$) of the median values among three groups (Table 3.3), and there was a significant difference between their activity values as obtained using the HPLC and spectrophotometric methods (t – test, $P < 0.001$). Because in the spectrophotometric method may have interference from compounds which have spectra similar to *p*-nitroaniline, and the retention time was averaged 2.5 minute. So that, using HPLC was selected to detect the elastase and its inhibitor in this study.

Table 3.3 The median values of HPLC and spectrophotometric determination of elastase activity in saliva samples were demonstrated in comparison.

	Median of elastase (μg/ml)	
	HPLC	Spectrophotometry
Normal (n=20)	0.88	2.96
Gingivitis (n=20)	0.59	3.10
Periodontitis (n=20)	7.05	37.87

Table 3.4 Composition of enzymes and enzyme inhibitors in saliva.

	Median (mean ±SD)		
	Normal (n=40)	Gingivitis (n=37)	Periodontitis (n=40)
Total proteinase activity (μg/ml)	225.0 (335.5±258.1)	100.0 (190.5±265.1)	900.0 (1746.6±2690.8)
(μg /mg protein)	156.0 (210.2±166.7)	60.0 (123.1±178.4)	346.3[a] (407.0±379.8)
Total proteinase inhibitor (μg/ml saliva)	154.9 (196.5±218.4)	28.2 (239.0±268.1)	10.4 (211.6±267.0)
(μg/mg dry saliva)	29.6 (43.1±42.2)	5.4 (45.2±52.4)	4.9[b] (40.1±45.5)
Cysteine proteinase activity (μg/ml)	2.4 (4.9±4.6)	6.5 (6.6±2.7)	7.9 (7.1±3.5)
(μg /mg protein)	2.0 (2.8±2.5)	3.5 (3.9±1.9)	2.9 (3.7±2.1)
Total cystatin (μg/ml saliva)	53.1 (51.0±28.2)	4.2 (4.4±1.9)	6.5 (6.5±2.0)
(μg /mg dry saliva)	8.3 (9.0±5.1)	0.7[b] (0.8±0.3)	1.0[b] (1.0±0.3)
Elastase activity (μg/ml)	0.9 (1.1±0.8)	0.6 (1.2±1.4)	7.1[b] (9.8±7.3)
(μg /mg protein)	0.6 (0.7±0.4)	0.4 (0.9±1.3)	3.6[b] (4.4±3.3)
Elastase inhibitor (μg/ml saliva)	20.3 (31.3±33.8)	44.0 (51.4±31.2)	43.4 (46.6±39.7)
(μg /mg dry saliva)	4.1 (3.7±2.6)	5.1 (5.7±2.7)	4.6 (4.6±2.4)

[a] significantly different (p<0.05) from the gingivitis group
[b] significantly different (p<0.05) from the normal group
elastase was taken by HPLC

3.7. The Electrophoresis of Whole Saliva

In order to confirm the enzyme level from salivary analysis, the sample were prepared and separated by gel electrophoresis. 50 μg of proteins in three groups (normal, gingivitis and periodontitis groups) was analyzed for 5 cases of each group.

Visual inspection of the gels showed that the whole saliva could be separated into three zones: Zone A, 215-45 KDa; Zone B, 45-20 KDa; and Zone C, < 20 KDa. The highest concentration of proteins was in Zone A. The trypsin (MW 25.0 kDa), elastase (MW 25.9 kDa), and papain (MW 23.4 kDa) concentration was in Zone B. Analysis found that the elastase level detected in gel electrophoresis and showed the same results by HPLC. Analysis of the periodontitis samples (Figure 3.11 C) found that the elastase levels in gel were higher than the normal (Figure 3.11 A) and the gingivitis samples (Figure 3.11 B).

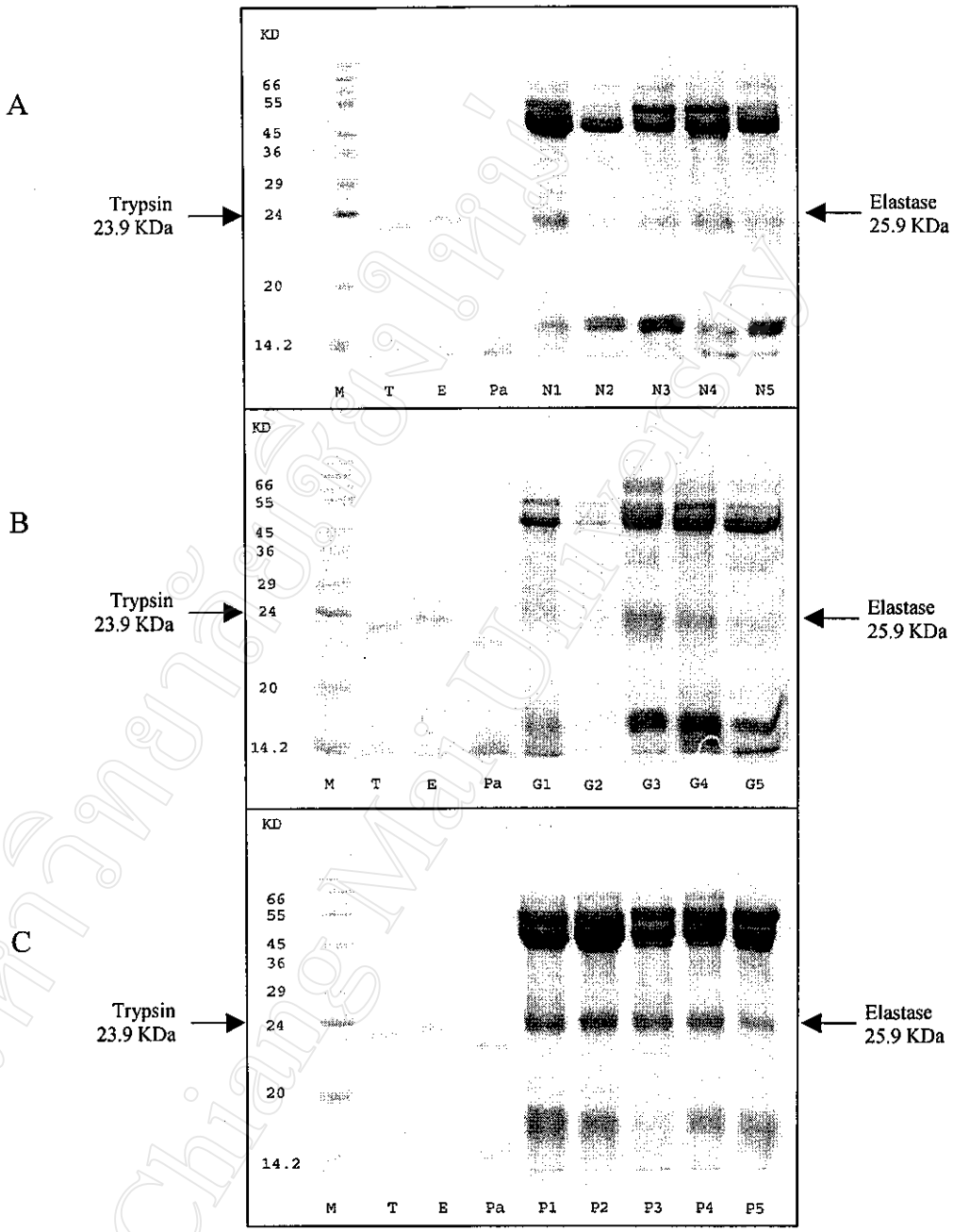


Figure 3.11 The pattern of saliva from different subjects was analyzed the level of elastase by SDS-PAGE (M = molecular weight marker; T = trypsin 10 μg; E = elastase 10μg; Pa = papain 10 μg; N = 50 μg protein of sample of saliva from normal subjects (Figure 3.11A) G = 50 μg protein of samples of saliva from gingivitis subjects (Figure 3.11B); P = 50 μg protein of sample of saliva from Periodontitis subjects (Figure 3.11C).

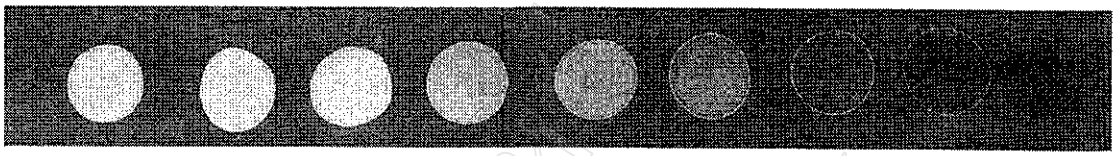
3.8. Trypsin Inhibitory Activity in Various Species of Thai-herbs

The gelatinolysis method was the test that used gelatin as a substrate for determining trypsin activity of individual extracts. The trypsin activity was represented by the detection of clear zones of gelatin when 50 μl of the mixtures were applied to the gelatin film, the release of enzyme and its activity could be visualized directly. Dilutions of trypsin (from 3 μg to 0.25 μg) were used to study its effect on the gelatin substrate on x-ray film. As show in Figure 3.12, a clear zone was observed at the site of application after the film was washed with running water. In contrast, trypsin (2 μg) mixed soybean trypsin inhibitor (from 3 μg to 0.125 μg) and a buffer control did not exhibit a clear zone, as shown in Figure 3.13. The exhibition zones were plotted against the concentration of soybean trypsin inhibitor (Figure 3.13), and calculated as a 50 percent of inhibition (10 μ g/ml)

The extractions of trypsin inhibitor from Thai-herbs were tested for their inhibitors by using the gelatinolysis method. The different concentration of extracted dilution was mixed with trypsin (2 μg) and was applied on the gelatin films. The films were incubated in incubator at 42°C for 1 hour. The inhibitory activity of each trypsin inhibitor was demonstrated by the decrease of clear zone as shown in Figure 3.14.

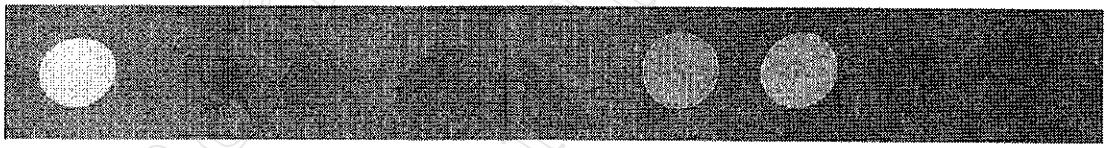
The trypsin inhibitory activities of all extract of trypsin inhibitors were calculated as a 50 percent of inhibition and as mg per g. dry weight in Table 3.5.

In the same way, extracts of various concentrations from Thai-herbs were tested with elastase (5 μg) or papain (150 μg) for inhibition by using the gelatinolysis method. The concentration of PMSF (60 μg/ml) and cystatin (6 μg/ml) were calculated as a 50 percent of inhibition. The inhibitory activities were shown in Table 3.5.



(μg trypsin)	3	2	1.5	1.0	0.75	0.50	0.375	0.250	0

Figure 3.12 The clear zone on the gelatin coating X-ray film showed the activity of various concentrations of trypsin diluted in 0.1 Tris-HCl buffer, pH 7.5. Each circle was applied a 50 μl onto a film and incubated at 42°C for 1 hour.



(μg trypsin)	2	2	2	2	2	2	2	-	-
(μg trypsin inhibitor)	-	4	2	1	0.5	0.25	0.125	0.125	-

Figure 3.13 The gelatin coating X-ray film demonstrated the inhibition effect of soybean extract solution when applied with trypsin. The minimum effective dilution of this inhibitor was showed between 0.5-0.25 μg where the clear zone started to reappear.

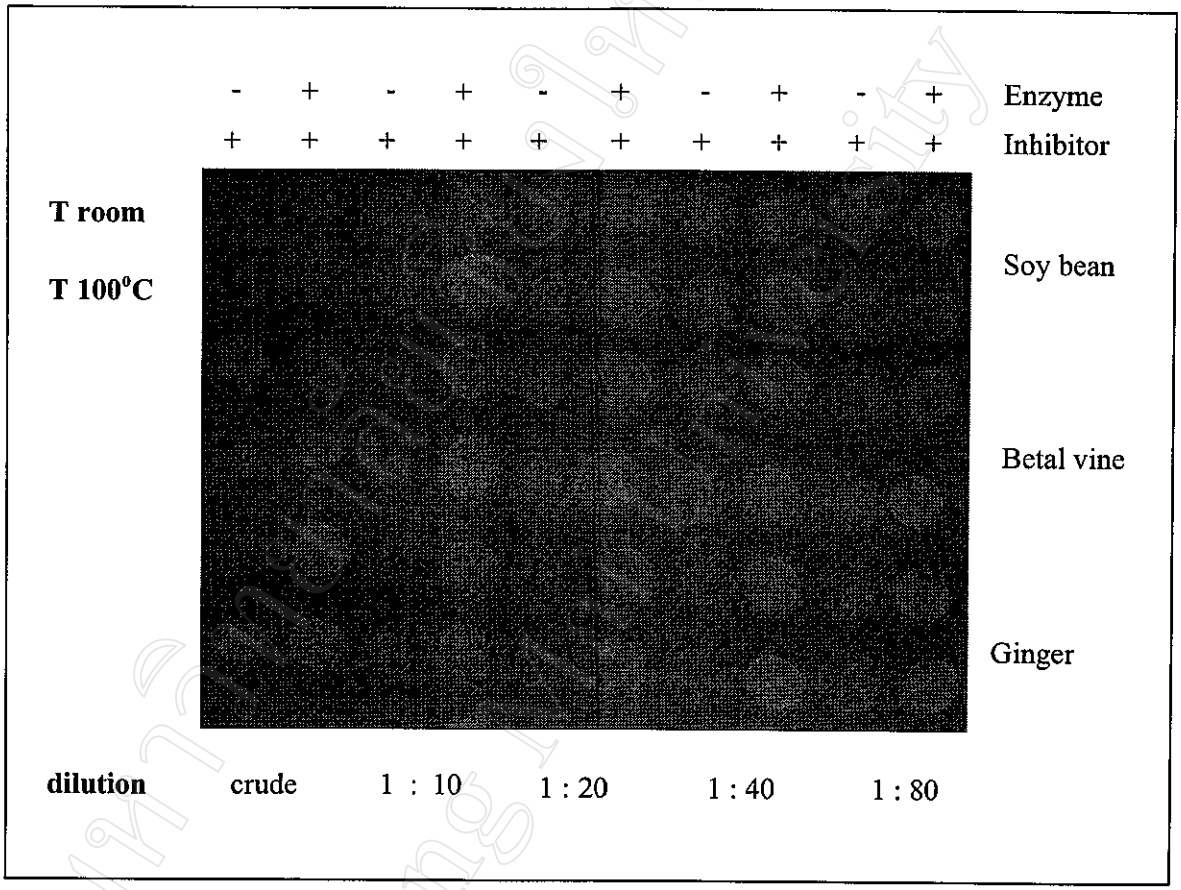


Figure 3.14 The clear zone on this gelatin film indicated the activity of inhibitor of carious dilutions of Thai herb extracts.

Table 3.5 Proteinase inhibitor of Thai herb extracts by unprocessed x-ray film method.

No.	Common name	Thai name	Botanical name	Enzyme inhibitor (mg/g dry weight)		
				Trypsin	Elastase	Papain
1	Betel vine	ใบพลู	Piper bettle Linn	NF	6.0	0.6
2	Guava leave	ใบฝรั่ง	Psidium guajva Linn	1.0	6.0	4.8
3	Black Tea	ชาดำ	Camellia sinesis	1.0	3.0	20.0
4	Green Tea	ชาเขียว		1.0	3.0	20.0
5	Female Salaid Pangporn	เสลดพังพอนตัวเมีย	Barleria lupulinalind	0.5	NF	0.6
6	Male Salaid pangporn	เสลดพังพอนตัวผู้		NF	1.5	NF
7	Phar talay jone	ฟ้าทะลายโจร	Andrographis panioulata nees	NF	NF	0.6
8	Lin choer	เห็ดหลินจือ		1.0	1.5	NF
9	Ulticaceae	ข่อย	Streblus asper lour	NF	1.5	NF
10	Garlic	กระเทียม	Allium sativum linn	0.5	3	0.6
11	Licorice	ชะเอม	Albizzia myriophylla Benth	1.0	1.5	0.6
12	Gingen	ขิง	Zingiber officinal Rose	NF	NF	NF
13	Lemongrass	ตะไคร้	Cymbopogon citratus stapf	NF	NF	NF
14	Greater Galomgal	ข่า	Alpinia galanga Swartz	0.5	1.5	0.3
15	Turmeric	ขมิ้นชัน	Curcuma Longer Linn	0.5	NF	1.2
16	Ceylon Cinnamon	อบเชยเทศ		1.0	3.0	1.2
17	Curry powder	ผงกระหรี่		1.0	1.5	0.6
18	Prao Hoam	เปราะหอม	Kaempferia galanga Linn	NF	1.5	0.3
19	Betel Nut	หมาก	Areca caechn Linn	9.6	1.5	20.0
20	Emblic myrabolan	มะขามป้อม	Phyllomthus emblica Linn	1.0	200.0	9.6
21	chun paad gleeb	จันทร์แปดกลีบ		1.0	6.0	0.6
22	Pepper vine	พริกไทย	Piper nigrum Linn	NF	3.0	0.3
23	Small egg plant	มะเขือแจ้เครือ	Solanum sanitwongsei Praib	NF	NF	NF
24	Nutmeg	จันทร์เทศ	Myristiea fragrans Hoult	NF	1.5	NF
25	Black paper	พริกไทดำ	Piper nigrum Linn	NF	NF	0.6
26	Fennel	ยี่หร่า		NF	1.5	NF
27	Siam cardamon	กระวาน	Amomon Krervanti Pierre	NF	NF	NF
28	Coriander seed	เมล็ดผักชี	Coriadrum sutivan Linn	1.0	1.5	NF
29	Pumpkin seed	เมล็ดฟักทอง	Cucurbita maring duch	1.0	NF	0.15
30	Indian mustard	เมล็ดผักกาด	Brassieg juncea Coss	1.0	1.5	0.6
31	Clove	ดอกกานพลู	Eugenia caryophyllata thunb	1.0	3.0	9.6
32	Soybean seed	เมล็ดถั่วเหลือง	Pheselous Limensis	9.6	NF	4.8

Data from assay with gelatin substrate by unprocessed x-ray film method.
NF = not found
The inhibitors were calculated as a 50% inhibition.

3.9. Determination of Total Proteinase Inhibitory Activity in Thai-herbs Extracts

The total proteinase inhibitory in Thai-herbs were found in twenty-eight, out of thirty-two species (see table 3.5).

The levels of trypsin inhibitory in the present study were varied from 0.5 to 9.6 mg/g dry weight. Thirteen species of Thai herbs, including leaves seeds and its stem showed trypsin inhibitors. Trypsin inhibitor in Betel nut and soybean showed higher than other species.

The levels of elastase inhibitory were varied from 1.5 to 200.0 mg/g dry weight. Twenty-two species of Thai herbs showed elastase inhibitory which emblic myrabolan had the highest inhibitor.

The levels of papain inhibitors were varied forms 0.6 to 20.0 mg/g dry weight. Twenty-two species of Thai herbs showed papain inhibitory. Three species showed higher inhibitors than other species i.e. black tea, green tea and betel nut.

It was noted that betel nut contained the highest trypsin and papain inhibitors. Emblic myrobolan had the highest elastase inhibitor.

3.10. Inhibitors with Synthesis Substrate

The crude extracts of Thai-herbs were determined for their trypsin inhibitor by using BApNA as a specific substrate. Thai-herbs were extracted with distilled water and heated at 100°C for 2 hours. The homogenates were centrifuged at 24,000 g for 30 min, and the supernatant fractions were tested. Ten kinds of selected extracts which had higher inhibitory activity were tested at various concentrations with 0.1 M Tris HCl buffer, pH 7.5. The concentrations of trypsin were tested for its substrate (BApNA). The trypsin activity was represented by the p-nitroaniline that was splited by trypsin. The different concentration of extracted dilution was mixed with trypsin 20 μg and preincubated at 37°C for 10 min. Remaining activities of trypsin were determined at 37°C for 20 min with respective BApNA (33 μg). The concentration of trypsin inhibitory activities (800 μg/ml) were calculated into 50 percent inhibition and as a mg per g dry weight. In addition, the crude extracts of Thai-herbs were determined for their papain and elastase inhibitory activity by using BANA, SAAVNA as a specific substrate, respectively. The elastase activity was taken by HPLC method. The concentration of cystatin (6.0 μg/ml) and PMSF (60.0 μg/ml) were calculated into 50 percent inhibition. The effect of enzyme inhibitors extracted from the selected Thai-herbs (mg/g dry weight) were shown in Figure 3.15, 3.16, 3.17 and Table 3.6. The concentration of Thai-herbs at 50%inhibition were shown in Table 3.7.The relative inhibitors were arranged in the patterns at room temperature.

The trypsin inhibitors: Black tea> Betel nut> Nut meg> Guave leave> Emblic myrobolan> Clove> Betel vine> Salaid pandporn> Garden pea > Pumpink seed.

The papain inhibitors: Betel nut> Nut meg> Black tea> Salaid pandporn> Guave leave> Betel vine> Clove> Garden pea> Emblic myrobolan >Pumpink seed.

The elastase inhibitors: Betel nut> Black tea> Guave leave> Salaid pandporn > Nut meg> Betel vine> Clove>Emblic myrobolan > Pumpink seed> Garden pea.

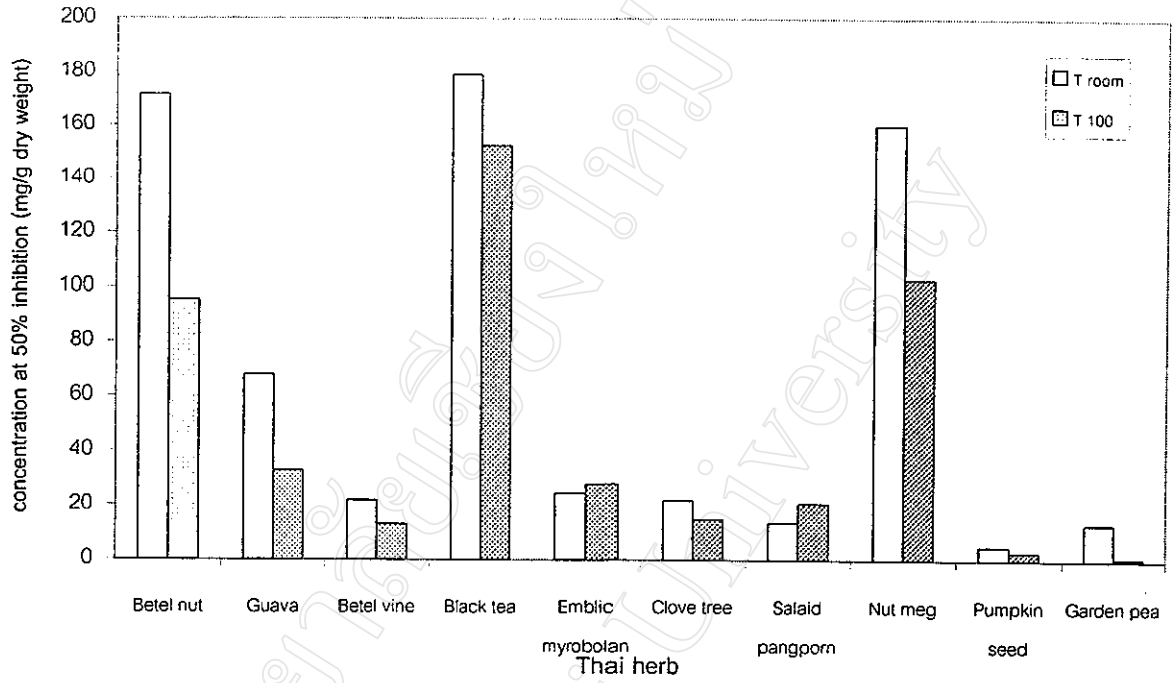


Figure 3.15 The effect of trypsin inhibitor extracted from the selected Thai-herbs (mg/g dry weight) on BApNA by spectrophotometric method was compared at room temperature and 100 °C.

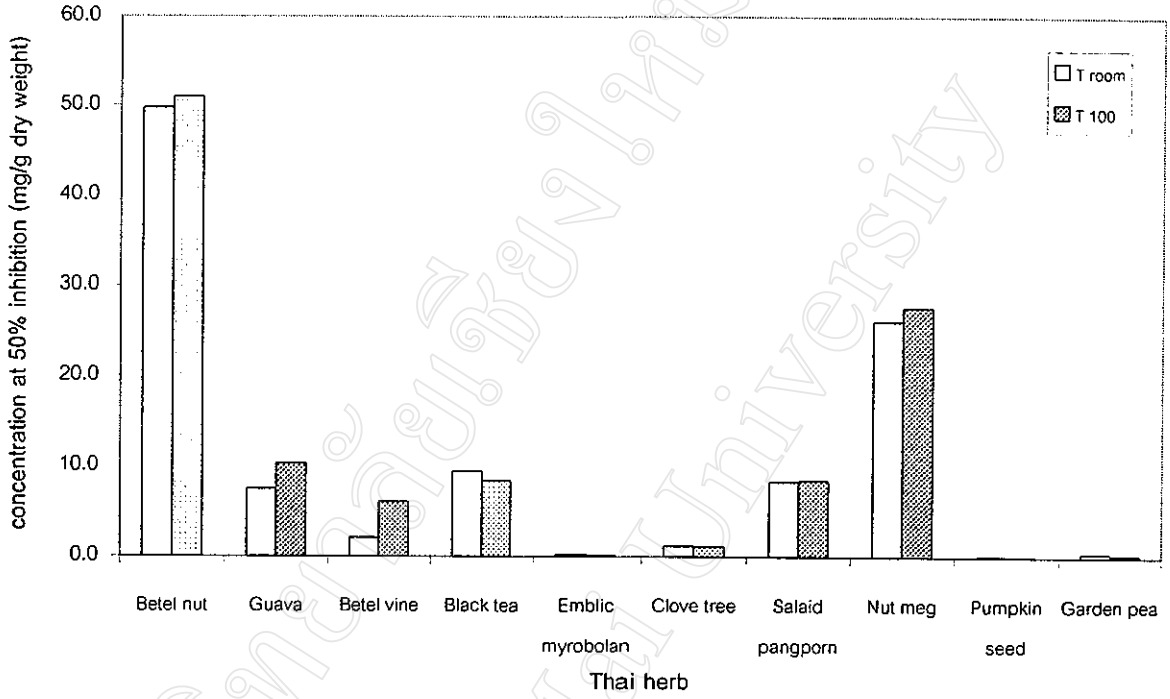


Figure 3.16 The effect of papain inhibitor extracted from the selected Thai-herbs (mg/g dry weight) on BANA by spectrophotometric method was compared at room temperature and 100 °C.

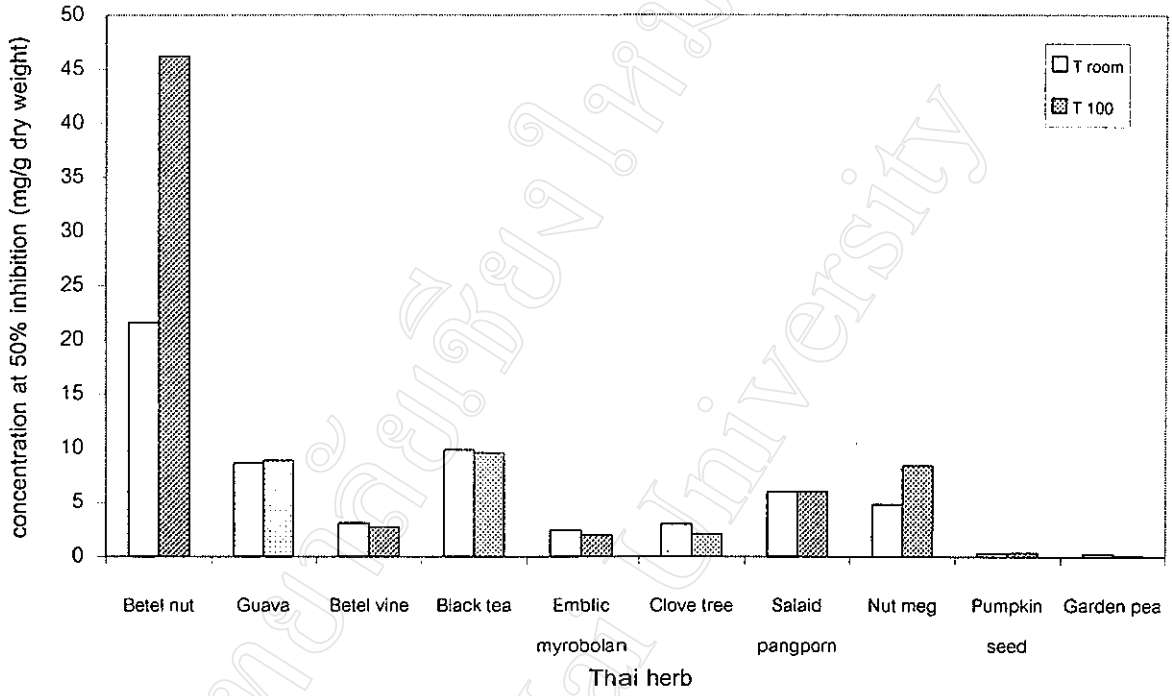


Figure 3.17 The effect of elastase inhibitor extracted from the selected Thai herbs (mg/g dry weight) on SAAVNA by HLPC method was compared at room temperature and 100 °C.

Table 3.6 Proteinase inhibitors (mg/g dry weight) of Thai herb extracts by spectrophotometry and HPLC.

	Enzyme inhibition (mg/g dry weight)					
	Trypsin		Papain		Elastase	
	T room	T 100^{0}C	T room	T 100^{0}C	T room	T 100^{0}C
Betel nut	171.43	95.24	49.69	50.92	21.60	46.20
Guava leave	67.81	32.76	7.46	10.29	8.64	8.88
Betel vine	21.71	12.95	2.06	6.06	3.12	2.76
Black tea	179.05	152.38	9.43	8.38	9.84	9.60
Emblic myrobolan	24.38	27.81	0.26	0.19	2.46	1.98
Clove	21.71	14.86	1.18	1.12	3.00	2.10
Salaid pangporn	13.71	20.57	8.28	8.44	6.00	6.00
Nut meg	160.00	102.86	26.11	27.60	4.80	8.40
Pumpkin seed	4.99	3.01	0.07	0.03	0.30	0.35
Garden pea	13.18	0.80	0.39	0.26	0.28	0.12

Data from assay with synthetic substrate by spectophotometry and HPLC.
The inhibitors were calculated as 50% inhibition

Table 3.7 Proteinase inhibitors (mg/ml) of Thai herb extracts by spectrophotometry and HPLC.

	Enzyme inhibitors (mg/ml)					
	Trypsin		Papain		Elastase	
	T room	T 100^{0}C	T room	T 100^{0}C	T room	T 100^{0}C
Betel nut	4.67	8.40	0.12	0.12	2.78	1.30
Guava	11.80	24.42	0.80	0.58	6.94	6.76
Betel vine	36.84	61.76	2.91	0.99	19.23	21.74
Black tea	4.47	5.25	0.64	0.72	6.10	6.25
Emblic myrobolan	32.81	28.77	23.53	32.25	24.39	30.30
Clove	36.84	53.85	5.09	5.37	20.00	28.57
Salaid pangporn	58.33	38.89	0.72	0.71	10.00	10.00
Nut meg	5.00	7.78	0.23	0.22	12.50	7.14
Pumpkin seed	160.31	265.82	82.77	176.15	200.00	169.49
Garden pea	60.69	995.26	15.47	23.53	217.39	500.00

Data from assay with synthetic substrate by spectophotometric and HPLC method.

The inhibitors were calculated as 50% inhibition

3.11. Determination of Enzyme Inhibitors in Periodontitis Saliva with the Highest Inhibitor of Thai herbs

The periodontitis saliva samples were tested with extract of betel nut, black tea and clove, which were prepared for the IC 50 dilution. Preincubation mixture with each enzyme before the substrate was added. The result of median values of enzyme inhibitors (μg /mg dry saliva) showed that total proteinase inhibitor periodontitis saliva with adding betel nut (13.36) or black tea (38.85) or clove (24.20) and cysteine protease inhibitor (cystatin) periodontitis saliva with adding betel nut (1.04) or black tea (1.02) or clove (0.99) were not statistically significant difference in periodontitis saliva (41.9, 0.98) (p = 0.875). In contrast, the elastase inhibitor periodontitis saliva with adding betel nut (13.43) or black tea (16.91) or clove (18.89) was statistically significant difference in periodontitis saliva (7.50) (p = 0.019). (Figure 3.18 and Table 3.8)

Examination by the SDS-PAGE gel indicated that the increase adding of tannin, which were common compounds of Thai herbs could be observed the change of molecular weight of elastase. So, they could be interacting with elastase activity as shown in Figure 3.19.

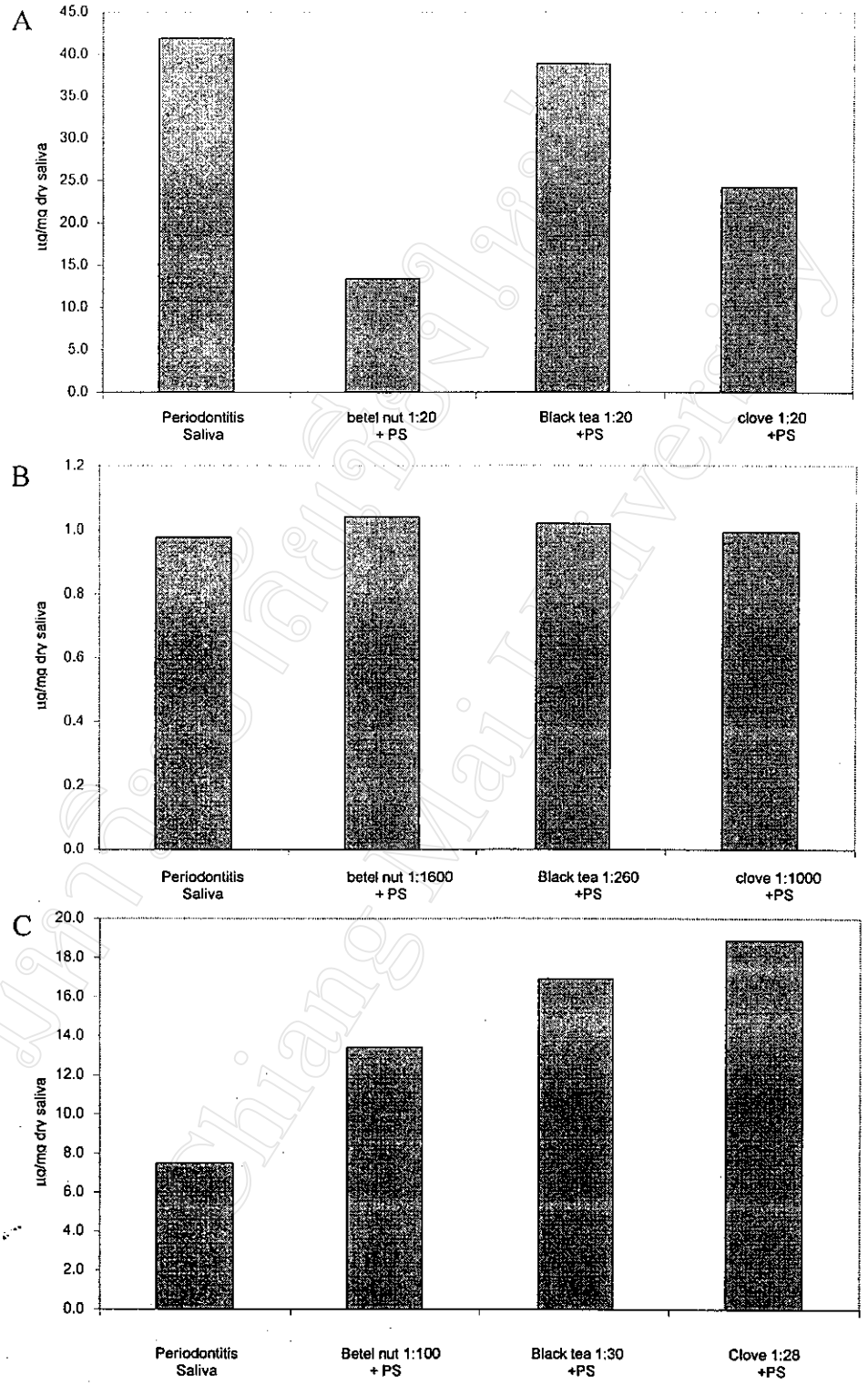


Figure 3.18 The enzyme inhibitors; total proteinase inhibitor (A), Cystatin (B) and elastase inhibitor (C), in periodontitis saliva and incubated with betel nut, black tea or clove; PS = periodontitis saliva.

Table 3.8 Enzyme Inhibitors of Periodontitis saliva with and without adding Thai herb extracts.

	Enzyme inhibitors (μg /mg dry saliva)		
	Total proteinase inhibitor	Cystatin	Elastase inhibitor
Periodontitis saliva (n=20)	41.90 (37.15 ± 26.99)	0.98 (1.05 ± 0.36)	7.50 (7.48 ± 2.59)
Periodontitis saliva + Betel nut (n=20)	13.36 (12.91 ± 6.06)	1.04 (1.08 ± 0.37)	13.43[a] (13.83 ± 5.88)
Periodontitis saliva + Black tea (n=20)	38.85 (40.15 ± 24.39)	1.02 (1.04 ± 0.31)	16.91[a] (17.98 ± 10.67)
Periodontitis saliva + Clove (n=20)	24.20 (26.11 ± 15.99)	0.99 (1.02 ± 0.30)	18.89[a] (17.41 ± 7.24)

[a] significantly different ($P<0.05$) from the periodontitis saliva without adding Thai herb extracts.
The inhibitors were calculated as 50% inhibition.

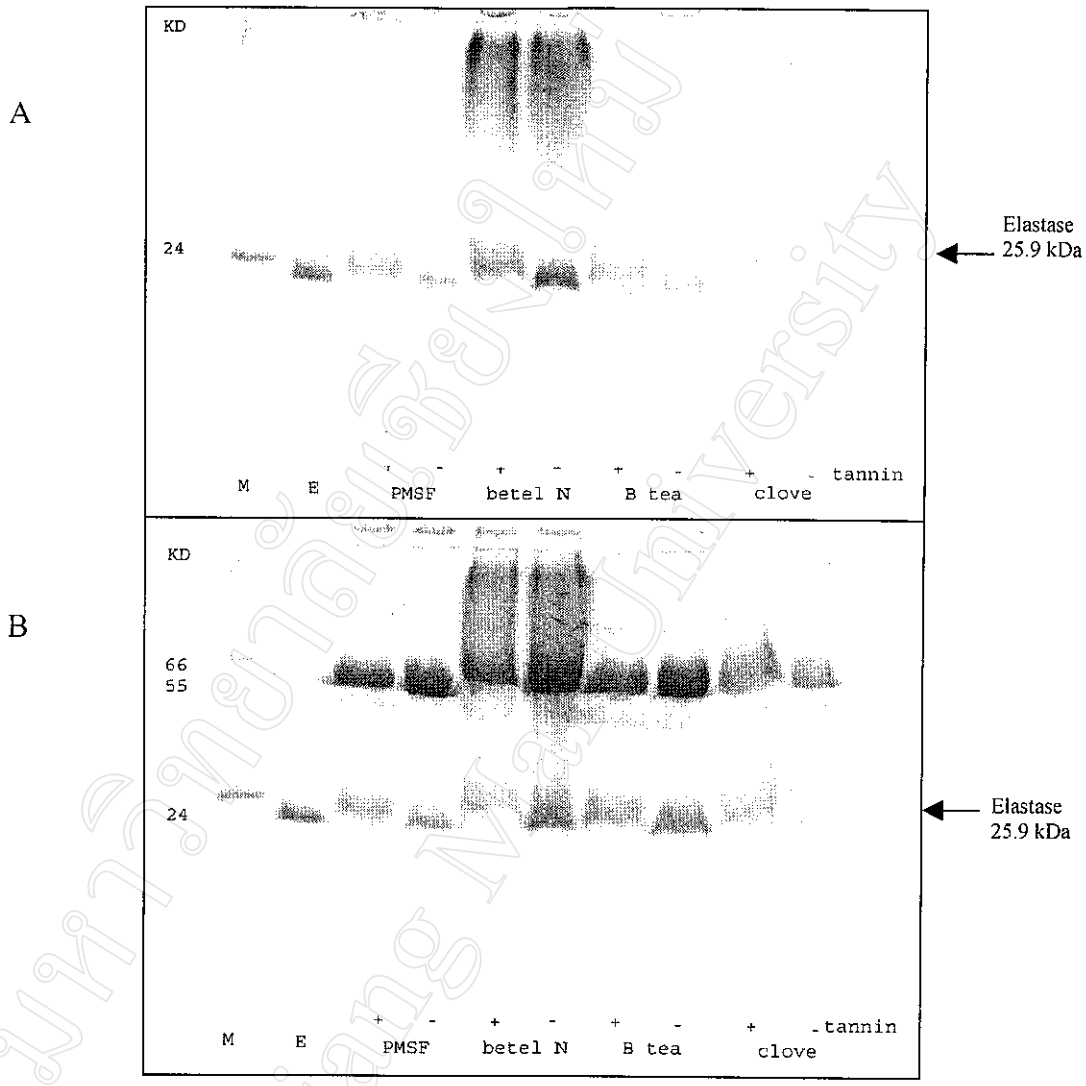


Figure 3.19 The SDS-PAGE gel of elastase (10 μg) activity was preincubated with Thai herbs (1mg), or PMSF with and without adding tannin (100μg) in the periodontitis saliva (50 μg) before incubated with SAAVNA (2.5 μg) at 37^0 C for 120 min (M = molecular weight marker; E = elastase activity); boiled periodontitis saliva (A), unboiled periodontitis saliva (B)