CHAPTER 3

RESULTS AND DISCUSSION

The results and discussion of this study is divided into 6 parts as the followings:

Part 1: Development of the proper elastic niosomal formulations loaded with the model drug (calcitonin)

1.1 Effects of niosomal concentrations and various dispersants on physical characteristics of elastic niosomes loaded with calcitonin

All niosomal dispersions were in translucent colloidal appearances with no sedimentation or layer separation. The vesicular sizes of calcitonin loaded niosomes were in the range of 138-292 nm. Niosomes loaded with calcitonin and dispersed in distilled water showed larger vesicular size and less negative zeta potential values than the blank niosomes. This may be due to the positive charge of calcitonin at pH 7.0 that can interact with the negative polarity of the niosomal membrane (from the hydroxyl group of cholesterol in the niosomal composition) resulting in less net negative of the system. However, the zeta potential values of niosomes loaded with calcitonin and dispersed in phosphate buffer (pH 7.0) remained unchanged (no significant difference at $p<0.05$). The phosphate buffer may contribute to the counter ions that balance the charges of the system. The elasticity (DI values) of various niosomal formulations was summarized in **Tables 14-15**. The DI values of the elastic niosomal membranes were higher than the non-elastic niosomes of 1.04 to 4.19 folds. Non-elastic niosomes also

showed some deformability owing to the influence of the surfactant (Tween 61) compositions. Tween 61 may accommodate at the high stress sites within the vesicles resulting in forming a highly curved area of the vesicles (Williams, 2003). The high curvature of the membrane surface gave high elastic bending energy according to the Hooke's Law (Du et al., 2006), thereby a more deformability of the vesicular membrane was obtained.

Table 14 Effects of various niosomal concentrations on vesicular deformability index

Formula no.	Non-elastic niosomes		20%v/v ethanol elastic niosomes	
	Blank	Loaded with calcitonin	Blank	Loaded with calcitonin
1	4.04±0.35	0.77±0.17	4.61±1.88	1.70±0.97
2	3.19±0.39	0.71±0.26	3.33±0.73	1.46±0.85
3	2.20±0.20	0.23±0.02	2.53±0.05	0.83±0.20

Note : For formula nos. 1-3, please see Table 13.

Table 15 Effects of dispersant types on vesicular deformability index

Formula no.	Non-elastic niosomes		20%v/v ethanol elastic niosomes	
	Distilled water	Phosphate buffer	Distilled water	Phosphate buffer
4	0.77±0.17	4.04±0.35	1.70±0.97	4.61±1.88
5	0.71±0.26	3.19±0.39	1.46±0.85	3.33±0.73
6	0.23±0.02	2.20±0.20	0.83±0.20	2.53±0.05

Note : For formula nos. 4-6, please see Table 13.

For the effects of various concentrations of niosomes (Tween 61 mixed with cholesterol) on vesicle elasticity in distilled water and phosphate buffer, the decrease concentrations (from 20, 10 to 5 mM) demonstrated the increase of vesicular elasticity. At the lowest concentration of the niosomes (5 mM), the packing properties of the surfactant (Tween 61) depend on the balance between the optimal cross-sectional surface area of the head groups (a_0), the volume of the hydrocarbon chains (v) and the maximum hydrocarbon chain length (l_c). According to the geometrical rule, the value of V/a_0l_c at more than 0.5 gives the possibility to form bilayer vesicles (Engberts et al., 1996). So, the lowest concentration at 5 mM of the niosomes was used. The existing of the bilayer vesicle at this concentration was confirmed by the TEM images. Moreover, the formulation of bilayer vesicles at this low concentration of the total lipid was also reported (Zhigaltsev et al., 2005). Vesicles formed from low concentration of Tween 61 mixed with cholesterol gave a decrease packing density of the surfactant molecules. This will lead to a decrease in the aggregation numbers and an increase in flexible spacer length (Cao et al., 2009). The formation of smaller vesicles with thinner membrane assembly was obtained (Gao et al., 2006). Therefore, vesicles formed from low concentration of Tween 61 mixed with cholesterol can certainly have larger degree of motion and give the more flexible vesicles.

1.2 Effects of dispersants on the maximum loading and entrapment efficiency of calcitonin in elastic niosomes

The maximum loading and entrapment efficiencies of calcitonin in niosomes by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was shown in **Figure 33**. All niosomal formulations which dispersed in phosphate buffer (at pH 7.0) gave the maximum loading of calcitonin at 0.5 mg/ml, whereas those dispersed in distilled water was at 0.2 mg/ml. Entrapment efficiency of calcitonin loaded in elastic (containing ethanol) and non-elastic niosomes was summarized in **Table 16**. The entrapment efficiencies of calcitonin in niosomes decreased as the concentration of calcitonin increased. When the amounts of the vesicles were fixed, the osmotic pressure difference between the outer and inner vesicular membranes depended on only the calcitonin concentrations. The high calcitonin concentration might cause some damage to the formed vesicles and make it easier for the drug to escape from the inner phase (Peltonen et al., 2004). The higher entrapment efficiency of calcitonin was observed in 20 mM phosphate buffer (pH 7.0) than in distilled water. The ionic strength in the buffer system may increase the ionic interaction between calcitonin and the vesicular membranes (Alsarra et al., 2004). Calcitonin in the buffer system can interact with the vesicular membranes more than in the distilled water system. The higher entrapment efficiency of calcitonin in elastic than non-elastic niosomal formulation was also observed owing to the aqueous solubility enhancement of calcitonin by ethanol that may facilitate the entrapment of calcitonin in the aqueous phase of the elastic niosomes.

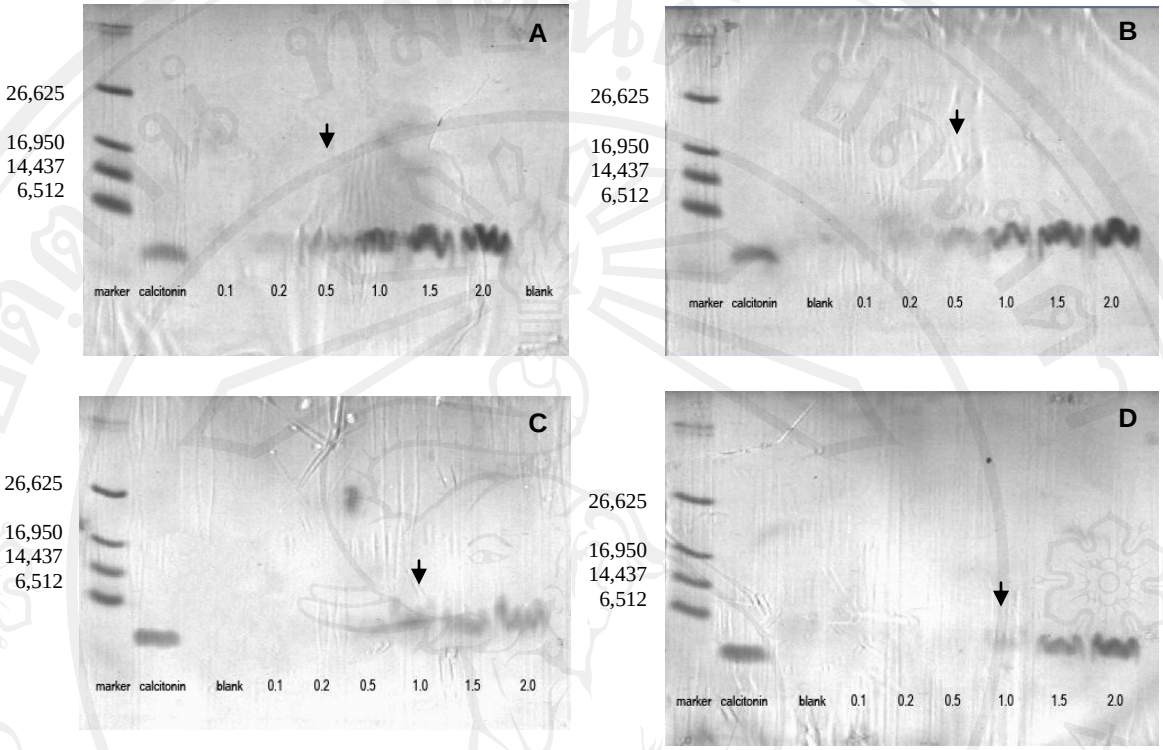


Figure 33 The maximum loading and entrapment efficiencies of calcitonin in niosomes by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); (A) Elastic niosomes loaded with various calcitonin concentrations (0.1-2.0 mg/ml) dispersed in distilled water, (B) Non-elastic niosomes loaded with various calcitonin concentrations (0.1-2.0 mg/ml) dispersed in distilled water, (C) Elastic niosomes loaded with various calcitonin concentrations (0.1-2.0 mg/ml) dispersed in 20 mM phosphate buffer (pH 7.0), (D) Non-elastic niosomes loaded with various calcitonin concentrations (0.1-2.0 mg/ml) dispersed in 20 mM phosphate buffer (pH 7.0); The arrow indicated the maximum loading of calcitonin loaded in niosomes in which the small amount of the free calcitonin band was first seen in the gel.

Table 16 Entrapment efficiency of calcitonin loaded in ethanol elastic and non-elastic niosomes

Concentrations of calcitonin	Distilled water		20 mM phosphate buffer (pH 7.0)	
	20%v/v ethanol elastic niosomes	non-elastic niosomes	20%v/v ethanol elastic niosomes	non-elastic niosomes
0.1	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.2	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
0.5	86.50±6.24	48.97±6.32	100.00±0.00	100.00±0.00
1.0	74.21±2.01	43.14±4.23	85.93±5.86	72.47±9.84
1.5	59.20±10.48	37.15±9.15	67.97±3.87	60.66±3.54
2.0	55.02±6.74	34.00±4.34	65.38±3.77	52.68±5.37

1.3 Effects of phosphate buffer concentrations on physical characteristics of elastic niosomes

The vesicular sizes did not depend on the phosphate buffer concentrations. When the phosphate buffer concentrations increased (5, 10, 20 and 30 mM), the negative zeta potential values were decreased (data not shown). Buffer at high concentration can result in a strong compression of the electric diffusion layer. The elasticity of the elastic niosomes loaded with calcitonin dispersed in 20 mM phosphate buffer (pH 7.0) was higher than those prepared in distilled water of 2.71, 2.28 and 3.05 folds for the niosomal concentration at 5, 10 and 20 mM, respectively **(Table 17)**. The ionic strength of the buffer system may affect the vesicle elasticity, as the decrease of the phosphate buffer

(pH 7.0) concentrations from 30, 20, 10 to 5 mM, the increase of vesicle elasticity was observed. Buffer at higher concentration may have more influence on the charge interactions between the vesicular membrane than at lower concentration. It has been indicated that at low ionic strength, the Debye length is large and the electrostatic repulsion between the membranes becomes effective (Claessens et al., 2007). This will lead to the decrease in the packing density of the surfactant molecules in the vesicles and the molecular assemblies with a large curvature according to the Hooke's Law as previously mentioned. Thus, niosomes at 5 mM dispersed in 5 mM phosphate buffer was selected for further study because of its high entrapment efficiency and vesicular elasticity.

Table 17 Effects of various concentrations of phosphate buffer on vesicular deformability index

Formula no.	Non-elastic niosomes		20%v/v ethanol elastic niosomes	
	Blank	Loaded with calcitonin	Blank	Loaded with calcitonin
7	0.99±0.28	6.45±2.36	4.15±2.04	6.79±2.03
8	1.03±0.04	4.65±0.42	1.60±0.40	6.23±1.13
9	1.00±0.57	4.35±1.90	0.45±0.09	5.52±2.36
10	1.71±0.40	6.91±1.55	3.88±2.20	4.58±1.74

Note : For formula nos. 7-10, please see Table 13.

1.4 Comparison of physical characteristics and cytotoxicity of elastic niosomes containing ethanol or the edge activators (NaC and NaDC)

1.4.1 Physical characteristics of elastic niosomes

Table 18 showed the vesicular sizes, zeta potential values and deformability index of various blank non-elastic and elastic niosomal formulations. The vesicular sizes of all ethanol, NaC and NaDC elastic niosomes were in the range of 108.30 nm to 126.33 μm and zeta potential values at -17.88 to -45.10 mv. Ethanol elastic niosomes showed larger vesicle sizes, but lower zeta potential values than the NaC and NaDC elastic niosomes. Higher ethanol concentrations gave larger vesicular sizes. This was in concordance with the previous investigation (Verma et al., 2004). The average size was decreased at low ethanol concentration (0-3.3%), whereas an increase in vesicular sizes was observed at high ethanol concentrations (3.3-30%). The decreased niosomal sizes may be due to the effect of the edge activator at high concentration that reduce the interfacial tension between the vesicular membrane leading to the decrease in the bilayer thickness (Liu et al., 2007a). However, when the concentrations of NaC and NaDC increased, not much influence on the zeta potential values was observed. Most NaC and NaDC elastic niosomes gave more negative zeta potential values (-39.92 to -45.10 mv) than the non-elastic niosomes (-38.62 mv) and ethanol elastic niosomes (-17.88 to -27.40 mv). This may be due to the anionic property of NaC and NaDC. The slight differences of the zeta potential values between NaC and NaDC niosomes might be from the different functional groups in their structures **(Figure. 17)**. NaC has one hydroxyl group more than NaDC which may affect zeta potential values (Lee et al., 2005a). Niosomes

Table 18 Physical characteristics and deformability index (DI) of various blank non-elastic and elastic niosomal formulations

Niosomal formulations	Vesicular size (nm)	Zeta potential (mv)	Deformability index (DI)
Non-elastic niosomes	120.53±9.64	-28.62±2.48	7.28±1.53
NaC elastic niosomes			
0.25% NaC	132.13±1.50	-39.92±1.48	11.32±1.29
0.5% NaC	129.00±5.46	-33.62±2.95	10.33±0.64
2.5% NaC	111.83±9.73	-34.68±0.56	9.75±3.46
5% NaC	108.30±1.15	-32.78±1.80	13.19±6.62
10% NaC	113.43±4.39	-38.24±1.38	2.19±2.14
NaDC elastic niosomes			
0.25% NaDC	123.30±9.42	-45.10±1.65	4.62±2.41
0.5% NaDC	137.27±5.95	-40.96±1.73	26.32±2.41
2.5% NaDC	129.83±11.42	-42.76±1.62	36.42±0.51
5% NaDC	126.33±2.85	-43.06±1.66	26.60±7.13
10% NaDC	115.73±5.85	-44.32±2.24	1.03±0.63
Ethanol elastic niosomes			
10% ethanol	181.03±10.81	-27.40±1.32	7.23±1.97
20% ethanol	231.33±11.77	-25.34±1.16	7.45±2.54
25% ethanol	298.97±16.61	-19.66±0.79	5.44±0.88
30% ethanol	1260.33±221.08	-17.88±0.99	4.17±0.69

Note : NaC = sodium cholate and NaDC = sodium deoxycholate

containing ethanol gave the zeta potential values less than those without ethanol. The zeta potential values decreased when the ethanol contents increased. This may imply the effect of ethanol on the reduction of the electrostatic repulsive energy of the vesicular surface (Liu et al., 2000). In general, the system which shows zeta potential values more than ±30 mV is normally considered stable. The ethanol elastic niosomes gave the zeta potential values of less negative than -30 mV. So, this system was probably not stable.

The vesicular sizes and zeta potential values of niosomes loaded with calcitonin were demonstrated in **Table 19**. The size of niosomes increased when loaded with calcitonin. The vesicular sizes of niosomes loaded with calcitonin were 150.98, 143.01 and 138.53 nm for non-elastic, NaC and NaDC elastic niosomes, respectively. Ethanol elastic niosomes loaded with calcitonin gave smaller size (194.69 nm) than the blank ethanol elastic niosomes, but larger than the NaC and NaDC elastic niosomes loaded with calcitonin. NaC and NaDC might be suitable to prepare the elastic niosomes loaded with calcitonin because they gave the smaller niosomes than ethanol.

The morphology of the niosomes was in the spherical shape observed under optical microscope. From negative-staining electron micrographs **(Figure 34)**, both non-elastic and elastic niosomes were in unilamellar structure with the vesicular size of less than 300 nm. All elastic niosomes appeared to have higher deformability structure than the non-elastic niosomes. The TEM of some elastic niosomal vesicles which had the elongated and self adaptable shape may be from the conformation change of the niosomal membrane affected by the neighbor niosomal vesicles (Haas et al., 1997). However, the non-elastic niosomes exhibited spherical shape. Perhaps, ethanol in elastic niosomes

Table 19 Physical characteristics and deformability index (DI) of the selected non-elastic and elastic niosomes loaded with calcitonin in comparing to their corresponding blank niosomes

Niosomal formulations loaded with calcitonin	Vesicular size (nm)		Zeta potential (mv)		Deformability index (DI)	
	Blank	Loaded with calcitonin	Blank	Loaded with calcitonin	Blank	Loaded with calcitonin
Non-elastic niosomes	120.53±9.64	150.98±4.23	-38.62±2.48	-27.92±4.23	7.28±1.53	1.60±0.62
5% NaC	108.30±1.15	143.01±5.27	-32.78±1.80	-31.15±4.53	13.19±6.62	21.59±0.91
2.5% NaDC	129.83±11.42	138.53±3.11	-42.76±1.62	-33.65±0.50	36.42±0.51	12.48±5.26
20% ethanol	231.33±11.77	194.69±41.49	-25.34±1.16	-18.98±1.70	7.45±2.54	5.78±4.14

Note : NaC = sodium cholate and NaDC = sodium deoxycholate

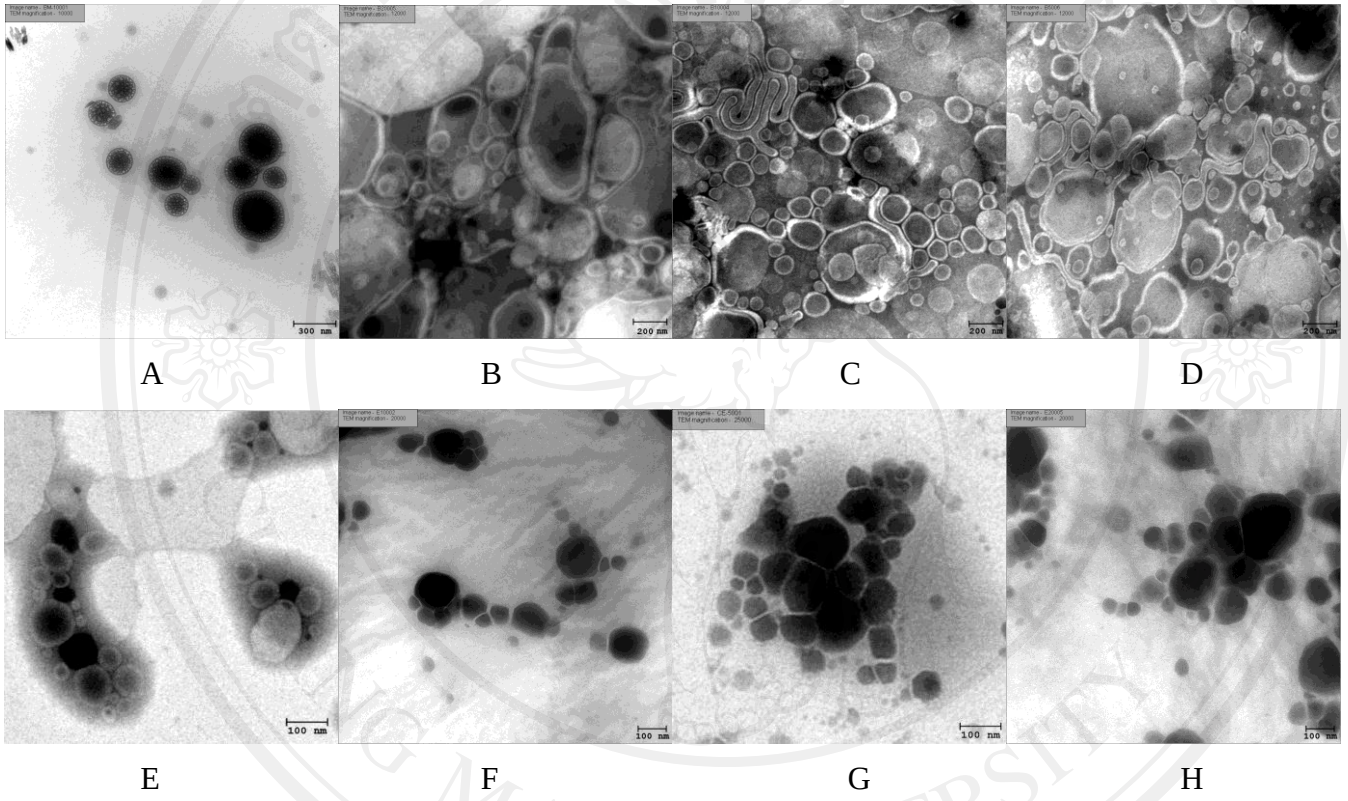


Figure 34 Negative-staining TEM images of the niosomal formulations (20000X) composed of Tween 61/cholesterol (1:1) dispersed in 5 mM phosphate buffer pH 7.0 (A), (B), (C) and (D) were blank non-elastic, 20%v/v ethanol elastic, 5% mole NaC elastic and 2.5% mole NaDC elastic niosomes, respectively, while (E), (F), (G) and (H) were non-elastic, 20%v/v ethanol elastic, 5% mole NaC elastic and 2.5% mole NaDC elastic niosomes loaded with calcitonin (0.22 mg/ml), respectively.

provided elasticity to the vesicular membrane by reducing the interfacial tension. The cholate molecules have a very strong tendency to increase the curvature of the niosomal membranes (Thurmond et al., 1991). Thus, these two cholates (NaC and NaDC) may have the elasticity effect on the spontaneous curvature and bending rigidity of the niosomal membranes.

1.4.2 Deformability index of elastic niosomes

The elasticity of NaC and NaDC niosomal membrane did not depend on the concentrations of the edge activators. However, the elasticity of these bilosomes was more than the ethanol elastic niosomes. Besides the interfacial tension reduction effects, the head groups of these two bile salts also intercalated between the surfactant molecules, producing the less packing density with more flexibility of the surfactant molecules. Thus, NaC and NaDC provided both interfacial and head group region mechanisms, whereas ethanol gave only the elasticity to the vesicular membrane by reducing the interfacial tension (Jain et al., 2007). In fact, previous syudy has also found that surfactants such as NaC and NaDC may interact with the lipid vesicles and change the conformation by fitting their lipophilic parts between the hydrocarbon chains, compensating the bulkiness of the head groups (Maghraby et al., 2000).

The blank elastic niosomes composed of 2.5% NaDC (36.42±0.51), 5% NaC (13.19±6.62) and 20% ethanol (7.45±2.54) which gave the highest elasticity (DI) in each elastic niosomal group of 5, 1.8 and 1.02 folds higher than their corresponding non-elastic niosomes (7.28±1.53), were selected to loaded with calcitonin. The NaC elastic niosome loaded with calcitonin gave the elasticity of 1.64 folds higher than its blank

niosome. This can be explained that the hydrophilic nature of the loaded calcitonin may enhance the elasticity of the vesicle (Manosroi et al., 2009). On the contrary, the elasticity of the blank NaDC, ethanol elastic and non-elastic niosomes was more than their corresponding niosomes loaded with calcitonin of 2.92, 1.29 and 4.55 folds, respectively. The positive charge of calcitonin at pH 7.0 may interact with the negative charge of the niosomes (from the cholesterol compositions) resulting in the decrease of the electrostatic repulsion between the vesicular membranes. This also led to the increase in packing density of the surfactant molecules in the vesicles and the molecular assemblies with a small curvature according to the Hooke's Law. In DI determination [$DI = j\ (rv/rp)^2$], the large vesicle sizes and the rigid property of the non-elastic niosomes loaded with calcitonin (the vesicle sizes ranging from 120.53±9.64 to 150.98±4.23 nm) can affect the DI value during the extrusion process through the polycarbonate membrane filter. According to the DI equation, the pore size and the weight of the extruded niosomal dispersion were fixed. The DI values were thus only affected by the vesicular sizes after extrusion (rv). Since the non-elastic niosome loaded with calcitonin which was in large sizes with rigid structures, only its small vesicle sizes could be extruded through the membrane resulting the low rv and DI values.

1.4.3 Entrapment efficiency (%EE) of calcitonin in elastic niosomes

The non-elastic, ethanol (20%v/v) elastic, NaC (5% mole) elastic and NaDC (2.5% mole) elastic niosomes gave the entrapment efficiencies of calcitonin (0.22 mg/ml) at 50.97±3.91, 51.95±9.47, 60.11±4.98 and 56.51±7.47%, respectively. The NaC and NaDC elastic niosomes indicated higher entrapment efficiency of calcitonin than the

ethanol elastic niosomes. The more negative charge from the hydroxy group of the two bile salts can interact with calcitonin which has the positive charge (PI=9.47) in phosphate buffer (pH 7.0) better than ethanol. Hence, calcitonin can be both adsorbed on the niosomal membrane by the electrostatic interaction and loaded in the aqueous layers in the vesicles from its hydrophilic property. NaC and NaDC elastic niosomes showed no significant difference of calcitonin entrapment efficiency despite their different structures. This may be due to their same HLB values of 16.7, which have the same affinity for calcitonin (Zaafarany et al., 2010).

1.4.4 Cytotoxicity of elastic niosomes loaded with calcitonin

The percentages of human skin fibroblast viability treated with blank and loaded non-elastic and elastic (ethanol, NaC and NaDC) niosomes with calcitonin were shown in **Figure 35**. The cells incubated with the buffer only gave more than 80% viability, even the concentration of the buffer was more than 0.5 mM. However, when the cells were incubated with calcitonin in buffer solution, a significant decrease of cell viability of only 20% was observed ($p<0.05$). The positive charge (PI=9.47) of calcitonin in phosphate buffer (pH 7.0) may be toxic to cells (Gajjara et al., 1990). At 0.05 mM, the blank non-elastic and elastic niosomes showed no cytotoxicity in comparing to buffer (no significant difference $p<0.05$). However, when the concentrations of the blank non-elastic and elastic niosomes were increased from 0.05 to 0.5 mM, cell viability was decreased. This may be affect by the increase permeability, lysis and solubilization of the lipid-protein-detergent association from the exposure of the cells to the surfactants (Tween 61, NaC and NaDC) (Moreno, 2000). Cell viability treated with NaDC niosomes

was higher than that treated with NaC niosomes, but no significant difference (p<0.05). Since high charge density is also related to cytotoxicity (Kolhatkar et al., 2007), the cell viability of NaDC elastic niosomes which had high charge density based on the zeta potential values was expected to be lower than that of the NaC elastic niosomes. However, no significant cell viability between NaDC and NaC elastic niosomes was observed. This may be due to the more amount of NaC (5% mole) in the NaC elastic vesicles than NaDC (2.5% mole) in the NaDC elastic vesicles. At 0.125 mM of niosomes, the blank NaC and NaDC niosomes showed significant higher cell viability than the blank ethanol elastic niosomes. For the blank non-elastic niosomes, the 20%v/v ethanol, 5% mole NaC and 2.5% mole NaDC niosomes gave cell viability of 58.96±0.91, 48.71±7.26, 67.35±5.43 and 71.45±7.50 %, respectively. The lowest cell viability was found in ethanol elastic niosomes, since ethanol can damage the cells by inducing oxidative stress in mitochondria DNA and leads to an impaired function of mitochondria and inhibits the synthesis of protein encoded by mitochondrial DNA in cells (Hoek et al., 2002).

In addition, non-elastic and elastic niosomes loaded with calcitonin gave higher cell viability than their corresponding blank non-elastic and elastic niosomes and the calcitonin solution. At 0.5 mM, niosomes loaded with calcitonin gave more than 55% of cell viability. When calcitonin at 0.22 mg/ml was loaded in non-elastic, ethanol elastic, NaC elastic and NaDC elastic niosomes, cell viability was increased to 3.02, 2.93, 3.29 and 3.65 folds, respectively in comparing to their corresponding blank niosomes. The neutralization of the positive charge of calcitonin at pH 7.0 by the negative charge of

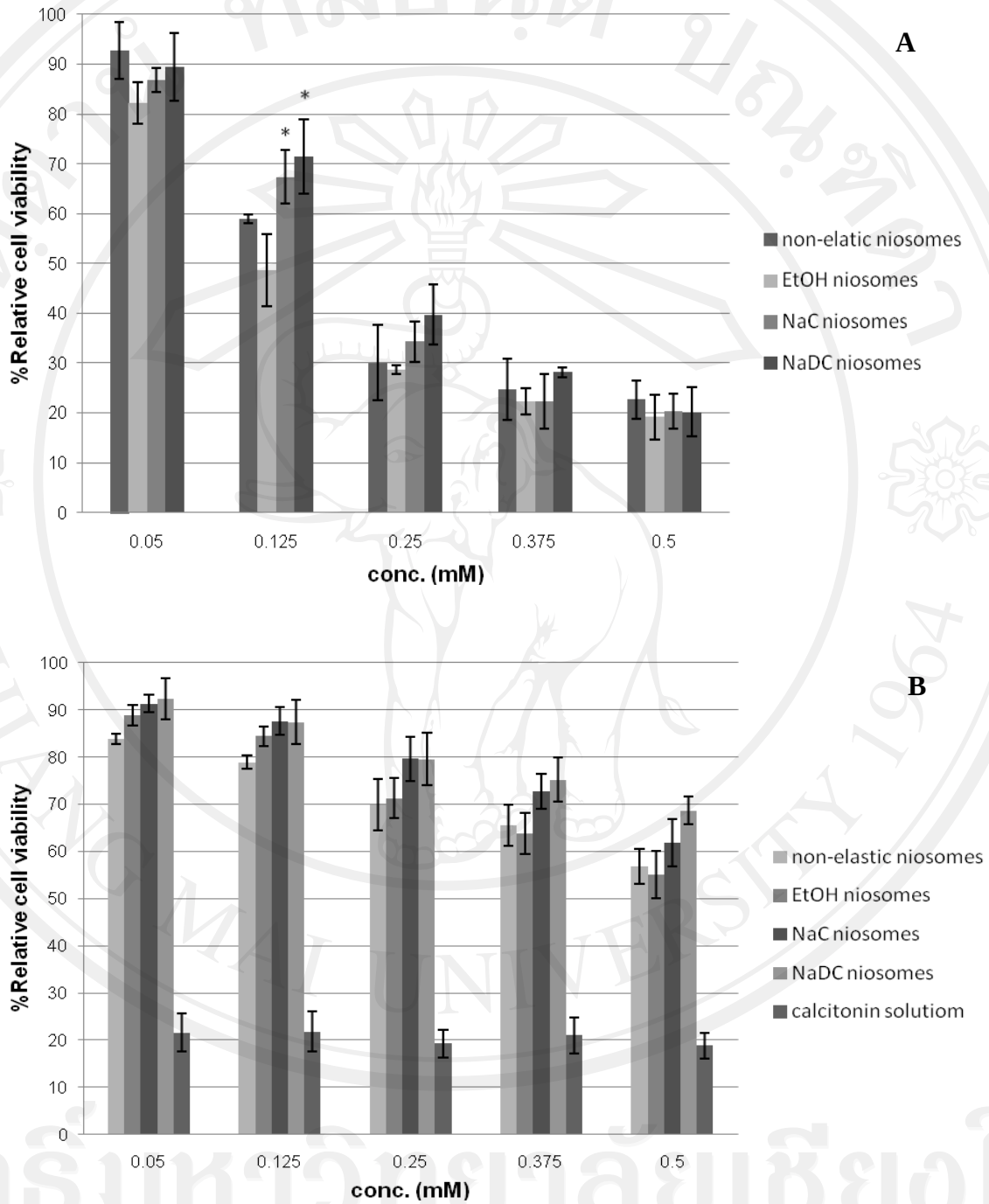


Figure 35 The percentages of human skin fibroblast viability by SRB assay of the blank niosomes (A) and calcitonin loaded in niosomes (B) (each value represents mean±S.D., n=3). * significant difference (p<0.05) in comparing to the blank ethanol niosomes

niosomes may result in charge density reduction, thereby decreasing of cytotoxicity. Moreover, when loaded with calcitonin, the negative zeta potential values and the cytotoxicity of the non-elastic and elastic niosomes decreased more than their blank niosomes. Thus, zeta potential values may relate to cell viability. The higher the zeta potential values of the niosomes, the stronger the interaction of the niosomal membrane with calcitonin can be expected. Furthermore, the decrease in cytotoxicity was also correlated with the binding process of cellular protein and the surfactant. The protein may act as a detoxifying agent on surfactant cytotoxicity (Benoit et al., 1987). In fact, the toxicity of niosomes is due to not just one, but many characteristics of the niosomal systems, such as surface charges, zeta potential values and protein binding process. The results from this study have suggested that the cytotoxicity of calcitonin can be reduced when loaded in elastic niosomes.

Part 2: Physical and chemical stability and transdermal absorption of elastic niosomes loaded with calcitonin

2.1 Characteristics of non-elastic and elastic niosomes loaded with calcitonin

The blank non-elastic and elastic niosomes were in translucent colloidal appearances, no sedimentation or layer separation with the vesicular size of about 55 nm. The size of the non-elastic and elastic niosomes increased (18-20 μm) when loaded with 2.0 mg/ml calcitonin. The non-elastic and elastic niosomes loaded with calcitonin showed flocculation, but were easy to redisperse to homogeneous dispersion by hand shaking. This may be from the low zeta potential values of niosomes, that the attraction

exceeds the repulsion thereby being flocculated. The non-elastic and elastic blank niosomes dispersed in 5 mM phosphate buffer, pH 7.0 exhibited negative zeta potential in the range of -25.8 to -27.5 mv. Niosomes loaded with calcitonin showed less negative zeta potential than the blank niosomes. The positive charge of calcitonin at pH 7.0 may interact with the negative property of the niosomal membrane (from the hydroxyl group of cholesterol) resulting in less net negativity of the system.

2.2 Physico-chemical stability at various storage temperatures of calcitonin loaded in elastic niosomes

The physical stability (vesicular size and zeta potential) of blank niosomes and niosomes loaded with calcitonin was presented in **Tables 20 and 21**. The blank non-elastic and elastic niosomes showed good physical stability determined by visual observation with no sedimentation, layer separation and color change at 4±2, 27±2 and 45±2 °C for 12 weeks. The average size of the vesicles and zeta potential values in all samples were increased after stored at 4±2, 27±2 and 45±2 °C for 12 weeks. The flocculation appearance of niosomes loaded with calcitonin was observed when kept at 4±2 and 27±2 °C for 4 weeks, but not at 45±2 °C. However, after stored at 45±2 °C for 1 week, the color of the niosomal dispersion became light yellow. The vesicles may be destroyed and converted to the surfactant and cholesterol at high temperature.

The percentages of calcitonin remaining in the niosomes when stored at 4±2, 27±2 and 45±2 °C for 4 weeks were shown in **Figure 36**. After 4 weeks, the percentages of calcitonin remaining in the solution at 4±2 and 27±2 °C were 69.95 and 3.11%, respectively. At 45±2 °C, calcitonin in solution can not be detected after stored in for 4

Table 20 Vesicular sizes (nm) and zeta potential (mv) of blank non-elastic and elastic niosomes after stored at various temperatures for 12 weeks

Niosomal formulations	Vesicular sizes (nm)				Zeta potential (mv)			
	At initial	After stored at			At initial	After stored at		
		4±2 °C	27±2 °C	45±2 °C		4±2 °C	27±2 °C	45±2 °C
Non-elastic niosomes	54.81±0.998	113±0.157	120.2±3.047	127.4±2.005	-27.5±1.85	-30.0±2.42	-42.0±0.67	-39.2±2.82
Elastic niosomes	54.53±0.709	107.1±1.985	116.6±0.959	115.0±5.584	-25.8±1.88	-30.8±1.74	-31.2±3.92	-39.8±2.18

Note : Each value represented mean±S.D. of three experiments

Table 21 Vesicular sizes (nm) and zeta potential (mv) of non-elastic and elastic niosomes loaded with calcitonin (2 mg/ml) after stored at various temperatures for 4 weeks

Niosomal formulations	Vesicular sizes (nm)				Zeta potential (mv)			
	At initial	After stored at			At initial	After stored at		
		4±2 °C	27±2 °C	45±2 °C		4±2 °C	27±2 °C	45±2 °C
Non-elastic niosomes	$2.016\times10^4\pm3205$	$1.697\times10^4\pm1784$	$4439\times10^4\pm230$	$2.359\times10^4\pm486.4$	-1.81±0.44	3.52±0.903	-27.5±1.17	-42.7±2.67
Elastic niosomes	$1.802\times10^4\pm2553$	$1.397\times10^4\pm537.9$	$7052\times10^4\pm265.3$	$1.433\times10^4\pm1565$	-4.19±0.72	4.30±0.454	-27.2±2.26	-12.8±1.61

Note : Each value represented mean±S.D. of three experiments

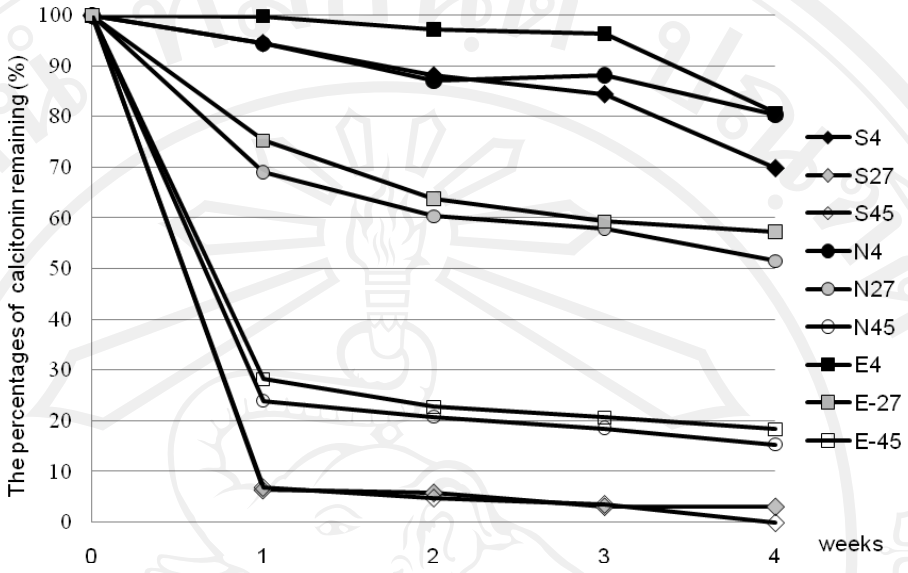


Figure 36 The percentages of calcitonin remaining in various systems when stored at various temperatures for 4 weeks; S4, S27, S45 = calcitonin solution in 5 mM phosphate buffer solution (pH 7.0); N4, N27, N45 = calcitonin loaded in non-elastic niosomes; E4, E27, E45 = calcitonin loaded in elastic niosomes and 4, 27, 45 = storage temperature at 4, 27 and 45°C, respectively.

weeks, while calcitonin loaded in non-elastic and elastic niosomes were found at 15.34 and 18.34%. At 4±2 and 27±2 °C for 4 weeks, the percentages of calcitonin remaining non-elastic niosomes were 80.40 and 51.57%, whereas calcitonin remaining in elastic niosomes were 80.76 and 57.31%, respectively. When loaded in non-elastic niosomes, calcitonin showed significantly ($p<0.05$) more remaining amount of 16.58 and 15.34 times than in solution when stored at 27±2 and 45±2 °C, respectively. Calcitonin loaded in elastic niosomes indicated higher remaining amount than in solution of 18.43 and 18.43 times when stored at 27±2 and 45±2 °C, respectively. All formulations

demonstrated better correlation between $1/[A_0]$ and times of the second order kinetics equation of $r^2 = 0.9643$ than the zero and first order kinetics equation ($r^2 = 0.94$ and 0.921, respectively). The shelf lives of calcitonin solution, calcitonin loaded in non-elastic niosomes and in elastic niosomes were 3.22, 5.67 and 6.09 days at 4±2 °C, 0.07, 1.47 and 1.68 days at 27±2 °C, and 0.07, 0.28 and 0.28 days at 45±2 °C, respectively. The half lives of calcitonin solution, calcitonin loaded in non-elastic niosomes and in elastic niosomes when stored at 4±2 °C were 29.43, 52.08 and 55.56 days, while stored at 27±2 °C were 0.37, 13.56 and 15.40 days and stored at 45±2 °C were 0.34, 2.39 and 2.79 days, respectively. At elevated temperatures, the increase fluidity and permeability of the vesicular membrane can result in the leakage of calcitonin from the vesicles thereby facilitating the chemical degradation of calcitonin by heat (Kim et al., 2009b). Salmon calcitonin is unstable in aqueous solution. Salmon calcitonin in 0.01 M phosphate buffer solution, pH 7.4 incubated at 37 °C gave a degradation peptide product after 7 days and complete degradation after 35 days of incubation (Tang et al., 2010). The 37% of calcitonin in 0.1 M phosphate buffer, pH 7.4 was degraded within 24 hours at 37 °C and 100% degradation after stored for 10 , 3 and 3 days at 4, 25 and 37 °C, respectively. The short half life of salmon calcitonin of approximately 8 days in 0.1 M phosphate buffer solution, pH 7.4 at 4 °C has been reported (Dani et al., 2001). In our study, calcitonin in solution showed higher half life of 29.43 days at 4 °C since calcitonin was in the lower buffer concentration (0.005 M). High buffer concentration gave high ionic strength of the system which may increase the degradation of salmon calcitonin by heat from the

facilitation of the calcitonin molecule to be exposed to the thermal energy (Geesink et al., 2000; Choe et al., 2008).

When loaded in either elastic or non-elastic niosomes, calcitonin was more stable than in aqueous solution of about 16 to 19 times when stored at high temperatures (27±2 and 45±2 °C) for 4 weeks. But, elastic and non-elastic niosomes showed no significant difference ($p<0.05$) in protecting calcitonin from the degradation by high temperatures. From various previous studies, the stability against high temperature of gallidermin (Manosroi et al., 2010b) and plasmid (Manosroi et al., 2010e) was improved when loaded in niosomes. In fact, several strategies to improve chemical stability of calcitonin have also reported. For examples, the methionin oxidation in position 8 residue of human calcitonin (hCT) can reduce the aggregation rate of hCT. The oxidized hCT was more stable than the native hCT at pH 4-7 of 1.5 times (Mulinacci et al., 2011). Cyclodextrins including 2-hydroxypropyl-βCD (HPβCD) and the randomly methylated-βCD (RMβCD) can not only inhibit the aggregation, but also increase the physical stability of salmon calcitonin (sCT) in aqueous solution at elevated temperature (55°C), but do not greatly affect the chemical stability of sCT in aqueous solution (Sigurjónsdóttir et al., 1999). Therefore, the entrapment of salmon calcitonin in non-elastic and elastic niosomes can be one of the strategies to improve chemical stability of calcitonin.

2.3 Transdermal absorption of calcitonin loaded in elastic niosomes

The cumulative amounts and fluxes per area of calcitonin various systems in whole skin and the receiver compartment solution following transdermal absorption

across the excised rat skin were shown in **Table 22**. With times, the cumulative amounts of calcitonin of all systems decreased in the whole skin while increased in the receiver compartment solution during the period of 6 h **(Figure 37)**. This may be due to the transcutaneous hydration gradient which acts as a driving force for calcitonin in the whole skin to the receiver compartment solution. Calcitonin loaded in elastic niosomes exhibited higher amount both in the whole skin (0.071±0.016 mg/cm^2) and in the receiver compartment solution (0.337±0.014 mg/cm^2) than that loaded in non-elastic niosomes and in the solution of 1.82 and 1.24; 1.06 and 1.38 times. It has been reported that elastic liposomes, composed of soybean phosphatidylcholine/cholesterol/sodium deoxycholate (NaDC) at 7:4:4 molar ratio improve the absorption of salmon calcitonin through intranasal administration (Chen et al., 2009). Similarly, elastic niosomes may act as penetration enhancers resulting from their compositions with the edge activator (NaC) that can disrupt the highly organized intercellular lipids from stratum corneum, thereby facilitating the calcitonin molecules to penetrate in and across the stratum corneum (Pirvu et al., 2010; Duangjit et al., 2011). Calcitonin solution also showed some cumulative amounts after 6 h in whole skin and the receiver compartment solution of 0.067±0.016 and 0.244±0.012 mg/cm^2, respectively. This may be owing to the carrier property of calcitonin of the amino acid sequence at positions 9-22 which contain several highly hydrophobic residues (three Phe residues, one Tyr and one Leu) resulting in the facilitating of skin penetration (Tréhin et al., 2004; Wagner et al., 2004; Meleleo et al., 2007).

Table 22 The cummulative amounts (mg/cm^2) and fluxes ($mg/cm^2/h$) of calcitonin from various systems in whole skin and receiver compartment solution following transdermal absorption across excised rat skin at 6 h by vertical Franz diffusion cells

Systems	Cumulative amounts (mg/cm^2)		Fluxes ($mg/cm^2/h$)	
	Whole skin	Receiver compartment solution	Whole skin	Receiver compartment solution
Calcitonin solution	0.067±0.016	0.244±0.012	0.011±0.002	0.041±0.002
Calcitonin loaded in non-elastic niosomes	0.039±0.006	0.271±0.004	0.006±0.001	0.045±0.001*
Calcitonin loaded in elastic niosomes	0.071±0.013	0.337±0.015*	0.012±0.005	0.056±0.002*

Note : Each value represented mean±S.D. of three experiments.

Calcitonin contents in all systems were at 2.0 mg/ml

- * significant difference ($p<0.05$) in comparing to the calcitonin solution system

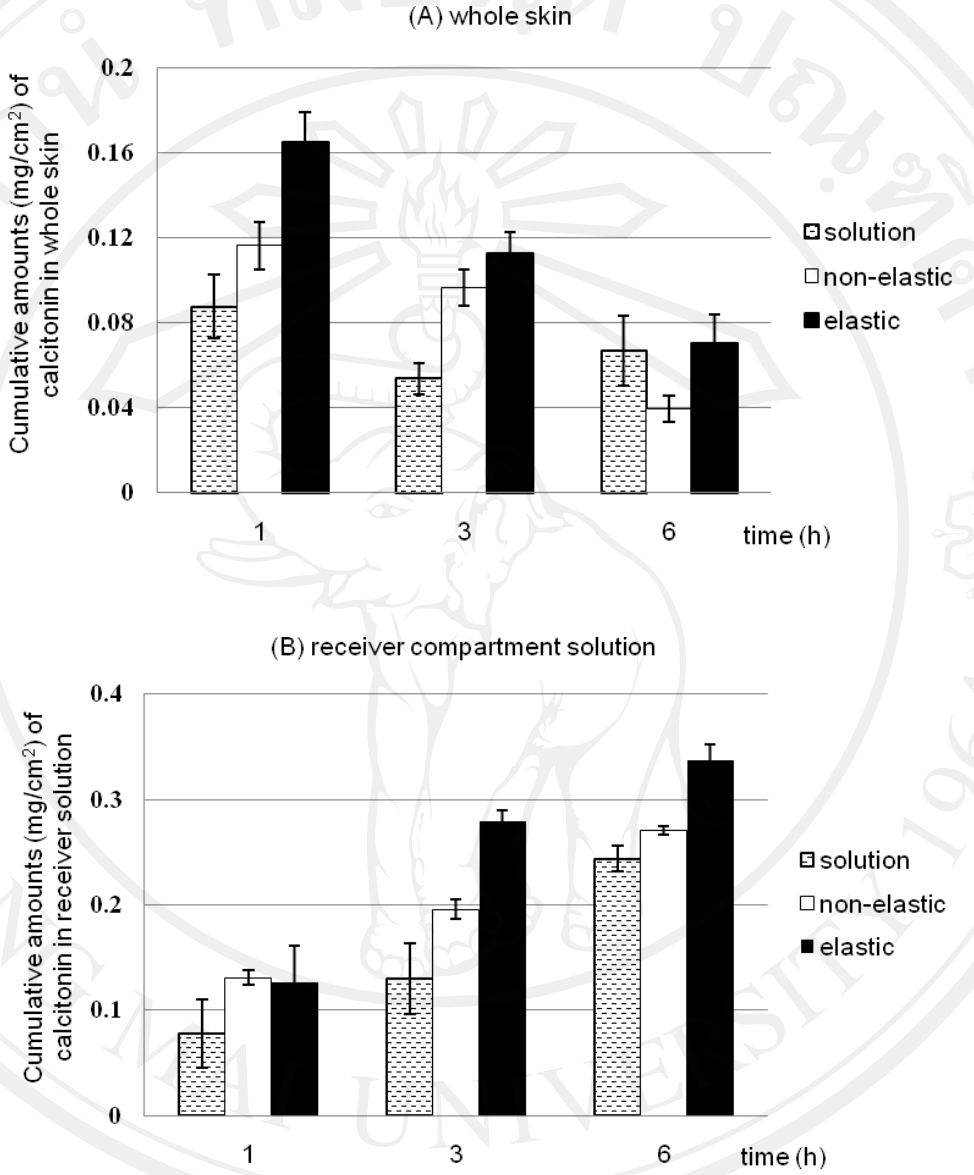


Figure 37 Cumulative amounts (mg/cm^2) of calcitonin from various systems in whole skin (A) and receiver compartment solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells (each value represents mean±S.D., *n*=3).

The fluxes of calcitonin in all systems were decreased with times during the period of 6 h **(Figure 38)**. Calcitonin loaded in elastic niosomes exhibited the highest flux after 6 h in the whole skin and the receiver compartment solution of 0.012±0.006 and 0.056±0.002 mg/cm^2/h which were 1.79 and 1.24 times more than calcitonin loaded in non-elastic niosomes and 1.09 and 1.36 times of calcitonin solution, respectively. The more free calcitonin in an aqueous system may substantially reduce the partition coefficient of the peptide between the skin and the vehicle, thereby decreasing the flux values (Kumar et al., 2011). After 6 h, the percentages of calcitonin in the receiver compartment solution of calcitonin in solution, calcitonin loaded in non-elastic niosomes and calcitonin loaded in elastic niosomes were 30.01±1.53, 33.32±0.47 and 41.44±1.83 % of the initial amount of calcitonin in the donor compartment, respectively. Calcitonin loaded in elastic niosomes showed significantly ($p<0.05$) higher percentage amount in the receiver compartment solution than that loaded in non-elastic niosomes and in solution. However, there was no significant difference of this amount between calcitonin loaded in non-elastic niosomes and calcitonin in solution. The higher deformability (data not shown) of the elastic niosomes than the non-elastic niosomes may result from the head groups of the NaC molecules which intercalate between the Tween 61 molecules in the niosomal membrane and decrease the packing density of the surfactant molecules. This will give the high flexibility of the niosomal membranes that can permit the vesicles to squeeze themselves through the skin pores which are much smaller than their sizes (Manosroi et al., 2009).

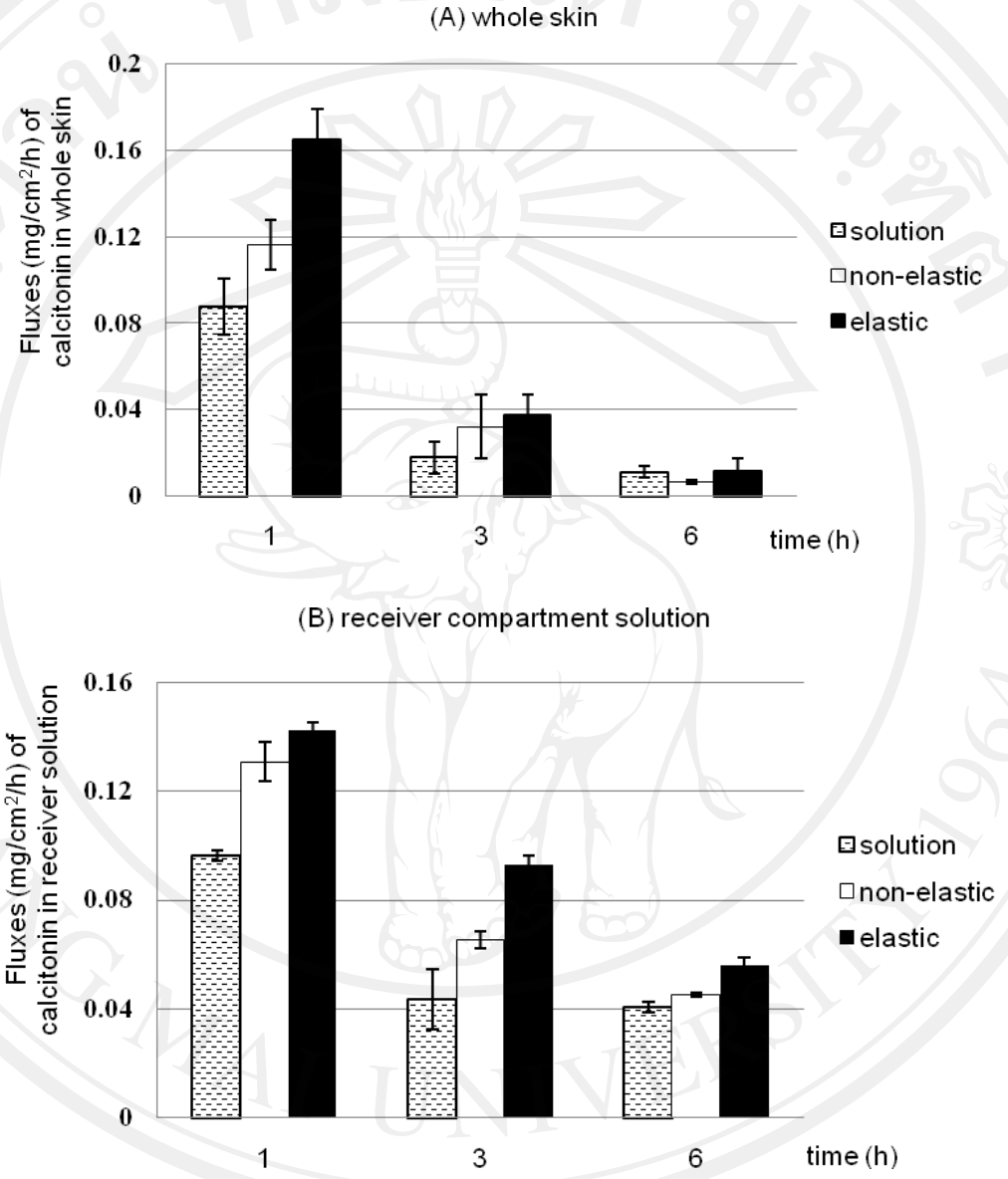


Figure 38 The fluxes (mg/cm^2/h) of calcitonin from various systems in whole skin (A) and receiver compartment solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells (each value represents mean±S.D., *n*=3).

Part 3: Preparation and biological activities of the extracted protese enzymes

3.1 Characteristics of the enzymes

Although the purification of the protease enzyme by the traditional precipitation (Doko et al., 1991; Adulyatham et al., 2006; Chaiwut et al., 2007) gave the purified enzyme with the contamination of other proteases, it was the easy and low cost method. In this study, the papain and bromelain were extracted by the simple precipitation with 95% ethanol and the saturated ammonium sulfate which gave the percentage yield of 16.76 and 0.97%, respectively. The low percentage yield of the extracted bromelain may be due to the high water contents (87%) of the pineapple juice (Sairi et al., 2004). After freeze dried, the extracted papain and bromelain were in white and yellow powder. The MW of the extracted enzymes were compared with their standards and the protein marker by the SDS-PAGE as shown in **Figure 39**. **Figure 39A** indicated the molecular weight of the papain at about 23 kDa. The lower bands might be from other proteases such as chymopapain, caricain and glycyl endopeptidase (El Moussaoui et al., 2001; Chaiwut et al., 2007). The band of papain (upper band) showed smaller area than other proteases (lower band). It has been reported that papain is a minor constituent among the papaya proteases (Pendzhiev, 2002; Nitsawang et al., 2006). For bromelain, its molecular weight was about 25 kDa **(Figure 39B)**. From the band of the enzyme on the SDS-PAGE, the purity of papain and bromelain was 77.68 and 44.95%, respectively. The tentative identification of the enzyme can be determined by the comparison of the retention times by HPLC between the standard and the extract

chromatograms (Myint et al., 1995; Hanachi et al., 2009). The retention times of the extracted enzyme were similar to those of the standard enzymes **(Figure 40)**.

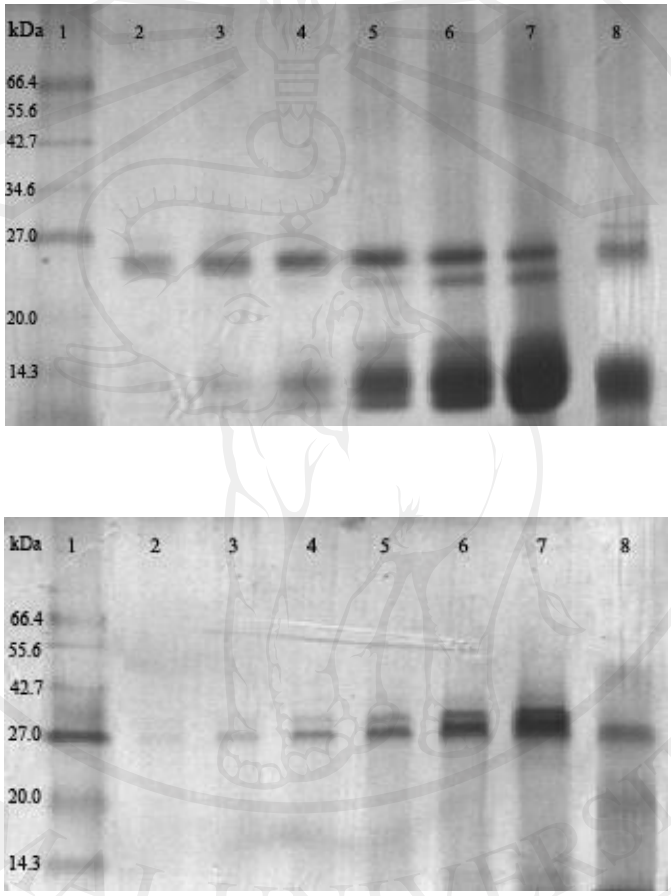


Figure 39 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the extracted papain (a), extracted bromelain (b) in comparing to the molecular weight marker (lane 1) and their standards at 1 mg/ml (lane 8); lanes 2-7 were the enzymes at 0.156, 0.312, 0.625, 1.25, 2.5 and 5 mg/ml, respectively

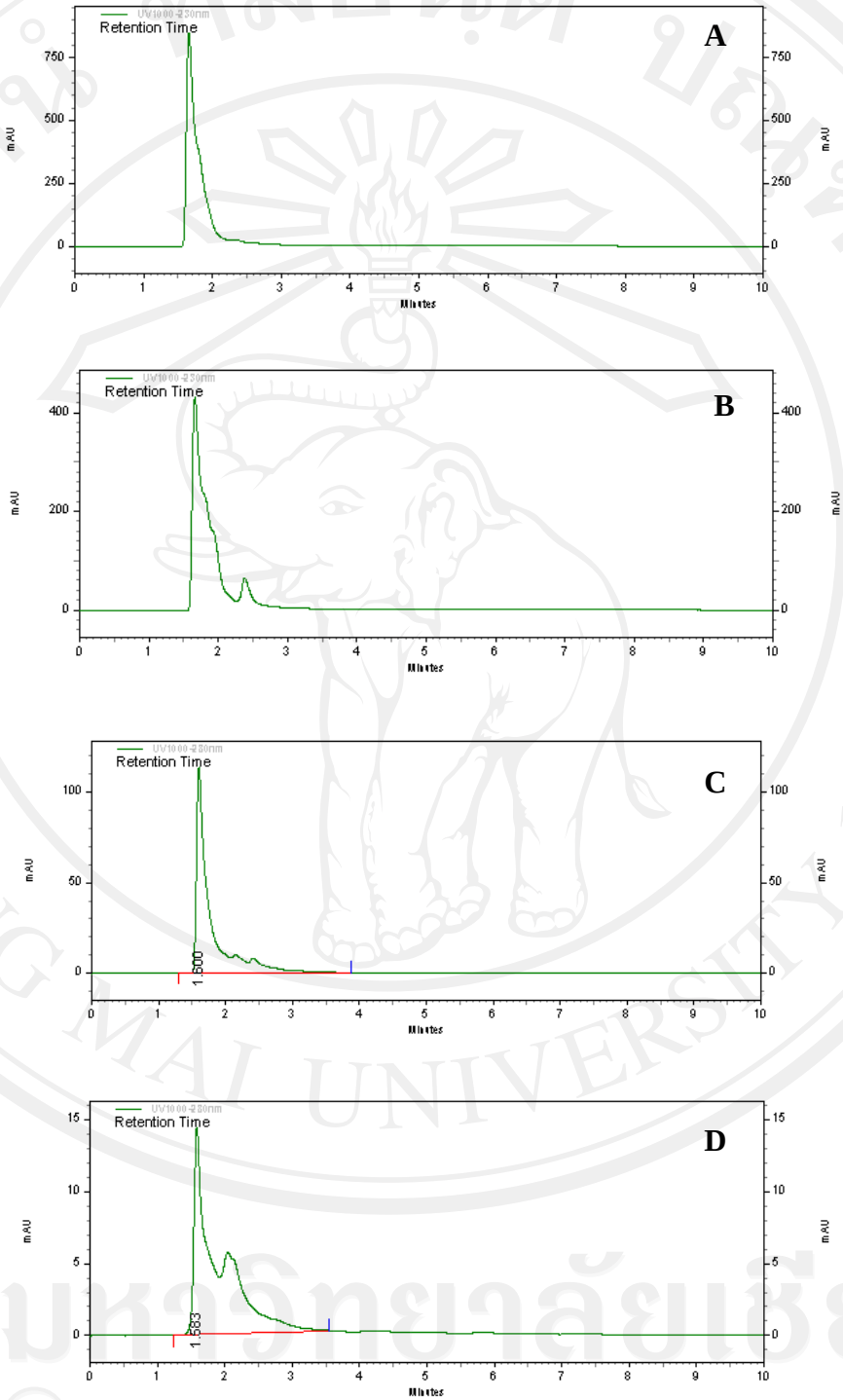


Figure 40 The HPLC chromatograms of the standard papain (A) extracted papain (B) standard bromelain (C) and extracted bromelain (D)

The extracted papain and bromelain demonstrated the major peaks with the retention times at 1.660 and 1.583 min, while their standard enzymes were at 1.658 and 1.600 min, respectively. The purity of papain and bromelain of the extracted enzymes determined by HPLC were 82.31 and 38.03%, respectively which were close to those determined by the SDS PAGE method.

The physicochemical properties including solubility and stability in chemicals (strong acid, weak acid, strong base, weak base, reducing agent, oxidizing agent and acid salt) of the two extracted enzymes were similar to their standards. All enzymes were soluble in distilled water but insoluble in alcohol and organic compounds such as glycerin and propylene glycol. The extracted enzymes were less chemical stable than the standard enzymes. For papain, the extracted enzyme was unstable to strong acid, weak acid, reducing agent and acid salt, whereas the standard enzyme was stable to all reagents except the strong acid. For bromelain, the extracted enzyme was unstable to strong acid, strong base and reducing agent, whereas the standard enzyme was unstable to strong alkali and reducing agent. This may be due to the effects from several other compounds in the extracted enzymes in accordance with many protein bands on the SDS-PAGE gel and the multiple peaks on the HPLC chromatogram.

3.2 Biological activities of the extracted protease enzymes

3.2.1 DPPH radical scavenging activity

Antioxidant activity of the extracted enzymes determined by DPPH radical scavenging was shown in **Table 23**. The SC_{50} value of the standard papain (1.71±1.45

Table 23 Antioxidant activities of the extracted enzymes in comparing to the standards

Sample	%yield	%purity		SC_{50}[a] (mg/ml)	IPC_{50}[b] (mg/ml)
		SDS-PAGE	HPLC		
Standard papain	-	-	-	1.71±1.45	0.05±0.003
Extracted papain	16.76±5.74	77.68±4.56	82.31±5.22	956.10±62.06	0.38±1.02
Standard bromelain	-	-	-	684.27±100.13	-
Extracted bromelain	0.97±0.16	44.95±3.88	38.03±2.04	-	-
Vitamin C	-	-	-	0.0023±0.001	0.052±0.01
Vitamin E	-	-	-	-	0.324±0.099

Note : Values represent mean±S.D. (n=4)

[a]SC_{50} = scavenging concentration at 50% activity (mg/ml)

[b]IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml)

mg/ml) was lower indicating of higher activity than the extracted enzyme (956.10±62.06 mg/ml) of 560 times. The previous study has reported that the papain extracts from Korean elk velvet antler showed antioxidative activity by scavenging free radicals (Kim et al., 2009a). The standard bromelain gave the SC_{50} value of 684.27±100.13 mg/ml, whereas the extracted bromelain showed no activity. The standard papain and bromelain enzymes gave lower activity than the standard vitamin C (SC_{50} = 0.0023±0.0001 mg/ml). The DPPH radical scavenging activity of the enzymes may be from the amino acid contents which can scavenge the DPPH radical by donation of the hydrogen atom to from a stable DPPH-H molecule (Haiwei, 2010). The standard papain indicated higher scavenging activity than the standard bromelain of 400 times owing to the contents of amino acids and peptides (Wu et al., 2003), that 20 amino acids found in the enzymes have the potential to interact with the free radicals. Also, the amino acids in the enzymes including Tryptophan (W), Tyrosine (Y), Methionine (M), Cysteine (C), Histidine (H) and Phenylalanine (F) have been proposed to contribute to antioxidant activity and be important free radical scavengers in the natural peptides (Elias et al., 2008; Udenigwe et al., 2011). The numbers of these amino acids in papain (39) was more than bromelain (35) of 5 **(Figure 41)**. The hydrogen atom donation by the amino acids in papain may be more than that in bromelain resulting in the high DPPH radical scavenging. The impurities of the extracted papain enzymes may cause lower scavenging activity than the standard enzymes (Smith et al., 1993; Gautam et al., 2010). The extracted bromelain which gave low percentage of purity (38-44%) did not show any scavenging activity.

1 IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNEYSEQEILDCDRRS 60

61 YGCNGGYPWSALQLVAQYGIHYRNTYPYEGVQRYCRSREKGPYAAKTDGVRQVQPYNEGA 120

121 LLYSIANQPVSVVLEAAGKDFQLYRGGIFVGPCGNKVDHAVAAVGYGPNYILIKNSWGTG 180

181 WGENGYIRIKRQTQNSYGVCGLYTSSFYPVKN 212

A

1 AVPQSIDWRDYGAVTSVKNQNPCGACWAFAAIATVESIYKIKKGILEPLSEQQVLDCAKG 60

61 YGCKGGWEFRAFEFIISNKGVASGAIYPYKAAKGTCKTDGVPNSAYITGYARVPRNNESS 120

121 MMYAVSKQPITVAVDANAAFQYYKSGVFNGPCGTSLNHAVTAIGYGODSIIYPKKWGAK 180

181 WGEAGYIRMARDVSSSSGICGIAIDPLYPTLEE 212

B

Figure 41 The amino acid sequences of papain (A) and bromelain (B). The beginning and the end of every part of the sequence were numbered.

3.2.2 Lipid peroxidation inhibition activity

The standard papain and the extracted papain showed lipid peroxidation inhibition at the IPC_{50} values at 0.05±0.003 and 0.38±1.02 mg/ml, respectively **(Table 23)**. The standard papain exhibited no significant differences (p<0.05) lipid peroxidation inhibition activity in comparing to vitamin C (0.052±0.01 mg/ml), while the extracted papain showed similar activity to vitamin E (0.324±0.099 mg/ml). However, both standard bromelain and the extracted bromelain did not give any lipid peroxidation inhibition activity. In fact, this activity might be from the hydrophobic amino acids which can inhibit the peroxidation formation (Je et al., 2007; Kim et al., 2008). In the amino acid sequence of the enzymes **(Figure 41)**, 35% hydrophobic amino acids in papain can exert high affinity to the linoleic acid. Thus, papain may interact with the

lipid molecules and scavenge lipid peroxyl radical by donating proton to lipid-derived radicals. In addition, the position of the hydrophobic amino acids, Leucine (L) at the N-terminal and Proline (P), Histidine (H) or Tyrosine (Y) in the peptide sequences have been reported to have high potent lipid peroxidation inhibitory activity (Suetsuna et al., 2002; Jun et al., 2006). The numbers of these amino acids in papain (31) were 5 more than in bromelain (26) **(Figure 41)**. Therefore, it is possible that these specific amino acid residues in the enzymes may play an important role in increasing the interaction between the peptides and the unsaturated fatty acids (linoleic acid) in the lipid peroxidation inhibitory assay. The standard papain exhibited better lipid peroxidation inhibition activity than the extracted papain. Similar to free radical scavenging activity, the impurity of the extracted enzyme may give this effect (Smith et al., 1993; Gautam et al., 2010).

3.2.3 Cytotoxicity of the extracted protease enzymes

Various concentrations of the enzymes were investigated for normal human fibroblast cytotoxicity by the SRB assay. When the concentrations of the enzymes increased from 10^{-9} to 100 µg/ml, cell viability was decreased. All samples at the concentration range of 10^{-9} to 25 µg/ml gave cell viability of more than 85% with no significant difference at $p<0.05$. At high concentration (100 µg/ml), the standard papain, extracted papain, standard bromelain and extracted bromelain showed %cell viability at 16.17, 15.61, 19.24 and 29.95, respectively **(Table 24)**. Since positively charges in the basic amino acids are also related to cytotoxicity, papain which has more number of basic amino acids (24) showed higher cytotoxicity than bromelain which has less number of

basic amino acids (21). When human skin contacted with papain at 0.2%w/v for 24 h, the large amount of the intercellular material in the stratum corneum was lost and most of the extracellular components were digested (Lopes et al., 2008). The basic amino acids including Lysine (K), Arginine (R) and Histidine (H) were apparently severe damage in human retinal pigment epithelial cells (Nakauchi et al., 2003). The cytotoxic effects of the extracted enzymes were less than those of the standard enzymes. This was possibly from the protective effect of some compounds such as glycoprotein (Lee et al., 2006). These compounds may have the protective effect on cell viability by the steric hindrance around the cells (Francica et al., 2010), thereby decreasing the membrane permeability and resulting in the reduced cytotoxicity of the extracted enzymes in comparison to the standard enzymes. Moreover, the extracted bromelain was less toxic than the extracted

Table 24 The percentages of cell viability on human skin fibroblast by the SRB assay of the extracted protease enzymes

Concentration of the enzymes (mg/ml)	%cell viability			
	Standard papain	Extracted papain	Standard bromelain	Extracted bromelain
0.00625	101.41±6.18	106.30±2.07	100.96±2.68	102.60±3.00
0.0125	94.80±3.27	99.85±1.78	94.65±7.46	99.47±7.20
0.025	85.95±12.35	95.14±7.39	90.82±10.88	88.21±11.30
0.05	48.96±8.97	65.10±8.27	59.95±4.77	63.48±0.52
0.1	16.17±0.28	15.61±0.35	19.24±0.42	29.95±1.77

Note : Cell viability (%) = (absorbance$_{\text{test cell}}$/absorbance$_{\text{control cell}}$) x100

papain. It may be due to the less purity of bromelain in the extracts. The highest concentration of the standard papain, extracted papain, standard bromelain and extracted bromelain that gave cell viability of more than 85% which were 85.95±12.35, 95.14±7.39, 90.82±10.88 and 88.21±11.30%, respectively was observed at 25 µg/ml. Thus, this concentration of all enzyme samples was selected for gelatinolytic activity on the MMP-2 study.

3.2.4 Gelatinolytic activity (zymography) on MMP-2

Figure 42 showed the relative MMP-2 stimulation by zymography of the extracted enzymes at 25 µg/ml in comparing to the negative (untreated cells) and positive (concanavalin A) control systems. The cells treated with the standard papain, extracted papain, standard bromelain and extracted bromelain indicated the relative MMP-2 stimulation of 2.01±0.14, 2.10±0.14, 1.63±0.27 and 1.71±0.12, respectively. However, the MMP-2 stimulatory activity of all enzymes was lower than that of concanavalin A (2.59±0.22) of 1.29, 1.23, 1.59 and 1.51 times, respectively. In fact, it is still unknown for the protease enzyme mechanism on the MMP-2 stimulation. One possible mechanism is that the protease may induce collagenase secretion (Werb et al., 1978). It has been demonstrated that papain has higher proteolytic activity and gives more amount of the secreated collagenase in the fibroblast than bromelain. Thus, papain gave higher stimulatory activity than bromelain. Also, the pH of our system (pH 7.0) may affect the activity, as papain has an optimum activity at pH 5-8 (Pendzhiev, 2002), while bromelain has high activity at an optimum pH at 4.5-5.5 (Kim et al., 1991). The pH of the system appeared to be favorable for papain. The extracted enzymes showed slightly higher

stimulatory activity than the standard enzymes but no significant difference (p>0.05), although the purity of both enzymes was different. This may be due to the addition effects from other protease enzymes existing in the extract such as chymopapain, glycyl endopeptidase and caricain in papaya latex (Pendzhiev, 2002; Chaiwut et al., 2007), and comosain and ananain in pineapple (Maurer, 2001; Tochi et al., 2008).

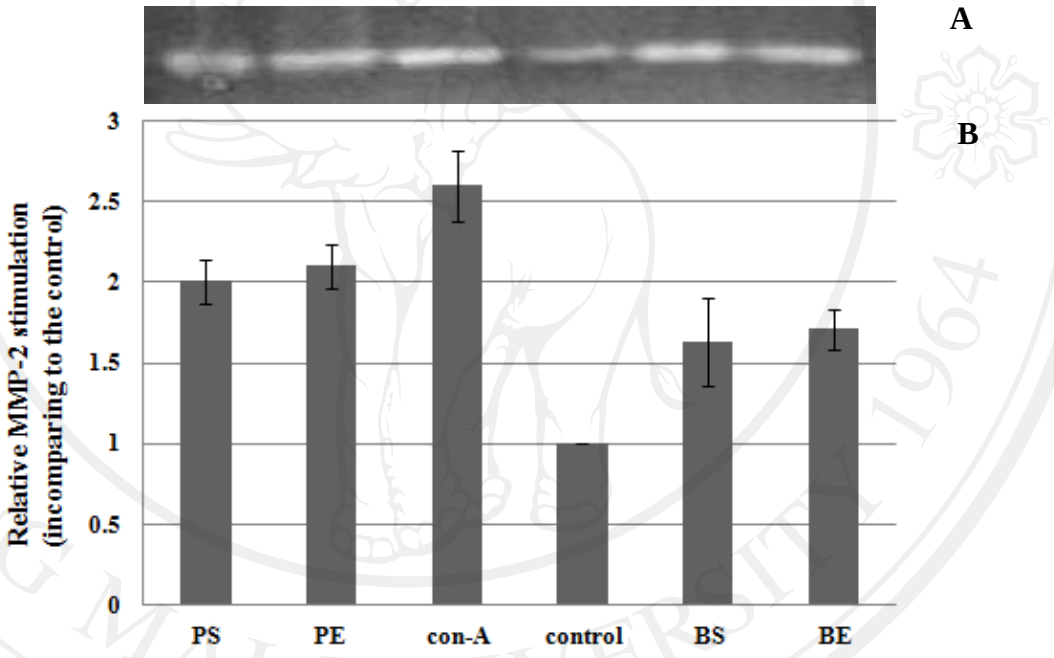


Figure 42 Gelatinolytic activity of the extracted protease enzymes in comparing to the control and concanavalin A. (a) zymograms and (b) MMP-2 stimulation relative to the control. PS= the standard papain, PE= the extracted papain, con-A= concanavalin A (positive control), BS= the standard bromelain and BE= the extracted bromelain

Part 4: Development of the elastic niosomes loaded with the extracted papain and bromelain

4.1 Physical characteristics of the blank and the non-elastic and NaC elastic niosomes loaded with the enzymes

All niosomal dispersions (blank non-elastic and elastic niosomes, non-elastic and elastic niosomes loaded with papain or bromelain) were in translucent colloidal appearances, with no sedimentation or layer separation. **Table 25** showed the vesicular sizes and zeta potentials of the non-elastic and elastic niosomes loaded with both the standard and extracted enzymes. The vesicular sizes of both the standard and extracted enzymes loaded non-elastic and elastic niosomes were in the range of 109.5 to 208.8 nm. Blank and the standard and extracted enzymes loaded elastic niosomes gave smaller sizes than those of the non-elastic niosomes. The decreased sizes of the elastic niosomes may be due to the effect of NaC which can reduce the interfacial tension between the vesicular membrane leading to the decrease in the bilayer thickness (Liu et al., 2007a). The sizes of the elastic niosomes were increased when loaded with papain, while decreased when loaded with bromelain. The charge interaction between the enzymes and vesicular membranes may be responsible for this effect. Papain which has the positive charge (PI=8.75) in the phosphate buffer solution at pH 7.0, and can be both adsorbed on the niosomal membrane from the electrostatic interaction and loaded in the aqueous layers in the vesicles from its hydrophilic property. Bromelain which has the negative charge (PI=4.6) in the phosphate buffer solution at pH 7.0 may be only loaded in the niosomes. Non-elastic and elastic niosomes loaded with the extracted enzymes gave higher

vesicular sizes than those loaded with the standard enzymes. The impurities containing in the extracted enzymes such as chymopapain (PI=10.6) and caricain (PI=10.9) in the papaya latex, comosain and ananain (PI>8.5) in the pineapple juice which had positive charges in the phosphate buffer solution at pH 7.0 may be bound on the niosomal membrane by the ionic interaction resulting in the larger vesicular size than those of the standard enzymes. The morphology of the niosomes was in spherical shape observed under TEM microscope **(Figure 43)**. All elastic niosomes were in unilamellar structure with the vesicular size of about 150-200 nm.

The zeta potential values of both the standard and extracted enzymes loaded in non-elastic and elastic niosomes were in the range of -14.7 to -30.1 mv. In general, blank non-elastic niosomes should not have any charge. However, they gave the negative zeta potential value of -27.4±1.59 mv. This might be from the preferential adsorption of the hydroxyl ions from the cholesterol molecules at the vesicular surface. The previous study has demonstrated that in the presence of enough cholesterol, the formation of the niosomal bilayer membranes occurred (relative humidity; RH ≈ 0.985). Upon further hydration, the lamellar *d* spacing increases monotonously with RH without reaching a finite swelling limit. Such indefinite swelling is due to the electrostatic repulsion between the opposing membranes. This is in good agreement with the previous zeta-potential measurements of niosomes showing that vesicles have a net negative charge (Pozzi et al., 2009). The blank elastic niosomes and elastic niosomes loaded with both the standard and extracted enzymes gave more negative zeta potential values than those of the non-elastic niosomes owning to the anionic property of NaC. When loaded with

Table 25 Vesicular sizes, zeta potential values and deformability index (DI) of the non-elastic and elastic niosomes loaded with the extracted papain and bromelain

Niosomal formulations	Vesicular size (nm)		Zeta potential (mv)		Deformability index (DI)	
	Non-elastic	Elastic	Non-elastic	Elastic	Non-elastic	Elastic
Blank niosomes	159.8±2.28	118.1±1.96	-27.4±1.59	-29.4±1.94	14.47±0.85	52.23±3.14
Niosomes loaded with the standard papain	166.2±2.20	138.8±1.79	-23.5±1.83	-25.7±1.12	18.67±6.11	25.17±0.25
Niosomes loaded with the extracted papain	208.8±2.27	143.9±0.416	-23.1±0.80	-26.6±0.74	16.43±0.27	29.74±8.77
Niosomes loaded with the standard bromelain	123.5±0.84	109.5±0.67	-19.7±2.28	-25.3±1.12	33.67±5.51	41.26±5.84
Niosomes loaded with the extracted bromelain	125.6±1.37	119.2±1.32	-14.7±2.57	-30.1±1.70	32.29±0.54	52.24±2.05

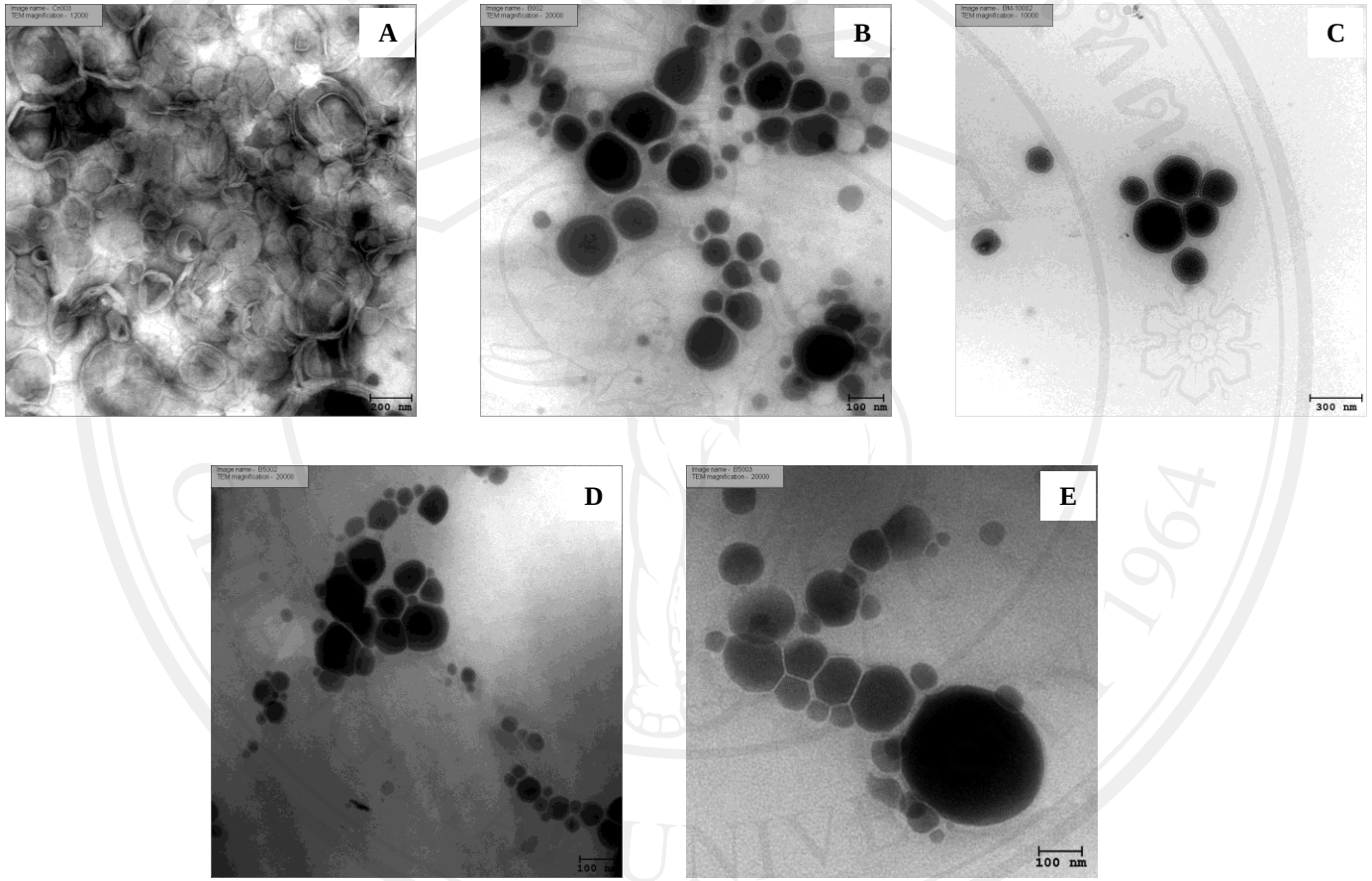


Figure 43 Negative-staining TEM images of the protease enzymes loaded in niosomes (20000X). (A): the blank elastic niosomes, (B) the standard papain loaded in elastic niosomes, (C) the extracted papain loaded in elastic niosomes, (D) the standard bromelain loaded in elastic niosomes and (E) the extracted bromelain loaded in elastic niosomes

both the standard and extracted enzymes, the zeta potential values of non-elastic and elastic niosomes were decreased owning to the positive charge of papain and the steric hindrance effects of some compounds such as glycoprotein containing in the extracted papain and bromelain on the niosomal surfaces. Thus, similar to the vesicular sizes, the impurity of the extracted enzyme may affect the zeta potential values of the loaded niosomes to be different from the standard enzymes.

4.2 Deformability index (DI) of the blank and the non-elastic and NaC elastic niosomes loaded with the extracted enzymes

Deformability of the non-elastic and elastic niosomes loaded with the extracted papain and bromelain were shown in **Table 25**. The DI values of the blank elastic niosomes were more than the blank non-elastic niosomes of 3.61 times. The head groups of NaC composed in the elastic niosomes may be intercalated between the surfactant (Tween 61) molecules, thereby decreasing the packing density of the surfactant molecules and being more flexible. Also, the surfactants may interact with the vesicles and change the conformation by fitting their lipophilic parts between the hydrocarbon chains, compensating for the bulkiness of the head groups (Maghraby et al., 2000). The elastic niosomes loaded with the standard papain, extracted papain, standard bromelain and extracted bromelain gave the DI values at 25.17±0.25, 29.74±8.77, 41.26±5.84 and 52.24±2.05 which were 1.35, 1.81, 1.22 and 1.61 times higher than those in the corresponding non-elastic niosomes, respectively. When loaded with the standard and extracted papain, the DI values of the elastic niosomes were decreased to 2.08 and 1.76 times, respectively. The positive charges of papain at pH 7.0 may interact with the

negative charges of the niosomes (from the cholesterol compositions) resulting in the decrease in electrostatic repulsion between the vesicular bilayers. Thus, this may lead to the increase in packing density of the surfactant molecules (Tween 61) in the vesicles and being less flexible. On the contrary, when loaded with the standard and extracted bromelain, the vesicle elasticity of the non-elastic niosomes were increased to 2.33 and 2.23 times, respectively. The negative charges of bromelain at pH 7.0 may increase the electrostatic repulsion between the negative vesicular bilayer leading to be more flexible.

4.3 The maximum loading and entrapment efficiency of the extracted enzymes in NaC elastic niosomes

The maximum loading of both the standard and extracted papain loaded in elastic niosomes was higher than in non-elastic. The maximum loading of the standard and extracted papain loaded in non-elastic and elastic niosomes were at 0.4 and 0.75; 0.45 and 0.95 mg/ml, respectively. At the maximum loading concentrations, the standard and extracted papain exhibited the entrapment efficiencies in the non-elastic and elastic niosomes at 37.20±3.61 and 45.14±2.07; 50.14±0.72 and 50.20±0.14%, respectively. The extracted papain showed higher entrapment efficiencies in the non-elastic niosomes than the standard papain. This was possibly due to the existing of other enzymes in the extracted papain which may contribute to the more positive charge of the system, such as chymopapain and caricain. In general, the entrapment efficiency of most water-soluble substances in the bilayer vesicles is low of not more than 10-20% (Peltonen et al., 2002). In this study, the entrapment efficiencies of papain in non-elastic and elastic niosomes were higher than this value. Also, the electrostatic interaction between the niosomes and

papain can affect the movement of the substance across the vesicular membrane (Manosroi et al., 2010c). The movement of the loaded enzymes across the vesicular membrane was dependent on not only the compositions of the bilayer, but also the characteristics of the enzymes (Gulatia et al., 1998). Carafe et al. has indicated that encapsulation of lidocaine and lidocaine hydrochloride leads to a faster diffusion with respect to the corresponding classical liposomal formulations. This effect can probably be related to the enhancing effect of the hydrophilic surfactant (Carafa et al., 2002), the charge of the loaded enzymes in the vesicles and the electrostatic interaction between the enzymes and the vesicle bilayers (Carafa et al., 2004). There were three reasons for the higher entrapment efficiencies of the standard and extracted papain in elastic niosomes than in non-elastic niosomes. First, the negative charges of NaC in the niosomal compositions may be presented on the vesicular membrane surface that can interact with the positive charge of papain. Papain can then be both adsorbed on the niosomal surfaces and entrapped in the aqueous layers of the vesicles (Manosroi et al., 2011a). Second, the negative charges of NaC may increase the hydrophilicity of the niosomal bilayers, thereby increasing the water intake to the vesicles and resulting in higher entrapment of papain which is a water soluble molecule. This did not happen in the non-elastic niosomal system which did not contain NaC. Third, the inclusion of a charged molecule (NaC) in the vesicular bilayers also can increase the stability of the niosomal dispersion (Uchegbu et al., 1998). Thus, the high entrapment efficiency percentage of papain in elastic niosomes perhaps was from the synergistic vesicular membrane structure stabilization by cholesterol and NaC to prevent the leakage of papain from the niosomes.

Without NaC in the loaded non-elastic niosomes, the membrane permeability and leakage of papain from the niosomes may be increased (Hashim et al., 2010).

In comparing the entrapment efficiencies between the two enzymes (papain and bromelain) at 0.5 mg/ml in elastic niosomes, the standard papain (41.98%) and extracted papain (48.77%) gave higher entrapment efficiencies than the standard bromelain (34.89%) and extracted bromelain (37.39%). Again, these different entrapment efficiencies between papain and bromelain in elastic niosomes may be from the different charges of the enzymes at pH 7.0, while the difference between the standard and the extracted enzymes may be from the impurities existing in the extracts.

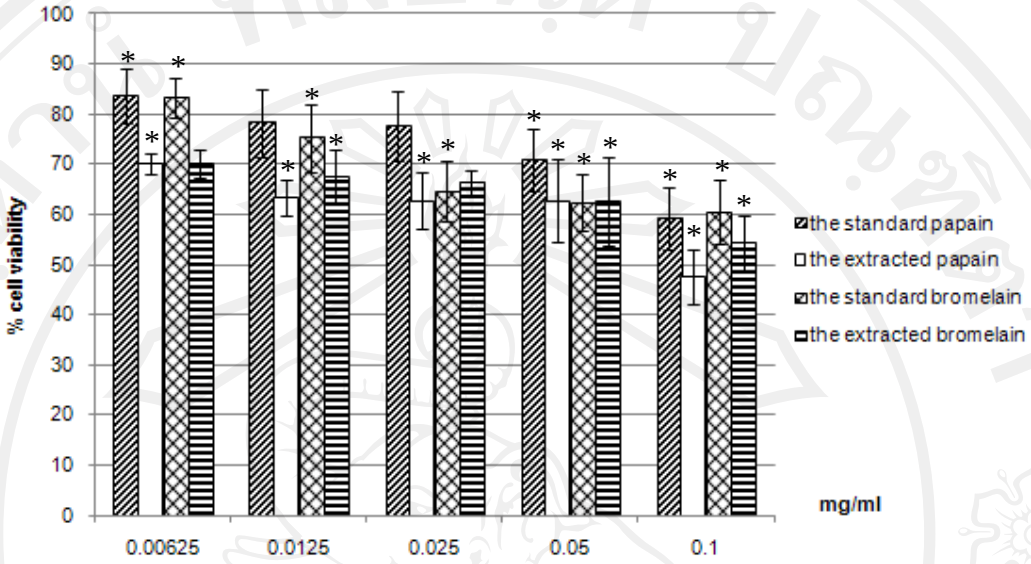
4.4 Cytotoxicity of NaC elastic niosomes loaded with the extracted enzymes on human skin fibroblast

The percentages of human skin fibroblast viability treated with the standard and extracted enzymes (papain and bromelain) were shown in **Figure 44A**. When the concentrations of the enzymes increased from 0.00625-0.1 mg/ml, cell viability was decreased. At high concentration (0.1 mg/ml), the standard papain, extracted papain, standard bromelain and extracted bromelain gave %cell viability at 59.23±6.06, 47.50±5.50, 60.40±6.36 and 54.26±5.57, respectively. It has been reported that when human skin contacted with papain at 0.2%w/v for 24 hours, large amounts of the intercellular materials in the stratum corneum were lost and most of the extracellular components were digested (Lopes et al., 2008). It has been demonstrated that bromelain exhibited cytotoxicity against fibroblasts at low concentration (0.1 µg/ml) (Honda et al., 2011). When the concentrations of the blank elastic niosomes increased from 0.03125 to

0.5 mM, cell viability was decreased. However, the blank elastic niosomes gave cell viability of more than 95%, even the concentration was at 0.5 mM. This may be affected by the increase permeability, lysis and solubilization of the lipid-protein-detergent association from the exposure of the cells to the surfactant compositions (Tween 61 and NaC) in the elastic niosomes (Moreno, 2000). In fact, the cell permeability can be detected by the permeability coefficient (*P*app) of the cellular uptake. It has been reported that the *P*app values of the drug across the human nasal epithelial cell increased with the increased ionic surfactant concentration in a dose dependent manner, and were significantly higher than the control (without the surfactant) (Lin et al., 2007).

The percentages of human skin fibroblast viability treated with the enzymes loaded in elastic niosomes were shown in **Figure 44B**. Both the standard and extracted papain and bromelain loaded in elastic niosomes at the concentration range of 0.00625-0.1 mg/ml gave no cytotoxicity with cell viability of more than 80%. The standard papain, extracted papain, standard bromelain and extracted bromelain at 0.1 mg/ml loaded in elastic niosomes showed %cell viability at 99.50±1.42, 99.79±5.09, 94.21±3.19 and 82.72±1.59, respectively, which were 1.68, 2.10, 1.56 and 1.52 times, respectively more than their corresponding free enzymes. The neutralization of the positive charges of the enzymes at pH 7.0 by the negative charges of the niosomes may result in charge density reduction, thereby decreasing the toxicity. The enzyme release from niosomes at the initial rapid phase appeared to be responsible for some cytotoxicity since the incubation time for the cytotoxic study was at 24 h and the initial and second release were at 6 and 48 h, respectively. Bromelain still appeared to be more toxic than papain when

A : Free enzymes



B : Enzymes loaded in elastic niosomes

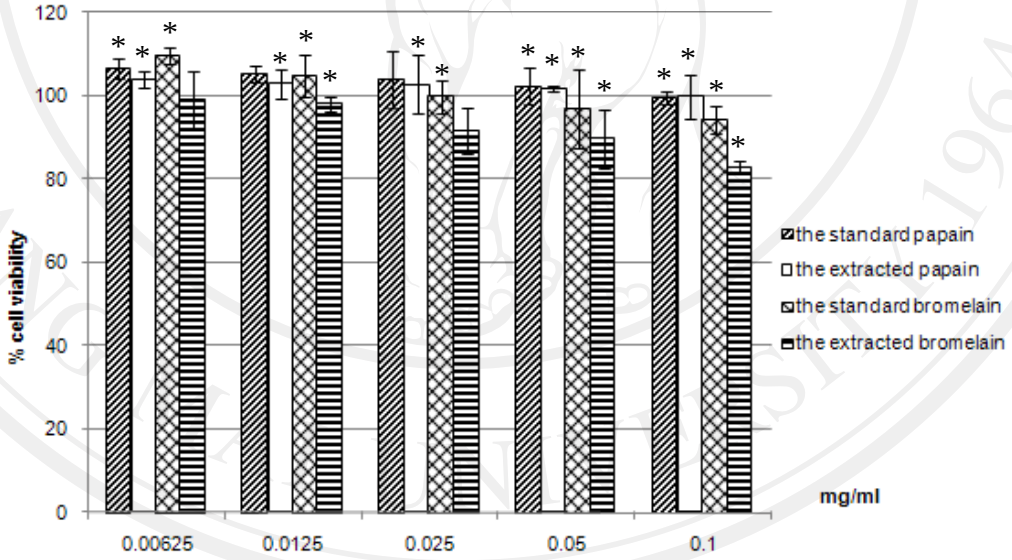


Figure 44 Cytotoxicity comparison of both the free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in NaC elastic niosomes on human skin fibroblasts by the SRB assay. (A): the free enzymes, (B): the enzymes loaded in elastic niosomes. Each value represented mean±S.D., n=4. * significant difference (p<0.05) in comparing between the free enzymes and enzymes loaded in elastic niosomes

loaded in elastic niosomes perhaps owing to the low entrapment efficiency of bromelain since more free bromelain, was left outside the niosomes and can expose to the cells. These results have suggested that the toxicity of papain and bromelain can be reduced when loaded in elastic niosomes.

4.5 Gelatinolytic activity (zymography) on MMP-2 of the extracted protease enzymes

Figures 45 and 46 showed the relative MMP-2 stimulation by zymography of the papain and bromelain loaded in elastic niosomes in comparing to the free enzymes, the untreated group, the negative (vitamin C) and positive (concanavalin A) control systems. All groups can stimulate MMP-2 activity more than the untreated groups and negative control groups. Vitamin C gave both pro MMP-2 and active MMP-2 inhibition activities with the relative MMP-2 stimulation values of 0.63±0.12 and 0.81±0.04, respectively. Cells treated with the standard papain, extracted papain, standard bromelain and extracted bromelain indicated the relative pro MMP-2 stimulation of 1.22±0.21, 1.39±0.21, 1.07±0.09 and 1.30±0.14, and the relative active MMP-2 stimulation of 1.28±0.15, 1.48±0.22, 1.10±0.23 and 1.14±0.18, respectively. When the enzymes were loaded in elastic niosomes, both the relative pro MMP-2 and active MMP-2 were slightly decreased, but not significant difference (p<0.05) in comparing to their corresponding free enzymes. The relative MMP-2 stimulation of the standard papain, extracted papain, standard bromelain and extracted bromelain loaded in elastic niosomes were 1.26±0.14, 1.34±0.15, 1.09±0.09 and 1.20±0.04 for the pro MMP-2, and 1.26±0.12, 1.41±0.23, 1.01±0.08 and 1.03±0.12 for the active MMP-2, respectively. The free or the adsorbed

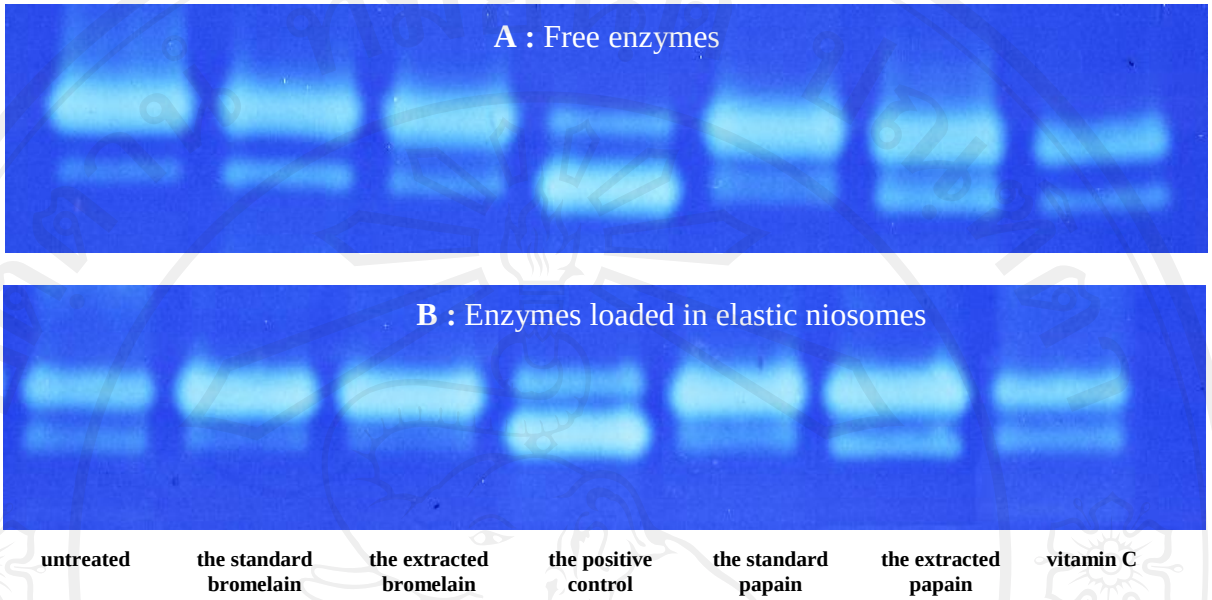


Figure 45 Zymograms of MMP-2 stimulation of both free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in elastic niosomes in comparing to the untreated cells, negative control (vitamin C) and positive control (concanavalin A). The positions of the pro MMP-2 (upper band) and the active MMP-2 (lower band) were indicated. (A): the free enzymes, (B): the enzymes loaded in elastic niosomes

enzymes on the niosomal membrane gave the MMP-2 stimulation activity at the first step and followed by the activity of the second step of the enzyme released from the inner niosomes (Manosroi et al., 2010b). This may be from the papain released from niosomes which was expected to be two phases with an initial rapid phase and followed by the slow release phase. The active MMP-2 stimulation activity of both free enzymes and enzymes loaded in elastic niosomes was lower than that of concanavalin A (3.44±0.16). However,

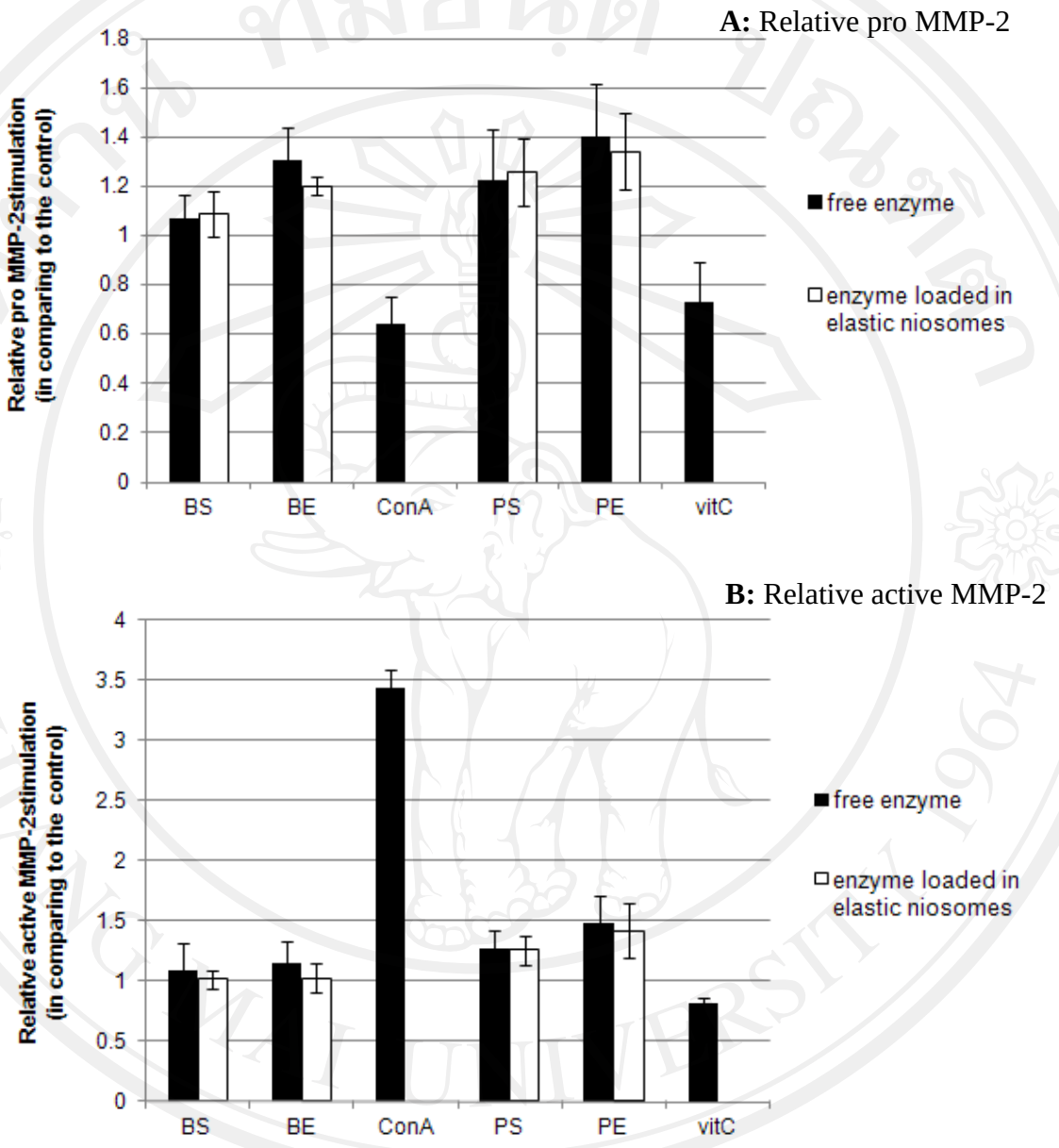


Figure 46 The relative pro MMP-2 and active MMP-2 stimulation of both free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in non-elastic and NaC elastic niosomes in comparing to the control. BS: standard bromelain, BE: extracted bromelain, PS: standard papain, PE: extracted papain. (A): the relative pro MMP-2, (B): the relative active MMP-2. Each value represented mean±S.D., *n*=3.

the pro MMP-2 stimulattion activity of both free enzymes and enzymes loaded in elastic niosomes was higher than that of concanavalin A (0.64±0.12), since concanavalin A is a MMP-2 activator which can convert the latent form (pro MMP-2) to the active form (active MMP-2). Thus, concanavalin A presented the decreased pro MMP-2 and the increased active MMP-2. The mechanism on the MMP-2 stimulation of these two proteases is still not known. One possibility is that the protease may induce the collagenase secretion (Werb and Aggeler, 1978) and activate the pro MMP-2 to change to the active MMP-2, resulting in the increase of both the pro MMP-2 and active MMP-2 expression on the zymogram. This result demonstrated that the MMP-2 stimulation activity of the standard and extracted papain and bromelain was still existing similar to their free enzymes even loaded in elastic niosomes.

4.6 Physical stability of elastic niosomes loaded with the papain (standard and extracted) and chemical stability of the papain (standard and extracted) loaded in elastic niosomes

According to the superior biological activities of papain to bromelain **(Table 26)**, free papain (standard and extracted) and papain (standard and extracted) loaded in non-elastic and elastic niosomes were selected to investigate for physico-chemical stability. The standard and extracted papain at the maximum loading concentrations loaded in non-elastic and elastic niosomes were physical stable with no sedimentation, no layer separation and no color change when stored at 4±2 for 8 weeks and at 27±2 °C for 3 weeks. After that, the flocculation appearance of the non-elastic and elastic niosomes loaded with the standard and extracted papain was observed at 4±2 and 27±2 °C. When

Table 26 Comparison of the biological activities of the standard and extracted protease enzymes (papain and bromelain)

Biological activities	Standard enzyme		Extracted enzyme	
	Papain	Bromelain	Papain	Bromelain
Free enzymes				
Free radical scavenging (SC_{50})[a]	1.71±1.45	684.27±100.13	956.10±62.06	-
Lipid peroxidase inhibition (IPC_{50})[b]	0.05±0.003	-	0.38±1.02	-
Cell viability at 0.1 mg/ml (%)	16.17±0.28	15.61±0.35	19.24±0.42	29.95±1.77
Relative MMP-2 stimulation	2.01±0.14	1.63±0.27	2.10±0.14	1.71±0.12
Enzymes loaded in elastic niosomes				
Cell viability at 0.1 mg/ml (%)	99.50±1.42	94.21±3.19	99.79±5.09	82.72±1.59
Relative MMP-2 stimulation	1.28±0.15	1.48±0.22	1.10±0.23	1.14±0.18

Note : [a]SC_{50} = scavenging concentration at 50% activity (mg/ml)

[b]IPC_{50} = inhibition peroxidation concentration at 50% activity (mg/ml)

kept at 45±2 °C, the non-elastic and elastic niosomes loaded with the standard and extracted papain did not show any aggregation for 12 weeks. However, the color of the niosomal dispersion became light yellow at 45±2 °C for 2 weeks. The vesicles may be destroyed and converted to the surfactant and cholesterol at 45±2 °C. The vesicular sizes and zeta potential values of non-elastic and elastic niosomes loaded with the standard and extracted papain were increased after stored at 4±2, 27±2 and 45±2 °C for 12 weeks from 215.8 to 5906 nm and -21 to -41 mv, respectively. The standard and extracted papain loaded in non-elastic and elastic niosomes kept at 27±2 °C gave larger vesicular sizes than those at 4±2 and 45±2 °C of 23.05 and 4.90, 7.11 and 3.17; 16.93 and 3.08, 2.88 and 4.44 times, respectively. The electrostatic interaction between the negative charge of the elastic niosomes and the positive charged papain may aggregate the vesicles.

For both the standard and extracted papain, the percentages remaining of free papain and papain loaded in non-elastic and elastic niosomes stored at 4±2, 27±2 and 45±2 °C for 12 weeks were demonstrated in **Figure 47**. Papain in non-elastic and elastic niosomes exhibited higher remaining amount than the free papain. Free papain and papain loaded in non-elastic and elastic niosomes at 4±2 °C were more stable than those at 27±2 and 45±2 °C. The standard papain solution kept at 45±2 °C for 2 weeks remained of only 47.44%, whereas papain loaded in non-elastic and elastic niosomes was at 56.43 and 67.37%, respectively. At high temperatures, the increase fluidity and permeability of the vesicular membrane may result in the leakage of papain from the vesicles, thereby facilitating the chemical degradation of papain by heat (Kim et al., 2009b).

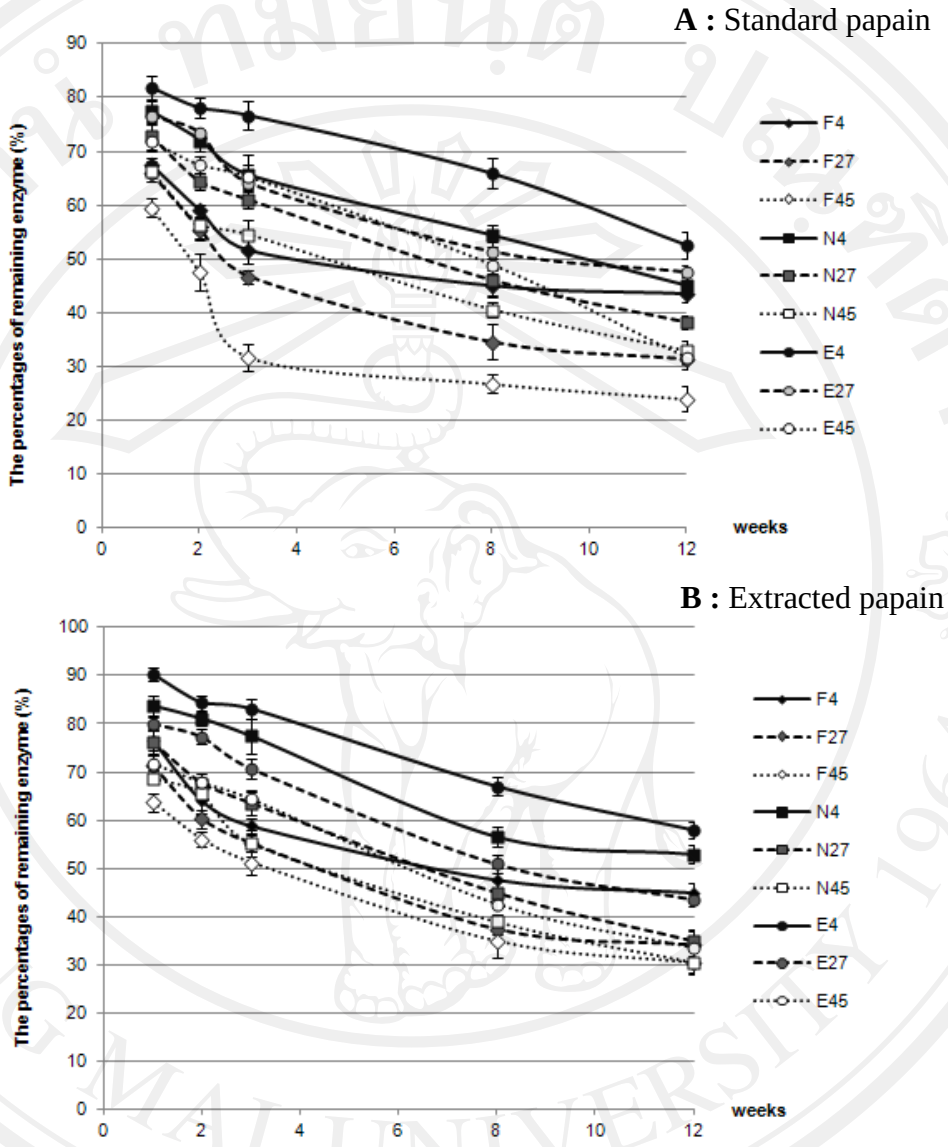


Figure 47 The percentages of remaining both free standard and extracted papain and the papain loaded in non-elastic and NaC elastic niosomes stored at different temperatures (4±2, 27±2 and 45±2 °C) for 12 weeks. (A): the standard papain, (B): the extracted papain; F4, F27 and F45 were the free papain kept at 4, 27 and 45°C; N4, N27 and N45 were papain loaded in non-elastic niosomes kept at 4, 27 and 45°C; E4, E27 and E45 were papain loaded in elastic niosomes kept at 4, 27 and 45°C, respectively.

After 8 weeks, the remaining percentages of the standard papain loaded in non-elastic and elastic niosomes were higher than the free standard papain of 1.20 and 1.46; 1.33 and 1.48; 1.51 and 1.82 times when kept at 4±2, 27±2 and 45±2 °C, respectively. The extracted papain loaded in non-elastic and elastic niosomes gave higher remaining amount of 1.19 and 1.40; 1.20 and 1.36; 1.11 and 1.21 times than the free extracted papain when stored at 4±2, 27±2 and 45±2 °C, respectively. In comparing non-elastic and elastic niosomes, the remaining percentages of the standard and extracted papain loaded in elastic niosomes were higher than those in non-elastic niosomes of 1.21 and 1.18; 1.11 and 1.13; 1.21 and 1.09 times when kept at 4±2, 27±2 and 45±2 °C, respectively. Both niosomes, especially the elastic niosomes appeared to protect the enzymes from degradation by high storage temperatures. NaC containing in the elastic niosomes can efficiently stabilize the structure of the niosomal membrane (Hashim et al., 2010). Therefore, loading in this elastic niosome can be one of the strategy to improve chemical stability of papain.

Part 5: Development of the PLGA nanospheres loaded with the standard papain

5.1 Characteristics of the standard papain loaded PLGA nanospheres

Papain loaded in PLGA nanospheres was prepared in the solvent system of acetone (5 ml) and 0.01 M hydrochloric acid (0.9 ml). Papain was expected to be stable in the mixed acetone/hydrochloric acid system which had the pH of 4.5 because the low concentration at 0.01M of hydrochloric acid was used. In fact, papain has been reported to be stable at pH 5.0 and becomes unstable at pH lower than 3.0 and above 11 (Norris et

al., 1970). In our first step of the double emulsion method, sonication which has been widely applied to produce stable emulsion (Hamed Mosavian et al., 2010) was used. Sonication can produce the stable emulsion containing small droplets which is critical for nanoencapsulation.

5.1.1 Particle size and zeta potential values

All PLGA nanosphere dispersions from the ESD and ESE methods were in colloidal appearance with milky white appearances, and no sedimentation or layer separation. After freeze drying, the white puffy PLGA nanosphere dry powder was obtained. The average particle size and zeta potential values of the blank PLGA nanosphere and those loaded with the standard papain by the ESD and ESE methods were demonstrated **(Table 27)**. The particle sizes of the blank PLGA nanospheres and those loaded with the standard papain by both methods were in the nanosize range (220 to 335 nm) and exhibited low polydispersity indicating a relatively narrow particle size distribution. The size of PLGA nanospheres slightly increased when loaded with papain. This may be from the encapsulated or adsorbed papain on the nanosphere surface. PLGA nanospheres which prepared by the ESE method gave smaller particle size and lower polydispersity index than the ESD method showing better of physical stability. The process variables such as PVA concentration and sonication may affect the particle size and the polydispersity index (Pereira-Lachataignerais et al., 2006). The ESE method had the sonication process in the emulsification step, while sonication was not used in the ESD method. The lower amount of PVA was also incorporated to prepare the nanospheres by the ESD (2%w/v) (Kawashima et al., 1998) than by the ESE (10%w/v)

Table 27 The comparison of particle sizes, zeta potential values and encapsulation efficiency (%EE) of PLGA nanospheres loaded with the standard papain prepared by the ESD and ESE methods

Samples	Method of preparation	Particle size (nm)		Polydispersity index (PDI)		Zeta potential (mV)		%EE
		BFD	AFD	BFD	AFD	BFD	AFD	
Blank nanospheres	ESD	317	335	0.317	0.539	-37.5±4.61	-34.8±11.7	-
	ESE	220	229	0.106	0.150	-33.7±5.68	-32.8±4.85	-
Papain loaded PLGA nanospheres	ESD	295	358	0.135	0.293	-36.2±6.82	-31.9±5.89	19.42±0.63
	ESE	229	232	0.052	0.151	-31.1±4.89	-29.6±7.18	43.03±0.15

Note : BFD = before freeze drying, AFD = after freeze drying and rehydration

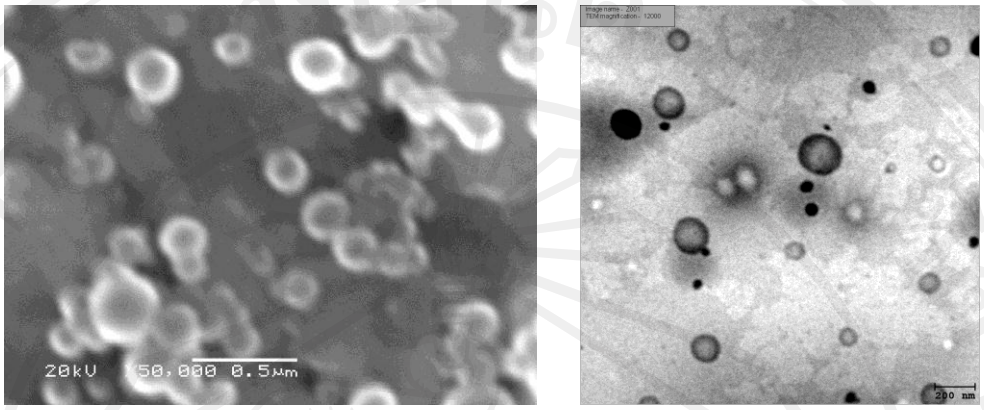
ESD = Emulsion solvent diffusion method in water

ESE = Water-oil-water (w/o/w) emulsion solvent evaporation method

(Tahara et al., 2009). It has been indicated that the high PVA concentration and the increase sonication time gave small particle size (Mainardes et al., 2005; Mukerjee et al., 2009). Several previous reports have also demonstrated that the increase of the PVA concentration in the external solvent/water interface resulting in the decrease of the interfacial tension (Galindo-Rodriguez et al., 2004), the significant increase in the net shear stress at a constant energy density during emulsification and the promotion for the formation of smaller emulsion droplets. The freeze dried nanospheres were readily redispersed in an aqueous medium by shaking manually, reproducing the original particle sizes before drying. It was assumed that PVA introduced into the system as dispersing agent was adsorbed on the surface of the nanospheres during freeze drying (Niwa et al., 1995). The PVA on the surface might improve the wettability of the nanospheres on redispersing.

5.1.2 Morphology of the standard papain loaded in PLGA nanospheres

The morphology and surface characterization of the standard papain loaded in PLGA nanospheres were observed by scanning electron (SEM) and transmission electron (TEM) microscopy. **Figure 48** showed the SEM and TEM microphotographs of the standard papain loaded in PLGA nanospheres prepared by the ESE method. The nanospheres were in spherical shape with the mean diameters of 250 nm and a smooth surface morphology.



A **B**

Figure 48 The scanning electron (A) and transmission electron (B) microphotographs of the PLGA nanospheres (composed of 100 mg PLGA and 10%w/v PVA403) loaded with 43 µg papain/mg PLGA nanospheres prepared by the ESE method (12000X)

5.1.3 Encapsulation efficiency of the standard papain loaded in PLGA nanospheres

Encapsulation efficiency of the standard papain loaded in PLGA nanospheres was summarized in **Table 27**. The encapsulation efficiencies of papain in the PLGA nanospheres prepared by ESD and ESE method were at 19.42±0.63 and 43.03±0.15%, respectively. The ESE method which gave about two times higher entrapment efficiency than the ESD method appeared to be the suitable method to load papain in the PLGA nanospheres. In the ESE method, papain which is a water-soluble peptide can be introduced into the internal aqueous phase of the multiple emulsions, resulting in an increase of encapsulation efficiency in the nanospheres. The encapsulation efficiency of water-soluble drugs in PLGA nanospheres by the ESD

method of less than 20% was reported (Kawashima et al., 1998; Yin et al., 2006). This is most probably due to the rapid diffusion of the enzymes into the water phase when their solution containing PLGA was poured into the aqueous medium. However, the high encapsulation efficiency of more than 20% was observed by the ESE method. This method has also been used to prepare water-soluble drug loaded nanospheres. It is supposed that the oil phase surrounding the internal water phase would prevent the leakage of the drug into the external water phase. Thus, the high encapsulation efficiency of more than 20% by the ESE method was obtained. In addition, the higher PVA concentration in the ESE method can affect the stability of the w/o emulsion providing a higher mass transferring resistance, thereby reducing the amount of the enzyme molecules diffusing from the internal into the external water phase during the emulsification (Sun et al., 2005). Also, when the polymer solution precipitated to form nanospheres, PVA could promote compatibility between the hydrophilic enzyme molecules and the hydrophobic polymer network, which is preferential for enzyme to locate within the nanosphere matrix.

5.2 The release profile of the standard papain from the PLGA nanospheres

The release profile of the standard papain from the PLGA nanospheres prepared by the ESD and ESE methods in 0.2 M phosphate buffer (pH 7.0) solution at 27±2 °C gave a 2-phase release profile as demonstrated in **Figure 49**. Papain released from the nanospheres prepared by the ESD method were 65, 82, 84 and 87% at 2, 6, 8 and 24 h, respectively and almost completed (90%) after 48 h. The slower release rate was observed in the ESE method which gave 48, 62, 63, 64 and 72% at 2, 6, 8, 24 and 48

h, respectively. The high PVA concentration of the ESE method can not only increase the viscosity of the external phase and the difficulty for the enzyme to diffuse out of the nanospheres, but also gave a more stable emulsion which can hinder the mass transfer of the enzyme to the surroundings (Yang et al., 2001).

The initial burst release of papain was observed within the first 6 h with the steep slope of 12.385 and 9.4899 $\%h^{-1}$ and followed by a plateau with the slope of 0.1678 and 0.2128 $\%h^{-1}$ of the ESD and ESE method, respectively. The first phase was faster than the second phase of about 73 and 45 times, respectively. The burst release in the first phase may be caused by a rather high mobility of the enzyme in the particles especially the small particle size, which has the short diffusion distance.

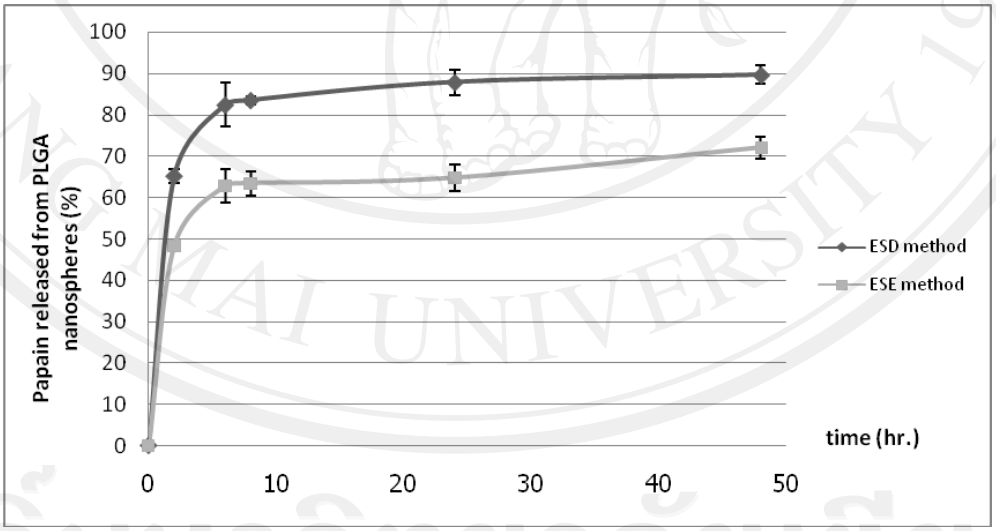


Figure 49 Comparision of the release profile of the standard papain from the PLGA nanospheres prepared by the ESD (emulsion solvent diffusion method in water) and ESE (w/o/w emulsion solvent evaporation method) in 0.2 M phosphate buffer (pH 7.0) solution at 27±2 °C

The slow release of papain from the nanospheres may be not only from the electrostatic interaction between the enzyme and the polymer (Singh et al., 1995), but also the charge interaction between papain which has the positive charge (PI=8.75) in phosphate buffer (pH 7.0) and the negative charge of the carboxylic-end group of the polymer on the nanoparticle surface. Other possibilities including the total content of the drug in the nanospheres which became less and less over the release time, the drug molecules located in the center of the nanospheres that have longer distance to diffuse and the crystallinity of the polymer which may have increased during the incubation time in the phosphate buffer may affect the slow release rate of papain in the second phase (Miyajima et al., 1997; Xu et al., 2006; Xu et al., 2008; Xu et al., 2009). **Table 28** showed the release kinetics of papain from the PLGA nanospheres. According to various kinetic models, the first phase of the enzyme release from the nanospheres prepared by the ESD and ESE method can be estimated for linearity fitting as the Higuchi's equation plot which gave the r^2 values of 0.9456 and 0.9554, respectively. These kinetics demonstrated the release of the enzyme from the matrix as a square root of time dependent process base on the Fickian diffusion which described the release by the pure diffusion of the enzyme from the nondegradable matrix (Shoaib et al., 2006). However, the second phase of the enzyme released from the nanospheres prepared by the ESE method was best fitted in the zero order kinetic with the r^2 value of 0.9409, while that by the ESD method was the Higuchi kinetics that gave the r^2 values of 0.9566. The release rate of papain from the PLGA nanospheres of the ESD and ESE method gave 13.73 % $h^{-1/2}$ and 10.47% $h^{-1/2}$ of the first phase and at 1.88% $h^{-1/2}$ and 1.56% h^{-1} of the second phase, respectively.

Table 28 The release kinetics of the standard papain from the PLGA nanospheres prepared by the the ESD and ESE method in 0.2 M phosphate buffer (pH 7.0) solution at 27±2 °C for 48 hours

		Zero order		First order		Higuchi	
		r^2	k_0 (h^{-1})	r^2	k_1 (h^{-1})	r^2	k_H ($h^{-1/2}$)
ESD method	First phase	0.7578	12.385	0.9047	0.2724	0.9456	34.375
	Second phase	0.8946	0.1678	0.9243	0.0124	0.9566	1.6275
ESE method	First phase	0.7769	9.4899	0.8614	0.1531	0.9554	26.147
	Second phase	0.9409	0.2128	0.9312	0.0067	0.8750	1.9254

Note : Zero order equation : $C = k_0 t$, where k_0 was the zero-order rate constant expressed in units of papain concentration and time, and t was the time in hours.

First order equation : $LogC = LogC_0 - k_t/2.303$, where C_0 was the initial concentration of papain and k was the first order constant.

Higuchi equation : $Q = k_H t^{1/2}$, where Q was the amount of papain released per unit surface area at time (h.), and k_H was the release rate constant

This different release behavior may be resulted from the differences of the nanosphere structures depending on the method of preparation. In the ESD method, the enzyme dispersed evenly in the matrix of the polymer due to the coprecipitation of the polymer and enzyme (Kawashima et al., 1998). The nanospheres prepared by the ESE method may construct a reservoir like structure (Matsumoto et al., 2005), that papain entrapped in the core of the nanospheres can be released by diffusing through the polymer shell indicating the zero order kinetics (Betancourt et al., 2008).

5.3 *In vitro* cytotoxicity of the standard papain loaded in PLGA nanospheres

The human skin fibroblasts incubated with the free papain at 10 µg/ml showed cytotoxicity on human skin fibroblasts of 16.17±0.28% cell viability with the IC_{50} value at 6.22 µg/ml. It has been shown that when human skin contacted with papain at 0.2%w/v for 24 hours, the large amount of the intercellular material in the stratum corneum was lost and most of the extracellular components were digested (Lopes et al., 2008). The blank PLGA nanospheres at more than 1 µg/ml gave of cell viability more than 80%. PLGA which is the main composition of the nanospheres is a biodegradable and biocompatible polymer, thereby being well tolerated by the cells. The percentages of the human skin fibroblast viability treated with the blank nanospheres and nanospheres loaded with papain prepared by both ESD and ESE methods were shown in **Figure 50**. The percentages of cell viability were compared between the two methods at different nanosphere concentrations. When the concentrations of the nanospheres loaded with papain increased from 0.001 to 10 µg/ml, cell viability was decreased. The loaded nanospheres at the concentration range of 0.001-0.1 g/ml gave no significant difference at

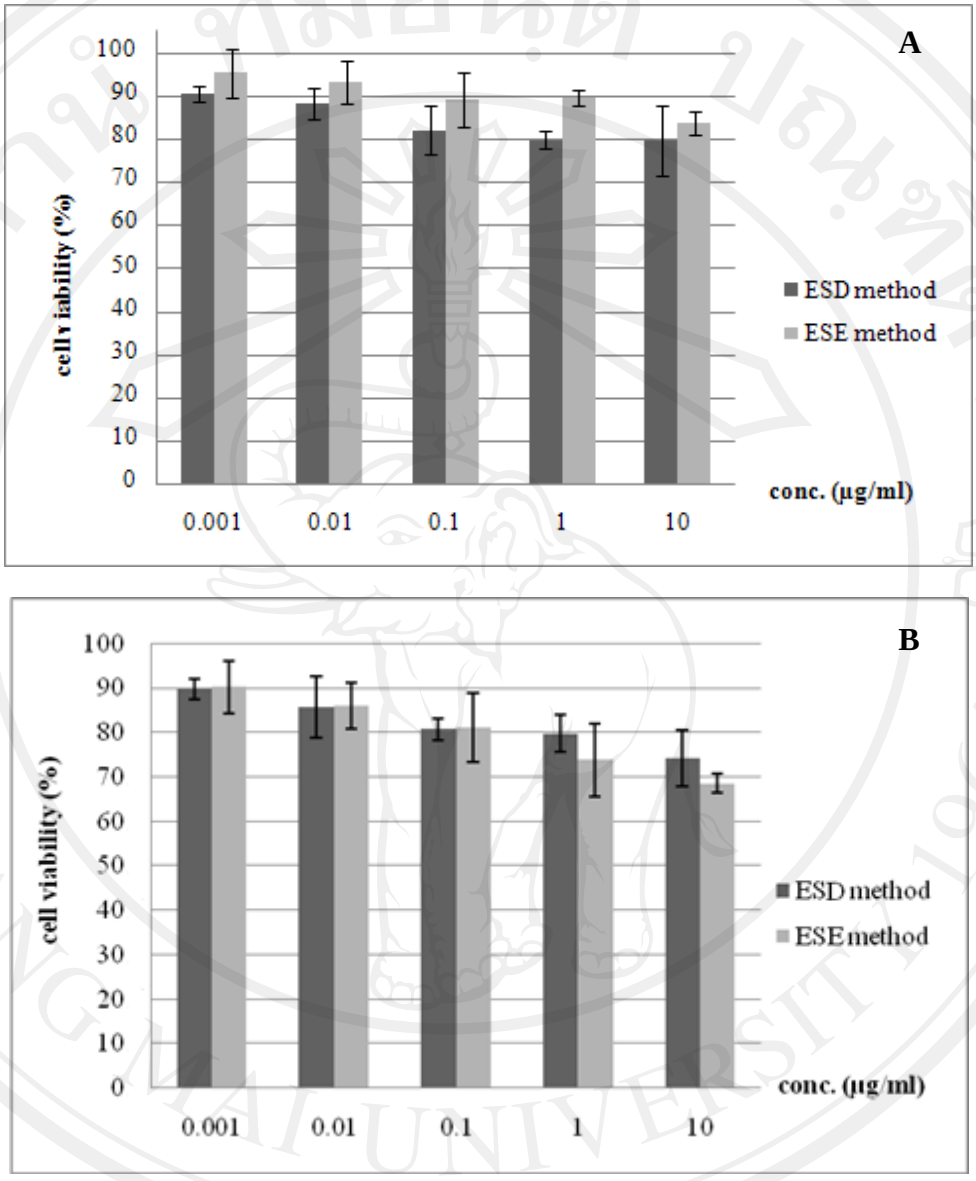


Figure 50 The percentages of human skin fibroblast viability by the SRB assay of (A) the blank nanospheres and (B) the PLGA nanospheres loaded with papain prepared by the ESD (emulsion solvent diffusion in water) method (19 µg papain/mg PLGA nanosphere) and the ESE (w/o/w emulsion solvent evaporation) method (43 µg papain/ mg PLGA nanosphere)

p>0.05 of cell viability of more than 80% in comparing to the untreated cells. At 0.001, 0.01 and 0.1 µg/ml of the PLGA nanospheres loaded with papain prepared by the ESD method gave cell viability at 89.62±2.31, 85.73±6.95 and 80.64±2.38%, respectively, while those by the ESE method were 90.15±5.82, 86.01±5.11 and 81.11±7.73%, respectively. At higher concentrations (1-10 µg/ml), PLGA nanospheres loaded with papain prepared by the ESD method gave slight higher cell viability (but no significant difference p>0.05) than those prepared by the ESE method. This may be due to the no difference in zeta potential values of the PLGA nanospheres loaded with papain prepared by both methods after freeze drying and rehydration (Kolhatkar et al., 2007). The zeta potential values of papain loaded PLGA nanospheres may relate to the cell viability. The larger the zeta potential values of the PLGA nanospheres, the stronger the interaction between the nanosphere membrane and the cell membrane leading to higher cytotoxicity (Qi et al., 2005).

Cytotoxicity of papain loaded in nanospheres prepared by these two methods was also compared. The papain contents in the nanospheres prepared by ESD were different from those by the ESE method (19 and 43 µg papain/mg PLGA nanosphere, respectively). When the IC_{50} values of the papain cytotoxicity were determined, the cytotoxicity of papain loaded in the nanospheres prepared by the ESD method was higher than those prepared by the ESE. Papain loaded in PLGA nanospheres prepared by the ESD method gave the IC_{50} values at 6.19 µg/ml which appeared to be more toxic than those by the ESE method with the IC_{50} values at 9.13 µg/ml. This may be due to the higher release rate of papain from the PLGA nanospheres prepared by the ESD method

than that by the ESE method as mentioned above **(Figure 49)**. The higher released papain in the cell culture medium of the PLGA nanospheres prepared by the ESD method result higher cytotoxicity to the cells than that of the nanospheres by the ESE method, which gave the lower released papain. The cell cytotoxicity of the free papain was more than papain loaded in the nanospheres prepared by the ESE method of 1.5 times, while no significant difference between the free papain and papain loaded in the nanospheres prepared by the ESD method. The results from this study has suggested that the cytotoxicity of papain was reduced when loaded in PLGA nanospheres prepared by the ESE method.

5.4 Physicochemical stability at various storage temperatures of papain loaded in PLGA nanospheres

The physico-chemical stability of papain loaded in PLGA nanospheres was performed in phosphate buffer at pH 5.0, while the released study of papain from PLGA nanospheres was done in the phosphate buffer at pH 7.0. Papain is expected to release from the PLGA nanospheres after penetration into the skin. The pH environments of the inner skin layer is similar to the physiological pH. So, the release study of papain from PLGA nanospheres was done at pH 7.0 (the physiological pH is in the range of 6.4 to 7.5) (Banks et al., 1976). However, the stability of papain has been studied at pH 5.0 since this pH value gave the highest activity of papain and this pH will be proposed to formulate the topical preparation of papain loaded in nanospheres (Pendzhiev, 2002). Papain loaded in PLGA nanospheres prepared by the ESE method determined by visual observation showed good physical stability with no sedimentation, layer separation and

color change at 4±2 and 25±2 °C for six weeks. At 45±2 °C, some aggregation of the particles was observed after one week. After stored for three weeks at 45±2 °C, the milky colloidal nanosphere dispersion became clear solution by visual observation and the particle size can not be detected by a laser particle size analyzer. The nanospheres were completely degraded possible due to the increase hydrolytic degradation of the PLGA polymer at high temperature (Jain et al., 2010).

The percentages remaining of papain in the PLGA nanospheres prepared by the ESE method stored at 4, 25 and 45°C for 6 weeks were shown in **Figure 51**. At 4 and 25°C, papain in the nanospheres exhibited higher remaining amount than in the solution. However, papain loaded in the nanospheres was unstable at 45°C because of the degradation of the PLGA polymer resulting in the leakage of the enzyme from the nanospheres and the destruction of the enzyme by heat. The percentages of papain remaining in PLGA nanospheres stored at 4 °C for 3 weeks significantly lower than those stored at 25 °C for 3 weeks and the percentages of papain remaining in phosphate buffer at 4 °C became lower at 2.5 to 6 weeks in comparing to that kept at 25 °C, because papain might form large insoluble materials and aggregation at low temperature (Wang, 1988). Another possible reason was also from the changing features of low temperature denaturation. After three weeks at 45°C, all PLGA nanospheres were completely degraded and the remaining papain was only the released enzyme in the supernatant. After six weeks at 4°C, the enzyme contents in the nanospheres (71.50%) were about 8 times higher than those in the solution (9.32%). The nanospheres can protect papain against aggregation at low temperature by increasing electrostatic repulsion between the

particular membranes. At 25°C, the enzyme remained in the nanospheres were 61.88% which were 3 times higher than those in the solution (22.66%). PLGA nanospheres can shield papain from thermal degradation at high temperature.

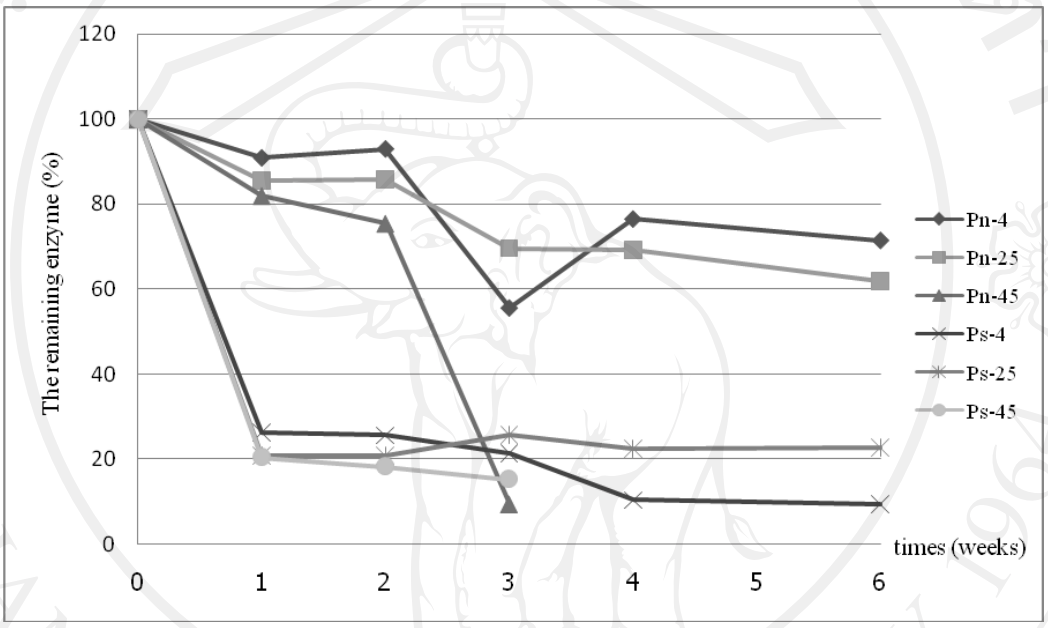


Figure 51 The percentages remaining of the free papain and papain loaded in PLGA nanospheres prepared by the ESE (w/o/w emulsion solvent evaporation) method stored at different temperatures (4±2, 25±2 and 45±2 °C) for 6 weeks; Pn-4: papain loaded in PLGA nanospheres kept at 4°C; Pn-25: papain loaded in PLGA nanospheres kept at 25°C; Pn-45: papain loaded in PLGA nanospheres kept at 45°C; Ps-4: papain solution in 0.2 M phosphate buffer (pH 5.0) kept at 4°C; Ps-25: papain solution in 0.2 M phosphate buffer (pH 5.0) kept at 25°C and Ps-45: papain solution in 0.2 M phosphate buffer (pH 5.0) kept at 45°C

The PLGA nanospheres appeared to protect the enzyme from peptide aggregation and degradation at low (4°C) and high (25°C) storage temperature. Thus, chemical stability and the shelf life of papain can be improved when loaded in PLGA nanospheres. This result agreed with the previous study which demonstrated that nafarelin stability was improved when loaded in nanospheres in comparing to the drug in the acidic medium (Niwa et al., 1994).

Part 6: Development of gel containing papain loaded in nanovesicles and nanoparticles for scar treatment

6.1 Physical characteristics of gel containing papain loaded in nanovesicles and nanoparticles

All prepared gel formulations were in white viscous creamy with smooth and homogeneous appearance. They were easily spreadable with acceptable bioadhesion. **Table 29** showed the physical characteristics of all gel samples. All gels gave the pH values in the range of 5.5-6 which is suitable for skin application. The vesicular sizes observed by zetasizer apparatus of nanovesicles and nanoparticles in all gel formulations were in the range of 220.7 to 520.2 nm which were larger than the sizes of the nanovesicular and nanoparticular dispersions not incorporated in gel (143 to 223 nm), and exhibited low polydispersity indicating a relatively narrow particle size distribution. These larger sizes when incorporated in the gels may be from the swelling of Carbopol polymer in an aqueous solution. The vesicle size observed in the gel formulations increased when incorporated both with the free papain and papain loaded in nanovesicles

Table 29 Physical characteristics of gel containing papain loaded in nanovesicles (non-elastic and elastic niosomes) and nanoparticles (PLGA nanospheres)

Formulations	pH value	Specific gravity (g/ml)	Viscosity (cP)	Vesicular size (nm)	Polydispersity index (PDI)	Zeta potential (mv)
Gel base (GB)	6	1.0576	92440	220.7±6.379	0.453±0.02	-40.8±0.934
Gel containing free papain (GS)	6	1.0709	67060	378.4±26.18	0.499±0.068	-53.7±2.20
Gel containing papain loaded in PLGA nanospheres (GPN)	6	1.0493	65410	332.5±12.6	0.562±0.053	-43.0±5.95
Gel containing papain loaded in non-elastic niosomes (GNN)	5.5	1.0074	83430	441.8±44.64	0.515±0.107	-44.5±2.71
Gel containing papain loaded in elastic niosomes (GEN)	5.5	1.0464	56220	520.2±36.30	0.593±0.034	-46.2±2.03

Papain was loaded at 1 mg/ml in all formulations.

PLGA nanospheres composed of poly (lactide-co-glycolic acid) as biodegradable polymers and polyvinyl alcohol as a dispersing agent.

Elastic niosomes composed of Tween 61/cholesterol/sodium cholate at 1:1:0.1 molar ratio, while non-elastic niosomes, no sodium cholate was added.

Values represent mean±SD of triplicate of three experiments.

and nanoparticles. The intercalation of the vesicles or particles in the matrices of Carbopol gel may give this effect. All gel formulations exhibited the negative zeta potential values in the range of -44.5 to -53.7 mv. The negative characteristics of the formulation may be from the carboxyl groups of Carbopol. However, the zeta potential of the gel formulations increased when incorporated with papain loaded in nanovesicles and nanoparticles owing to the negative charge on the nanovesicular and nanoparticular surface. In fact, papain has the positive charge in phosphate buffer solution at pH 7.0. In the previous study, the purity of the extracted papain determined by SDS-PAGE and HPLC were 77.68 and 82.31%, respectively. Thus, the impurity of the extracted enzymes such as chymopapain (PI=10.6) and caricain (PI=10.9) containing in the papaya latex may give the negative charge and result in the higher zeta potential of gel containing free papain than the gel base. The viscosity of gels was decreased when incorporated with the nanovesicles and nanoparticles. This may be due to the effect of the surfactant molecules surrounding each Carbopol particle that may form an intrapolymeric micelles and break the interpolymer connections, resulting in the reduction of gel viscosity (Barreiro-Iglesias et al., 2001).

6.2 Transdermal absorption through rat skin

The cumulative amounts and fluxes per area of gel containing free papain and papain loaded in various systems (non-elastic niosomes, elastic niosomes and PLGA nanospheres) in whole skin and the receiver solution following transdermal absorption across the excised rat skin were shown in **Figures 52 and 53**. During the period of 6 h, the cumulative amounts of papain from gel containing papain loaded in elastic niosomes

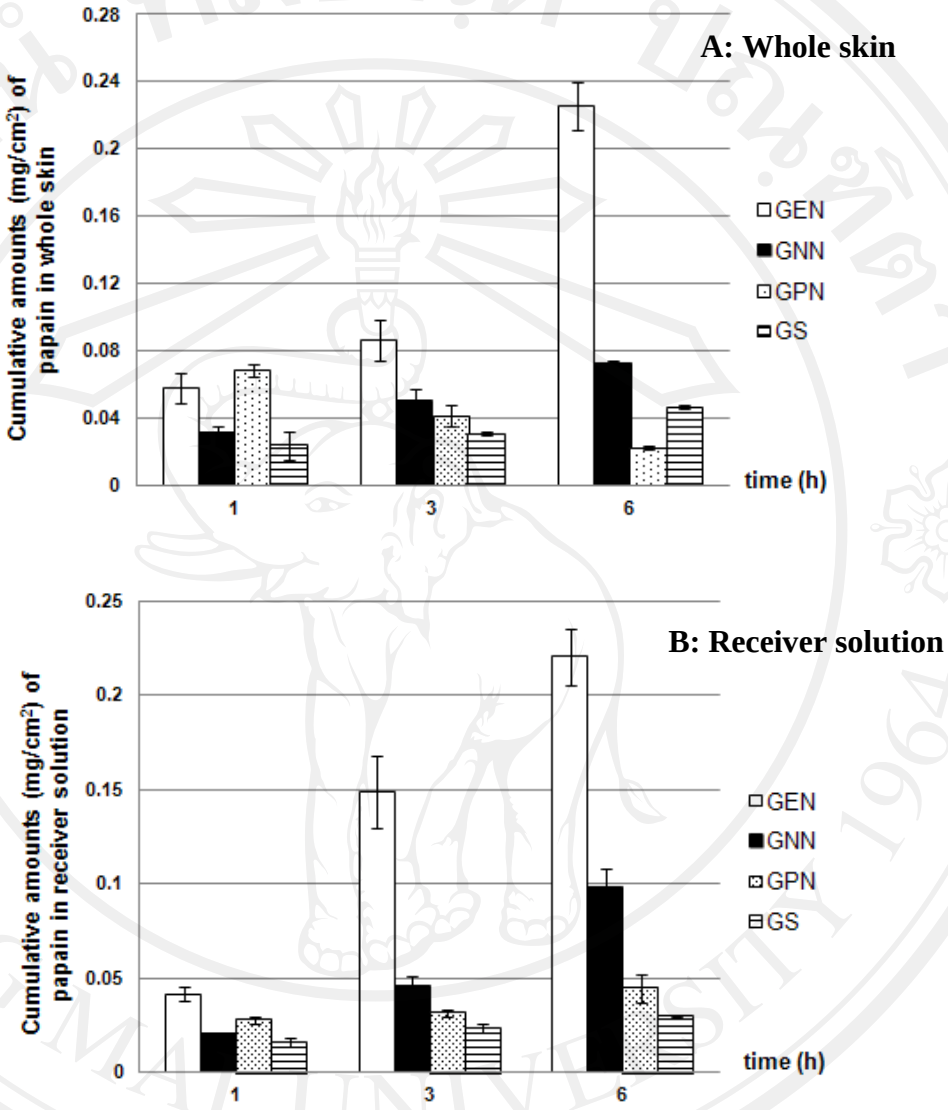


Figure 52 Cumulative amounts (mg/cm^2) of papain from various formulations in whole skin (A) and receiver solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells (each value represents mean±S.D., *n*=3). GEN = gel containing papain loaded in elastic niosomes, GNN = gel containing papain loaded in non-elastic niosomes**,** GPN = gel containing papain loaded in PLGA nanospheres and GS = gel containing free papain. All formulations contained papain at 1 mg/ml.

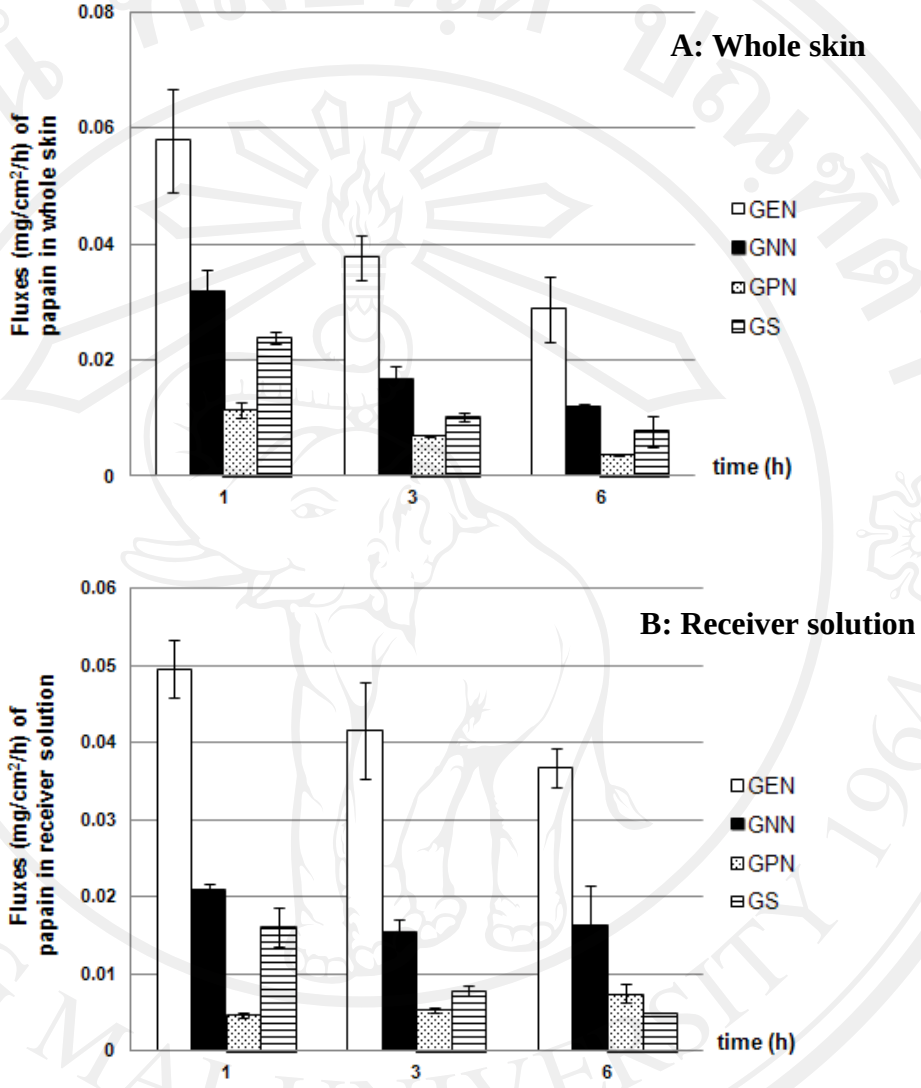


Figure 53 The fluxes (mg/cm^2/h) of papain from various formulations in whole skin (A) and receiver solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells (each value represents mean±S.D., *n*=3). GEN = gel containing papain loaded in elastic niosomes, GNN = gel containing papain loaded in non-elastic niosomes**,** GPN = gel containing papain loaded in PLGA nanospheres and GS = gel containing free papain. All formulations contained papain at 1 mg/ml.

(GEN), gel containing papain loaded in non-elastic niosomes (GNN) and gel containing free papain (GS) were increasing both in the whole skin and the receiver compartment solution. However, the cumulative amounts of papain from gel containing papain loaded in PLGA nanospheres (GPN) were decreasing in the whole skin but increasing in the receiver solution. This may be due to the transcutaneous hydration gradient which can act as the driving force for papain in the whole skin to move to the receiver compartment solution (Manosroi et al., 2012a). This can be explained that the non-elastic and elastic niosomes can form trans-epidermal osmotic gradient and can penetrate into the epidermis by hydration force, while the PLGA nanospheres are only able to penetrate into the superficial layers of the SC and from there the loaded papain may be released into the deeper skin layer (Desai et al., 2010). Futhermore, the penetration can also be due to the adhesion of the vesicle to the skin surface followed by the destabilization and finally the fusion with the SC lipid matrix. It has been demonstrated that elastic vesicles were often localized and accumulated in the channel like regions which are located within the intercellular lipid lamellae of the skin surface (Loan Honeywell-Nguyen et al., 2002). GEN exhibited the highest papain amount both in the whole skin and the receiver solution in comparing to GNN, GPN and GS. GEN gave the cumulative amounts of papain after 6 h in the whole skin and the receiver solution at 0.226±0.014 and 0.220±0.015 mg/cm^2 which were 3.12 and 2.24 times higher than GNN (which gave 0.072±0.001 and 0.098±0.010 mg/cm^2), 10.26 and 4.93 times than GPN (which gave 0.022±0.001 and 0.045±0.007 mg/cm^2), and 4.85 and 7.38 times than GS (which gave 0.046±0.016 and 0.030±0.0004 mg/cm^2), respectively. During the period of 6 h, the

fluxes of papain in GEN, GNN and GS decreased both in the whole skin and the receiver compartment solution. Nevertheless, the fluxes of papain in GPN decreased in the whole skin, but increased in the receiver solution. GEN exhibited the highest flux after 1 h in the whole skin and the receiver solution of 0.058±0.009 and 0.049±0.002 mg/cm^2/h which were 1.82 and 2.38 times of GNN (which gave 0.032±0.004 and 0.021±0.001 mg/cm^2/h), 5.06 and 10.63 times of GPN (which gave 0.011±0.001 and 0.005±0.0003 mg/cm^2/h) and 2.43 and 3.07 times of GS (which gave 0.024±0.001 and 0.016±0.0002 mg/cm^2/h), respectively.

As known, the vesicular systems were able to promote skin penetration of papain by a variety of mechanisms including the intracellular, intercellular, and transappendageal of the free papain, the penetration enhancing process of the niosomal components and the adsorption of the vesicles to the SC. However, there is still considerable debate whether elastic vesicles act as the carrier systems by penetrating intact through the skin. In this study, the highest penetration of papain was observed in the elastic niosomes containing NaC which may act as an edge activator. During deformation, the NaC molecules tended to accumulate at the site of the increased stress, due to their propensity for the curved structures, thus reducing the energy required for changing the shape (Morrow et al., 2007). For other possible mechanism, the head groups of the NaC molecules may intercalate between the Tween 61 molecules and decrease the packing density resulting in the high flexibility of the niosomal membranes which can permit the elastic vesicles to squeeze themselves through the skin pores that are much smaller than their diameters. The distribution of papain in the skin layer from

the present study suggested that papain did not only remain in association with the niosomal material in the upper and middle layers of the skin, but also penetrated through the lower skin layer. This result may be advantageous for the treatment of hypertrophic scars or keloids by papain because papain can be found in the dermis where excessive collagen is accumulated as the scars.

The transdermal fluxes of free papain was lower than that loaded in nanovesicles and nanoparticles due to the hydrophilic property of papain which may substantially reduce the partition coefficient between the skin and papain thereby decreasing the flux values (Kumar et al., 2011). The higher transdermal flux of papain loaded in nanovesicular and nanoparticular formulations than the free papain may be due to the synergistic effect of the increase partition coefficient between the skin and papain, the alteration of the skin barrier properties from the interaction of cholesterol or PLGA polymer in the nanovesicles and nanoparticles, respectively with the skin lipid component and the elasticity of the vesicular membrane (Maurya et al., 2010).

When incorporated in gels, the cumulative amounts and transdermal flux of papain loaded in elastic niosomes (GEN), non-elastic niosomes (GNN) and free papain (GS) were higher than those not incorporated in gel formulations of 17.48, 3.40 and 6.76; 13.35, 3.40 and 6.76 times in the whole skin while 3.32, 2.77 and 1.89; 3.33, 2.77 and 1.89 times in the receiver solution. The gel structure may promote the penetration of papain across the SC from the occlusion effects of the gel composition thereby enhancing skin hydration and consequently increasing the absorption and penetration of papain across the skin (Cevc et al., 2008; Manosroi et al., 2010b).

6.3 Rabbit skin irritation

The calculated PII values of all formulations except GS (PII = 0.78, slight irritation) and the positive control (20% sodium lauryl sulfate, PII = 1.89, slight irritation) on rabbit skin by the closed patch test were in the range of 0.00-0.44 **(Table 30)**. Some irritation may be from the papain since the gel base gave no irritation (PII = 0.22). In fact, the allergic reaction to skin contacted with papain has also been reported (Ezeoke, 1985; Epstein, 2000). The common side effects of topical formulation of papain are temporary burning sensation and mild skin irritation. The blank elastic niosomes (BEN, PII = 0.22), non-elastic niosomes (BNN, PII = 0.11) and PLGA nanospheres (BPN, PII = 0.33) showed no skin irritation. GEN, GNN and GPN gave lower PII values than GS. The decrease irritation of GEN, GNN and GPN may be due to the encapsulation of papain in the bilayer of niosomes or the matrix of nanospheres that reduces the direct contact of the free papain to the skin (Kaur et al., 2010). Papain loaded in elastic niosomes (PEN), papain loaded in non-elastic niosomes (PNN) and papain loaded in PLGA nanospheres (PPN) also showed no skin irritation with the PII range of 0.33-0.44. When papain was loaded in nanovesicles and nanoparticles (PEN, PNN and PPN), they showed the highest erythema index after 24 h of application. However, after 72 h, the erythema indexs of all sites were similar to initial and the erythema sign from all samples disappeared. During the peroid of 24 h, the erythema may be from the free papain which was not loaded in nanovesicles and nanoparticles. When incorporated in the gel formulation, the PII values of papain loaded in PLGA nanospheres, non-elastic niosomes and elastic niosomes (0.44, 0.44 and 0.33) were decreased to 0.22, 0.33 and 0.11,

Table 30 Primary irritation index (PII) and category of irritation based on PII of various gel formulations

Formulations	Primary irritation index (PII)				Category of irritation
	24 h	48 h	72 h	average	based on PII
Phosphate buffer solution (pH 7.0)	0.00	0.00	0.00	0.00	Negligible
Blank PLGA nanospheres (BPN)	0.00	0.67	0.33	0.33	Negligible
Blank non-elastic niosomes (BNN)	0.33	0.00	0.00	0.11	Negligible
Blank elastic niosomes (BEN)	0.67	0.00	0.00	0.22	Negligible
Papain loaded in PLGA nanospheres (PPN)	0.67	0.33	0.33	0.44	Negligible
Papain loaded in non-elastic niosomes (PNN)	0.67	0.33	0.33	0.44	Negligible
Papain loaded in elastic niosomes (PEN)	0.67	0.33	0.00	0.33	Negligible
Gel base (GB)	0.33	0.33	0.00	0.22	Negligible
Gel containing free papain (GS)	1.67	0.67	0.00	0.78	Slight irritation
Gel containing papain loaded in PLGA nanospheres (GPN)	0.67	0.00	0.00	0.22	Negligible
Gel containing papain loaded in non-elastic niosomes (GNN)	0.67	0.33	0.00	0.33	Negligible
Gel containing papain loaded in elastic niosomes (GEN)	0.33	0.00	0.00	0.11	Negligible
20% sodium lauryl sulfate (positive control)	2.67	1.67	1.33	1.89	Slight irritation
Untreated area (negative control)	0.00	0.00	0.00	0.00	Negligible

Grading scale for skin irritation effect following OECD Test Guideline 404

Papain was at 1 mg/ml in all formulations.

PLGA nanospheres composed of poly (lactide-co-glycolic acid) as biodegradable polymers and polyvinyl alcohol as a dispersing agent.

Elastic niosomes composed of Tween 61/cholesterol/sodium cholate at 1:1:0.1 molar ratio, while non-elastic niosomes, no sodium cholate was added.

Values represent mean±SD of triplicate of three experiments.

respectively indicating of no skin irritation. Thus, the gel containing the Carbopol polymer may reduce the direct contact between the free papain and the skin (Manosroi et al., 2011b).

6.4 The physico-chemical stability of gel containing papain loaded in nanovesicles

The cumulative amounts of papain from gel containing papain loaded in elastic niosomes (GEN) and non-elastic niosomes (GNN) were increased with times. These formulations were selected to determine the physico-chemical stability in comparing to the gel base (GB) and gel containing the free papain (GS).

The GB, GNN and GEN gave good physical stability with no sedimentation, no layer separation and no color change at all temperatures (4±2, 27±2 and 45±2°C) for 3 months. However, the color of GS became light yellow when kept at 45±2°C for 2 months. The vesicular sizes, zeta potential values, viscosity of various gel formulations when kept at 4±2, 27±2 and 45±2°C for 3 months were shown in **Table 31**. The vesicular sizes of GB, GS, GNN and GEN were increased from initial at 12.06, 15.27 and 33.96; 4.20, 6.42 and 2.46; 1.77, 2.82 and 1.50; 1.46, 1.58 and 1.18 times when stored at 4±2, 27±2 and 45±2 °C for 3 months, respectively. The zeta potential values of GB, GNN and GEN at initial were not significantly different after kept at 4±2, 27±2 and 45±2°C for 3 months, while the zeta potential values of GS decreased from initial. All gel formulations showed the decrease viscosity from initial after 3 months at all temperatures especially when kept at 45±2°C. It has been reported that the viscosity of Carbopol gel was decreased with increased temperatures (Barry et al., 1979). High temperature may cause the breaking down of the polymer structure of Carbopol.

The percentages of papain remaining in GS, GNN and GEN stored at different storage temperature for 3 months were demonstrated **(Figure 54)**. The percentages of papain remaining after 3 months in all formulations (GS, GNN and GEN) at higher storage temperature were less than at the lower temperature owning to the chemical degradation of papain by heat especially GS which gave the remaining amount of only 59.74±4.19%. The free papain in GS did not have niosomes to protect from heat degradation. However, all formulations exhibited the remaining of papain more than 50% at different storage temperatures for 3 months. The matrices of Carbopol gel may be able to protect the thermal degradation of papain. In addition, when incorporated in gel, papain loaded in non-elastic and elastic niosomes was more stable against heat than that not incorporated in gel of about 1.62, 2.39 and 2.64; 1.52, 1.95 and 2.42 times when stored at 4±2, 27±2 and 45±2 °C for 3 months, respectively (Manosroi et al., 2012b). The gel structure may retard the leakage of papain from the nanovesicles, thereby preventing the degradation from the thermal effects (Glavas-Dodov et al., 2002; Ruel-Gariépy et al., 2002). Papain in GNN and GEN exhibited higher remaining amount than GS of 1.10, 1.27 and 1.35; 1.13, 1.29 and 1.35 times when stored at 4±2, 27±2 and 45±2 °C after 3 months, respectively. However, the percentages of papain remaining in GEN were slightly higher than those in GNN, but not significant. This result suggested that both GNN and GEN formulations were the proper topical formulations because of their superior physical and chemical stability when stored at 4±2, 27±2 and 45±2 °C for 3 months.

Table 31 Vesicular size, zeta potential values, viscosity of various gel formulations when kept at 4±2, 27±2 and 45±2°C for 3 months

Formulations	At initial	3 months		
		4±2 °C	27±2 °C	45±2°C
Vesicular sizes (nm)				
Gel base (GB)	220.7±6.379	2661±108.2	3371±204.4	7495±269.2
Gel containing free papain (GS)	378.4±26.18	1588±470.0	2428±280.9	929.1±168.5
Gel containing papain loaded in non-elastic niosomes (GNN)	441.8±44.64	781.7±85.3	1247±48.5	661.6±127.6
Gel containing papain loaded in elastic niosomes (GEN)	520.2±36.30	758.5±38.4	823.9±77.1	615.8±80.2
Zeta potential values (mv)				
Gel base (GB)	-40.8±0.934	-40.0±2.04	-40.4±4.88	-45.2±4.14
Gel containing free papain (GS)	-53.7±2.20	-32.4±6.67	-32.8±3.59	-43.4±3.57
Gel containing papain loaded in non-elastic niosomes (GNN)	-44.5±2.71	-38.7±2.42	-37.5±2.79	-36.6±2.03
Gel containing papain loaded in elastic niosomes (GEN)	-46.2±2.03	-43.0±1.84	-45.1±2.61	-48.6±1.98
Viscosity (cP)				
Gel base (GB)	92440	45600	53540	43840
Gel containing free papain (GS)	67060	42800	34580	20900
Gel containing papain loaded in non-elastic niosomes (GNN)	83430	66920	40680	28600
Gel containing papain loaded in elastic niosomes (GEN)	56220	59880	48090	33640

Papain was at 1 mg/ml in all formulations.

PLGA nanospheres composed of poly (lactide-co-glycolic acid) as biodegradable polymers and polyvinyl alcohol as a dispersing agent.

Elastic niosomes composed of Tween 61/cholesterol/sodium cholate at 1:1:0.1 molar ratio while non-elastic niosomes, no sodium cholate was added.

Values represent mean±S.D. of triplicate of three experiments.

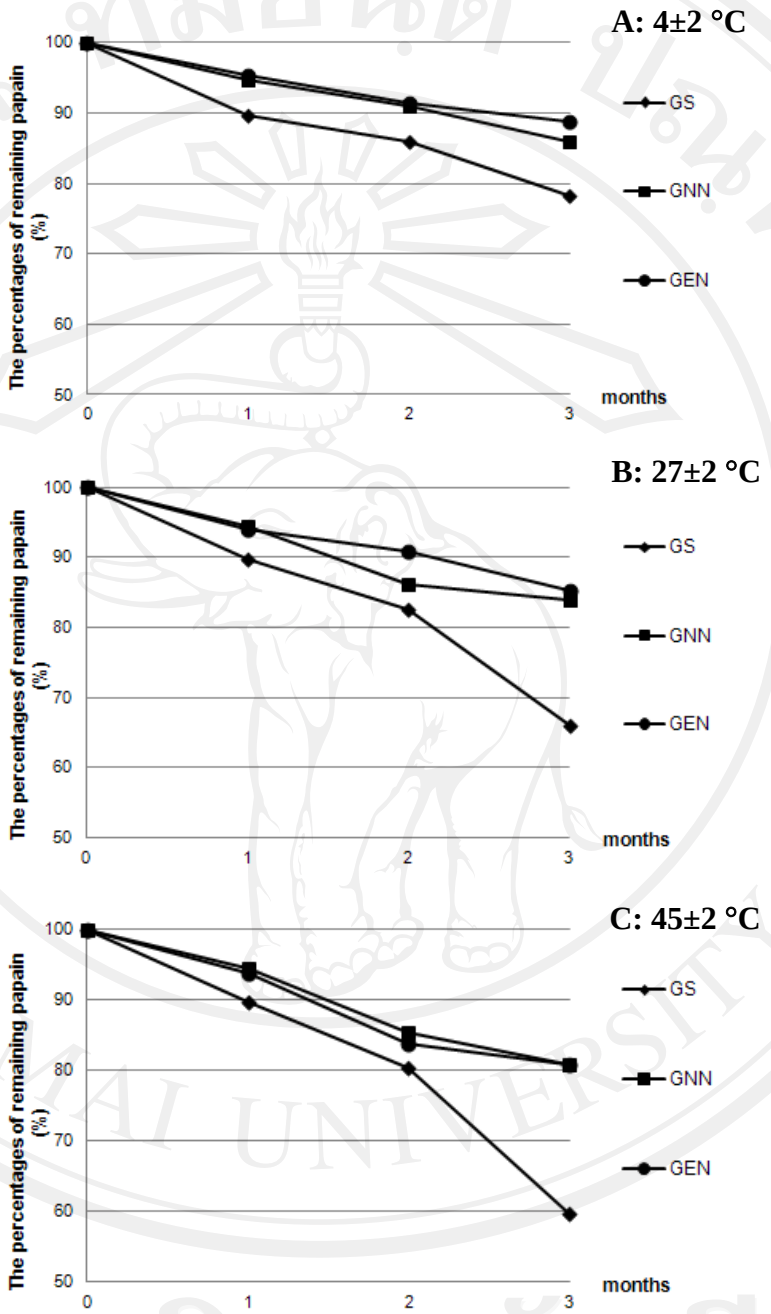


Figure 54 The percentages remaining of papain in various gel formulations when stored at different temperatures (A: 4±2, B: 27±2 and C: 45±2 °C) for 3 months. GS, GNN and GEN were the gel containing free papain, non-elastic niosomes and elastic niosomes, respectively. Papain was at 1 mg/ml in all formulations.

6.5 Hypertrophic scar model in the rabbit ears

After 14 day of application, the scars applied with GNN, GEN and Mederma® gave the moderate score of contour, whereas the scars applied with GB and GS still showed the severe hypertrophic scar. The percentage of hypertrophic scar reduction of various gel formulations were shown in **Figure 55.** The height of the scars decreased after applied with all formulations. After 7 and 14 days of application, the scar applied with GNN, GEN and Mederma® demonstrated significantly (p<0.05) higher hypertrophic scar reduction than GB, while GS showed no significance. After 28 days of application, the scar applied with GEN exhibited the percentage of hypertrophic scar reduction at 29.17±1.87% which was higher than GB, GS, and GNN of 10.20, 2.73 and 2.31 times, respectively. However, the scar applied with GEN showed slightly lower percentage of hypertrophic scar reduction than Mederma®, but no significant difference (p<0.05). The high skin penetration of elastic niosomes may result in the high amount of papain in the hypertrophic scars to degrade the accumulative collagen. However, GEN showed higher scar reduction than Mederma® of 2.17, 1.59 and 1.11 times after 7, 14 and 21 days of application, respectively. Mederma® is a topical gel with a botanical extract derived from onion (*Allium cepa Linn.*). The principal constituent of *Allium cepa* is quercetin, a bioflavonoid noted for its anti-proliferative effects on both normal and malignant cells and for its antihistamine release effects. The previous study has demonstrated that treatment of hypertrophic scars with Mederma® could block histamine release, decrease inflammation, decrease collagen production, subsequently resulting in reduced scar volume (Saulis et al, 2002b).

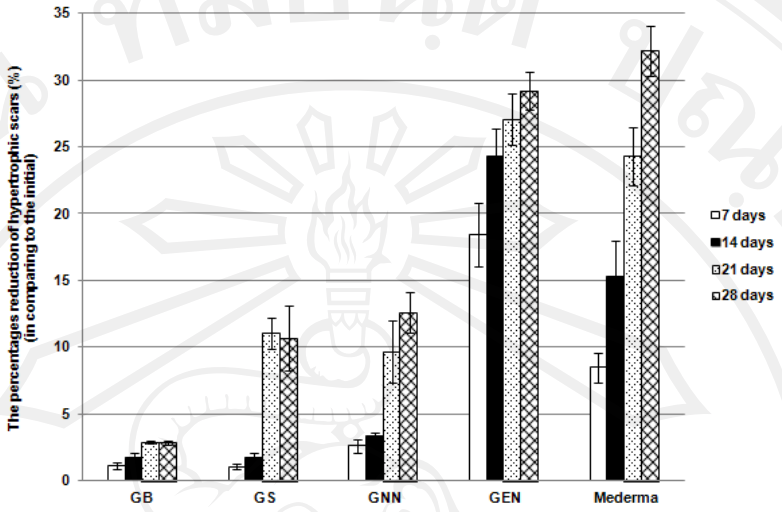


Figure 55 The percentages (mean±S.D.) of hypertrophic scar reduction of various gel formulations after 7, 14, 21 and 28 days of application. GB, GS, GNN and GEN were the gel base, gel containing free papain, papain loaded in non-elastic niosomes and elastic niosomes, respectively.

Histological staining of the scar tissues was carried out at day 28 after gel application. The hematoxylin-eosin-stained cross-section of the scars treated with various gel formulations was shown in **Figure 56.** The regularly arranged and small collagen fibres were observed in the unwounded (normal) skin tissue. The scar tissue of the control group treated with GB demonstrated more irregular arrangement of the collagen fibres. After the treatment with GS, GNN, GEN and Mederma® for 28 days, the collagen fibres were arranged more regularly than the control group. The numbers of the collagen fibres and the height of the scars treated with GEN were significantly decreased in comparing to the control group, which were similar to the normal skin. This result has

indicated the *in vivo* hypertrophic scar reduction activity enhancement of papain when loaded in niosomes, especially the elastic niosomes.

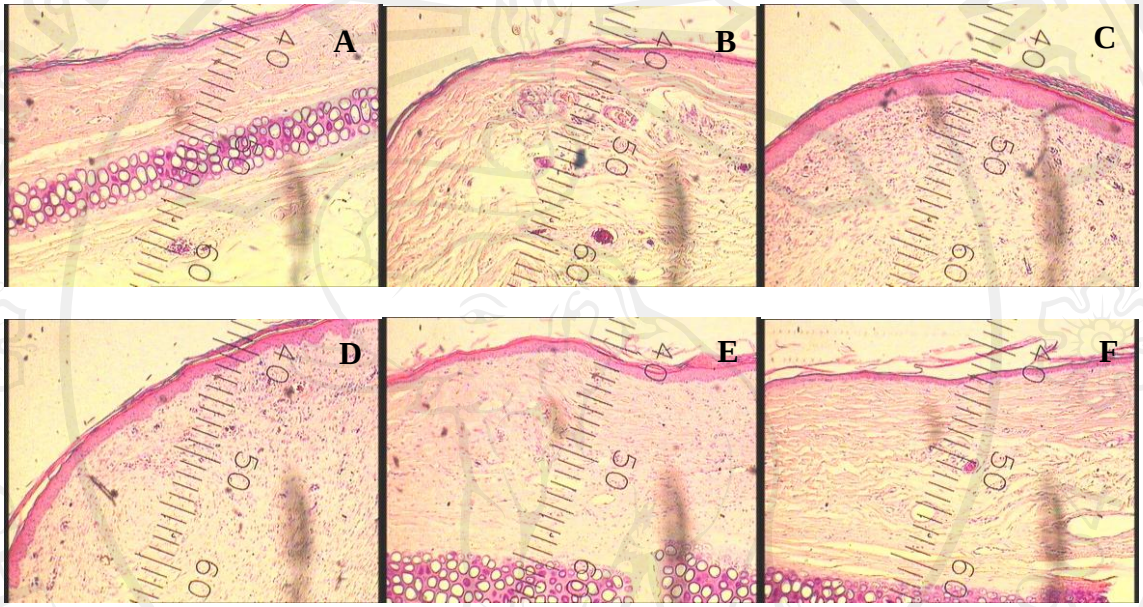


Figure 56 The hematoxylin-eosin-stained cross-section of the induced scars of the rabbits' ear skin treated with various gel formulations after 28 days of application. (A) unwound (normal) skin, (B) scar tissue treated with the gel base, (C) scar tissue treated with the gel containing free papain, (D) scar tissue treated with the gel containing papain loaded in non-elastic niosomes, (E) scar tissue treated with the gel containing papain loaded in elastic niosomes and (F) scar tissue treated with Mederma® (the commercial product containing onion extract) (original magnification ×40).