CHAPTER 4

CONCLUSION

The results from this study can be concluded as the followings:

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

1.1 The decrease concentrations of niosomes (Tween 61 mixed with cholesterol) from 20, 10 to 5 mM and the phosphate buffer concentrations (pH 7.0) from 30, 20, 10 to 5 mM increased the vesicular elasticity.

1.2 The entrapment efficiency of calcitonin was higher in niosomes dispersed in 20 mM phosphate buffer (pH 7.0) than in distilled water.

1.3 The blank elastic niosomes composed of 2.5% mole NaDC and 5% mole NaC niosomes loaded with calcitonin exhibited the highest elasticity of 36.42±0.51 and 21.59±0.91 which were 5 and 13.49 folds of their corresponding non-elastic niosomes, respectively.

1.4 The NaC (5% mole) elastic niosomes showed the highest entrapment efficiency of calcitonin at 60.11±4.98%

1.5 There was no significant difference (p<0.05) in cell viability on human skin fibroblast of calcitonin loaded in NaC (5% mole) and NaDC (2.5% mole) elastic niosomes.

1.6 Thus, the 5 mM elastic niosomes in 5 mM phosphate buffer appeared to be the most appropriate niosomal system to load the peptides.

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

2.1 The chemical stability of calcitonin loaded in non-elastic niosomes was 1.15, 16.58 and 15.34 folds which in elastic niosomes was 1.15, 18.43 and 18.43 folds more stable than in solution when stored at 4±2, 27±2 and 45±2 °C, respectively.

2.2 Calcitonin in all formulations was found in the whole skin and the receiver compartment solution investigated by vertical Franz diffusion cells at 37±2 ºC for 6 h. Calcitonin loaded in elastic niosomes exhibited higher amount both in the whole skin (0.071±0.016 mg/cm^2) and 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.

2.3 Thus, the chemical stability and skin permeation of salmon calcitonin were enhanced when loaded in niosomes especially in elastic niosomes.

2.4 The 5 mM elastic niosomes in 5 mM phosphate buffer was selected to load with protease enzymes (papain and bromelain).

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

3.1 The percentage yields of extracted papain and bromelain were 16.76 and 0.97%w/w and the MW which estimated by the protein markers were 23 and 25 kDa,

respectively which is the same as the MW of their standards. The purities of the extracted papain and bromelain determined by HPLC were 82.31 and 38.03% which were similar to those by the SDS-PAGE that gave 77.68 and 44.95%, respectively.

3.2 The standard and extracted papain exhibited higher antioxidant activity than those of bromelain both in free radical scavenging (SC_{50}) and lipid peroxidation inhibition (IPC_{50}) activity. However, the extracted papain showed lower antioxidant activity than the standard papain.

3.3 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. All samples at the concentration range of 10^{-9} to 25 µg/ml gave cell viability of more than 85%.

3.4 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. The extracted papain and bromelain showed the MMP-2 stimulatory activity on zymograms similar to their standards, but lower than concanavalin A.

3.5 Thus, the protease enzymes (papain and bromelain) from papaya latex and pineapple fruits which gave similar collagenolytic activity to their standards can be extracted by the simple precipitation with 95% ethanol and saturated ammonium sulfate.

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

4.1 The elastic niosomes loaded with the standard papain, extracted papain, standard bromelain and extracted bromelain showed the DI values of 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 their corresponding non-elastic niosomes, respectively.

4.2 The maximum loading of the standard and extracted papain 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 indicated the entrapment efficiencies in 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.

4.3 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 more viability, respectively in comparing to their corresponding free enzymes.

4.4 Both the relative pro MMP-2 and active MMP-2 of both enzymes loaded in elastic niosomes were slightly decreased, but not significant difference ($p<0.05$) in comparing to the free enzymes. Thus, the MMP-2 stimulatory activity of papain and bromelain was still existing similar to their free enzymes, even loaded in elastic niosomes.

4.5 The remaining percentages of the standard and extracted papain loaded in elastic niosomes were higher than the free papain of 1.46 and 1.40; 1.48 and 1.36; 1.82

and 1.21 times and higher than the enzymes loaded in non-elastic niosomes of 1.20 and 1.19; 1.33 and 1.20; 1.51 and 1.11 times when kept at 4±2, 27±2 and 45±2 °C, respectively after 8 weeks.

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

5.1 The particle sizes of the PLGA nanospheres unloaded and loaded with papain by both methods were in the nanosize range (220 to 335 nm). Papain loaded in PLGA nanospheres prepared by the ESE method gave superior characteristics (small particle size and low polydispersity index) to the ESD method.

5.2 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.

5.3 The release profile of papain from the PLGA nanospheres prepared by both methods demonstrated an initial burst release for 6 h and followed by the sustain release for 48 h. Papain loaded in the nanospheres prepared by the ESD method showed more rapid initial release than those by the ESE method.

5.4 The free papain showed cytotoxicity on human skin fibroblasts with the IC_{50} value of 6.22 µg/ml, while papain loaded in PLGA nanospheres prepared by the ESE method, showed an increase IC_{50} value of 9.13 µg/ml which was 1.5 times less toxic than the free papain.

5.5 Papain loaded in the PLGA nanospheres showed higher chemical stability than papain in solution of 8 and 3 times when kept for 6 weeks at 4 and 25°C, respectively.

5.6 Since the ESE method gave superior characteristics and encapsulation efficiencies than the ESD method, the ESE method was selected for prepared papain loaded in PLGA nanospheres.

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

6.1 The gel containing papain loaded in nanovesicles (non-elastic niosomes, elastic niosomes and PLGA nanospheres) gave the pH values of about 5.5-6 which is suitable for topical application and the specific gravity at 1.0074-1.0709 g/ml. The vesicular sizes of all gel formulations were in the range of 220.7 to 520.2 nm.

6.2 The GEN gave superior cumulative amounts and fluxes to GNN of 3.10, 2.38 and 2.24, 2.25 times; GPN of 10.08, 7.78 and 4.92, 4.93 times and GS of 4.86, 3.71 and 7.38, 7.38 times in the whole skin and the receiver compartment solution, respectively.

6.3 The GB, GNN and GEN gave good physical stability. Papain in GNN exhibited higher remaining amount than GS of 1.10, 1.27 and 1.35 times, whereas the remaining amount of papain in GEN was 1.13, 1.29 and 1.35 times more than GS when stored at 4±2, 27±2 and 45±2 °C after 3 months, respectively.

6.4 The calculated PII values of all formulations in rabbit skin irritation closed patch test were in the range of 0.00-0.44 which indicated no irritation, except GS (PII = 0.78, slight irritation).

6.5 After 28 days of application, the scar applied with GEN exhibited higher reduction of hypertrophic scars than GB, GS, and GNN of 10.20, 2.73 and 2.31 times, respectively.

In summary, papain which was extracted from papaya latex by 95% ethanol precipitation demonstrated the potential of scar treatment because of its highest MMP-2 stimulatory activity. The extracted papain loaded in low cytotoxic elastic niosomes did not only reduce the toxicity of papain on skin human fibroblasts, but also enhance the chemical stability which exhibited higher remaining contents than the free enzyme when kept at various temperatures (4±2, 27±2 and 45±2 °C) for 8 weeks. The gels incorporated with papain loaded in elastic niosomes exhibited high *in vitro* transdermal penetration through rat skin and *in vivo* hypertrophic scar reduction in rabbit ears with no irritation. Furthermore, gel containing papain loaded in elastic niosomes is expected to be used as both for prevention and treatment of hypertrophic scar and keloids, because papain can scavenge the free radicals in the inflammatory phase for the prevention and can stimulate collagenolytic activity after hypertrophic scar and keloids formation for the treatment.

For further research suggestions, the mechanism of papain on MMP-2 stimulation might be thus valuable to investigate. Papain may stimulate the MMP-2 synthesis and secretion. However, one possible mechanism of the papain on MMP-2 stimulation may be from the increasing of the activation processes by converting the latent form of MMP-2 (pro MMP-2) to an active form (active MMP-2). However, the scar treatment effectiveness in human depends on several factors including sex, age and

genetics as well as the age and location of the scars. In addition, for more convincing commercialization, the developed gel containing papain loaded in elastic niosomes should be further clinically investigated for scar reduction activity in human volunteers.