# CHAPTER IV

# RESULTS AND DISCUSSION

## 1. Calibration Curve of Standard Protein (Assay by Bradford Method)

The relationships between concentrations of standard protein (BSA) in PBS pH 7.4 and absorbances at 595 nm was shown in Table 3, their corresponding Beer's law plots were depicted in Figure 14. The correlation coefficient of this straight line was 0.9999.

Bradford method is simply and more sensitive method for determining concentration of solubilized protein, only 10 µl/ml of protein can be detected. The linear range of the assay is 8.0 µl /ml to approximately 80.0 µl/ml for microassay procedure. Bradford method can be used in the presence of sugars, 2- mercaptoethanol, and dithiotheritol which may interfere with Lowry method. Alternatively, the Lowry method can be used in the presence of detergents and sodium hydroxide, this two components known to interfere with the Bradford method.

In this study, Bradford method was used to determine concentration of protein in KSCN extract and in supernatant of liposome suspension, there were free proteins which was unencapsulated or released from liposome vesicles.

Table 3 Relationship between concentration of standard protein (BSA) in PBS pH 7.4 and absorbance at 595 nm (Assay by Brandford method)

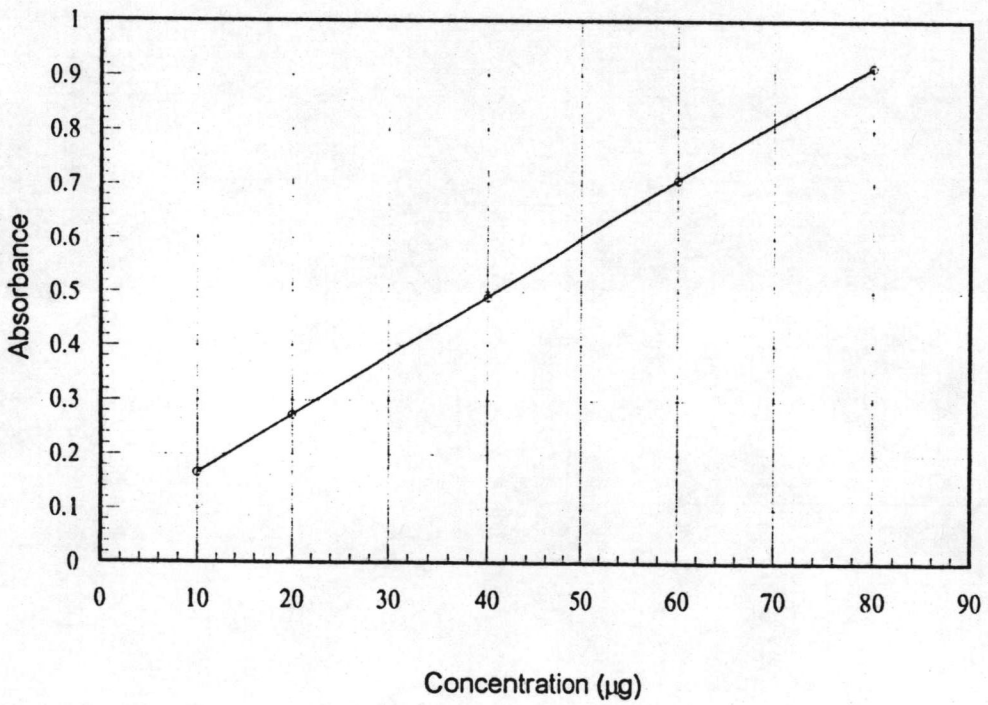
| Concentration | Absorbance [a] |
|---|---|
| 10.0 | 0.166 |
| 20.0 | 0.272 |
| 40.0 | 0.490 |
| 60.0 | 0.708 |
| 80.0 | 0.915 |

$y = 0.0589 + 0.0107x$

$r^2 = 0.9999$

a , average of three determinations

Figure 14 Calibration curve of standard protein in PBS pH 7.4 at 595 nm (Assay by Bradford method)



**2. Calibration Curve of Standard Protein.**

**(Assay by Lowry Method)**

The relationships between concentrations of standard protein (BSA) in PBS pH 7.4 and absorbances at 655 nm was shown in Table 4, their corresponding Beer's law plots were depicted in Figure 15. The correlation coefficient of this straight line was 0.9999.

The straight line do not start from origin because in this assay the amount of sodium dodecyl sulfate was high. This error can be eliminated by using blank control.

In this study, Lowry method was used to determine concentration of protein that remained in liposome vesicles. Protein in liposome vesicles were determined by ruptured liposome vesicles with Triton x-100.

Table 4 Relationship between concentration of standard protein (BSA) in PBS pH 7.4 and absorbance at 655 nm. (Assay by Lowry method)

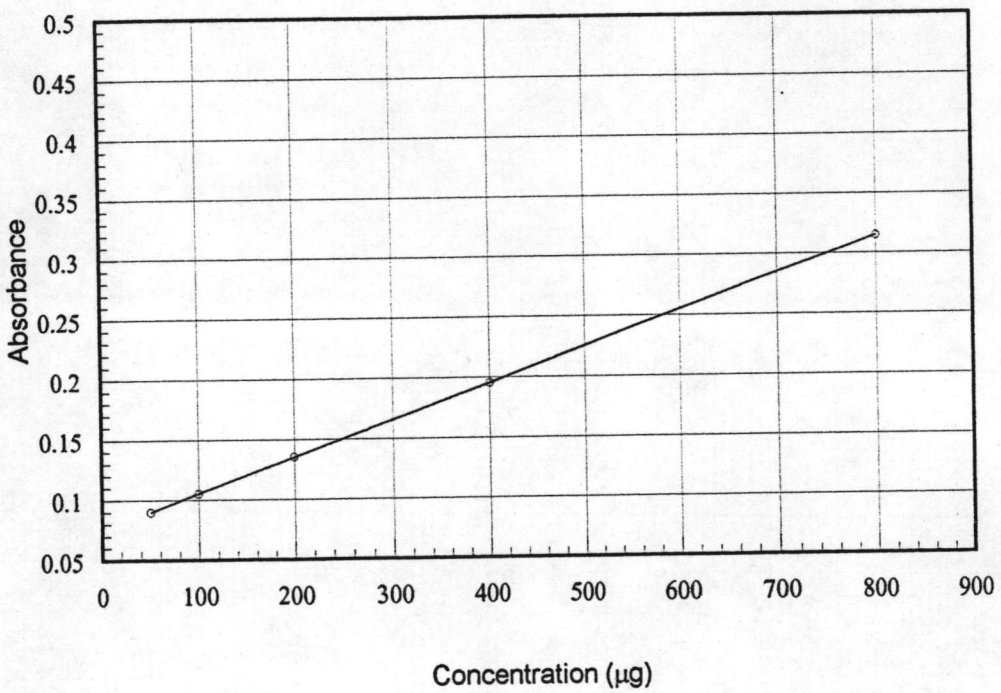
| Concentration | Absorbance [a] |
|---|---|
| 50.0 | 0.090 |
| 100.0 | 0.105 |
| 200.0 | 0.135 |
| 400.0 | 0.195 |
| 800.0 | 0.315 |

$y = 0.0749 + 0.0003x$

$r^2 = 0.9999$

a , average of three determinations

Figure 15 Calibration curve of standard protein in PBS pH 7.4 at 655 nm (Assay by Lowry method)



## 3. Preparation of Protein Extract from *P. multocida*

### 3.1 Culture Organism

The culture used in antigen extraction is *Pasteurella multocida* serotype 8:A (2T35), obtained from Animal Vaccine Research Unit, Chulalongkorn University. It is a low virulent and mutant strain that developed for using in vaccine study, especially. This organism grows on tryptose blood agar (TBA) and give white to yellow color colony. When inoculated in brain heart infusion (BHI) broth, it grows better in shaker incubator than static incubator at 37°C . The collected cells from 5000 ml BHI were 18.403 gm. (3.68 gm/L).

### 3.2 Preparation of Potassium Thiocyanate Extraction

The collected cells of 18.403 gm were extracted by 10 ml of 0.5 M potassium thiocyanate and 0.08 M sodium chloride pH 6.3. The resulted dialysate, protein antigen was 75 ml. They were concentrated by freeze drying method and stored in closed containers, and kept at -20°C until used.

## 4. Protein Determination of *P. multocida* Antigen Extracts.

The potassium thiocyanate (KSCN) extract of *P. multocida* was a subcellular antigen containing various components such as protien, carbohydrate, lipopolysaccharides (LPS), DNA, and RNA. Since the main component was protein, it was determined by dye-binding method which is a simple and accurate method for determining concentration of solubilized protein. In this study, the average protein concentrations from three determinations was 1199.50± 8.10μg/ml.

## 5. Preparation of Liposomes Containing Protein Extract form *P. multocida* by Double Emulsion Technique.

Liposomes containing protein extract from *P.multocida* were prepared by double emulsion technique that based on the method of Kato, A., Arakava, M., and Kondo, T. (24) .This technique was similar to the emulsification technique of Fumiyoshi Ishii (67) that mentioned in the part of the review literature. This technique was selected for preparing liposomes containing protein extract from *P. multocida* because of several benifit factors. The first, it enabled the polymer to become associated with surface of each bilayers in the multilamellar structure, resulted in a high stability. The second, because of its simplicity and reproducibility, the large scale production could be developed.

### 5.1 Study of the Cholesterol Content on Liposome Properties.

#### 5.1.1 Preparation of liposomes with various molar ratios of egg yolk lecithin to cholesterol

White colloidal vesicles of liposomes containing protein extract from *P.multocida* were obtained by double emulsion technique with various molar ratios of 1:0, 7:2, and 1:1 of egg lecithin and cholesterol. They were suspended in PBS pH 7.4. The sedimentation takes time for several days. The appearance of liposome suspensions gave no difference among three formulations, when detected with eye.

5.1.2 Studies of physicochemical properties of liposomes

The physicochemical properties of liposomes containing protein extract from *P. multocida*, which prepared from various molar ratio of lecithin to cholesterol were shown as following:

(a) Entrapping efficiency

Table 5 showed entrapping efficiency of protein extract from *P. multocida* in liposomes. The molar ratio of 1:1 of egg yolk lecithin to cholesterol gave the higher entrapment of 45.26 ± 1.25% followed by the molar ratio of 7:2 of egg yolk lecithin to cholesterol which was 42.11 ± 1.38. The 1:0 molar ratio gave a lowest entrapment efficiency which was 37.47 ± 1.15. They could explain that the increasing cholesterol content the more entrapping efficiency of protein extract from *P. multocida*.

Table 5 Effect of cholesterol content on entrapping efficiency.

| Lipid composition (Lecithin : Chol) | Percent of entrapment |
|---|---|
| 1 : 0 | 37.47±1.15 |
| 7 : 2 | 42.11±1.38 |
| 1 : 1 | 45.26±1.25 |

Protein extract from *P. multocida* are hydrophilic substance since entrapment was depended upon the volume of aqueous phase that encapsulated during liposomes formation. The role of cholesterol on entrapping efficient may be explained on the basis of swelling effect of the lipid membrane. The former report showed that 10 water molecules are bound to each lecithin polar group. Low concentration of cholesterol causes the packing of lipid chains to change from a tilted configuration to a vertical configuration, so that the bilayer thickness is also increased but amount of bound water is not changed. At the higher cholesterol concentrations, the more amount of water was bound.

(b) Microscopic appearances

Figure 16, 17 and 18 showed microscopic appearance of liposomes prepared from the various molar ratio of 1:0, 7:2, and 1:1 of egg yolk lecithin to cholesterol, respectively. Micrograph of prepared liposomes appear heterogeneous of small and round vesicles, some of the large vesicles show the lamellar structure. They showed no difference under microscopic observation but they showed difference when measuring the particle size by Mastersizer which base on the principle of laser ensemble light scattering as shown in Table 6.

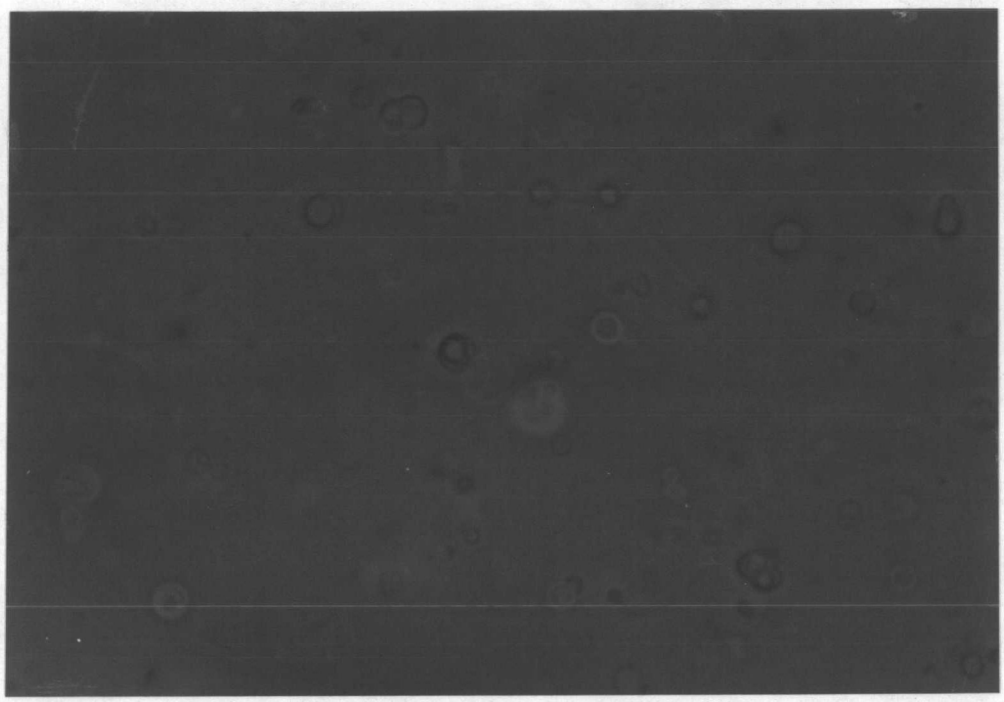


Figure 16 Micrograph of liposomes with 1 : 0 molar ratio of lecithin to choloesterol (magnification 1000x)

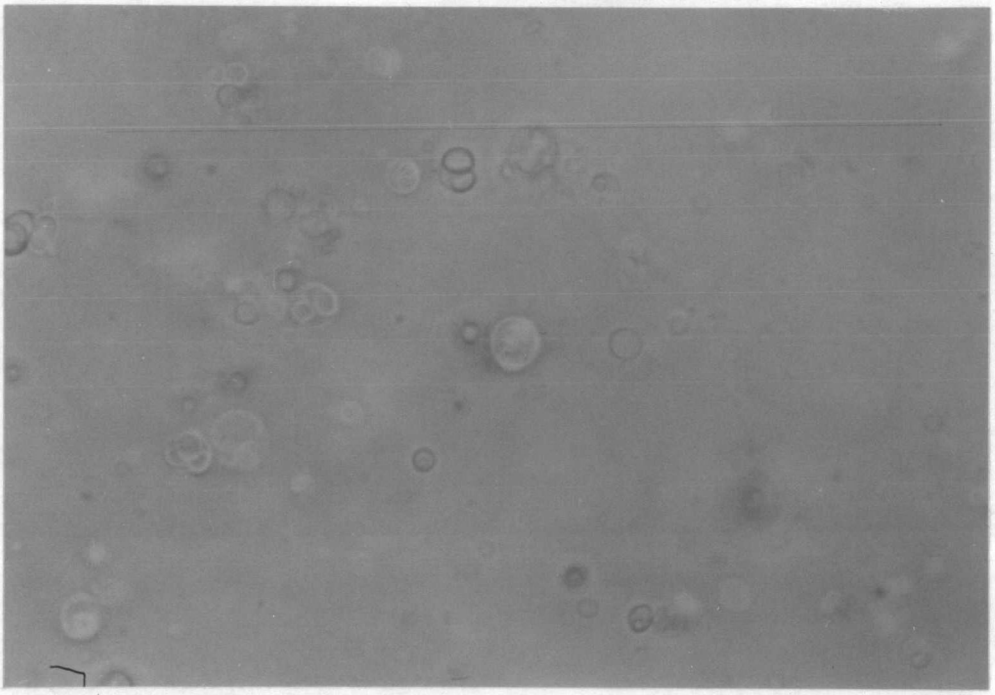


Figure 17 Micrograph of liposomes with 7 : 2 molar ratio of lecithin to choloesterol (magnification 1000x)

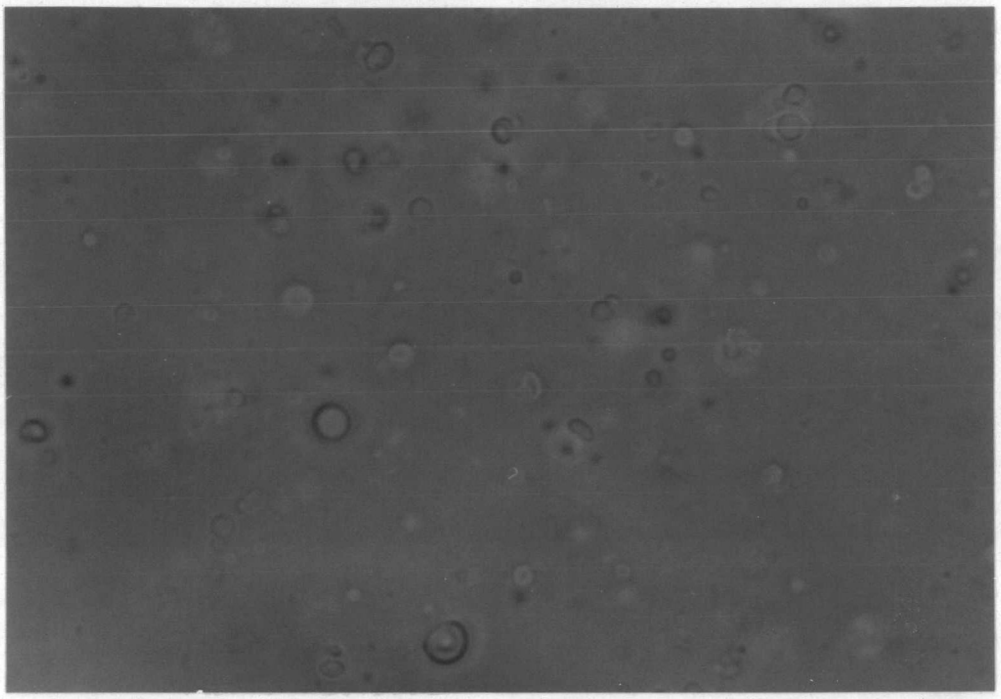


Figure 18 Micrograph of liposomes with 1 : 1 molar ratio of lecithin to choloesterol (magnification 1000x)

(c) Particle size analysis

Table 6 showed the effect of molar ratio of egg yolk lecithin to cholesterol content on the particle size of liposomes containing protein extract from *P.multocida* measured by Mastersizer which based on the principle of laser ensemble light scattering.

The molar ratio of freshly prepared of 1:0 and 7:2 of egg yolk lecithin to cholesterol showed the same median diameter of 3.81-3.83 micron. The molar ratio of freshly prepared of 1:1 of egg yolk lecithin to cholesterol showed the median diameter of 5.92 micron. The influence of the amount of cholesterol on the liposomes revealed that the incorporation of more cholesterol would yield larger particles. Corresponding to the previous finding, the particle diameter increased when the trapping efficiency was increased.

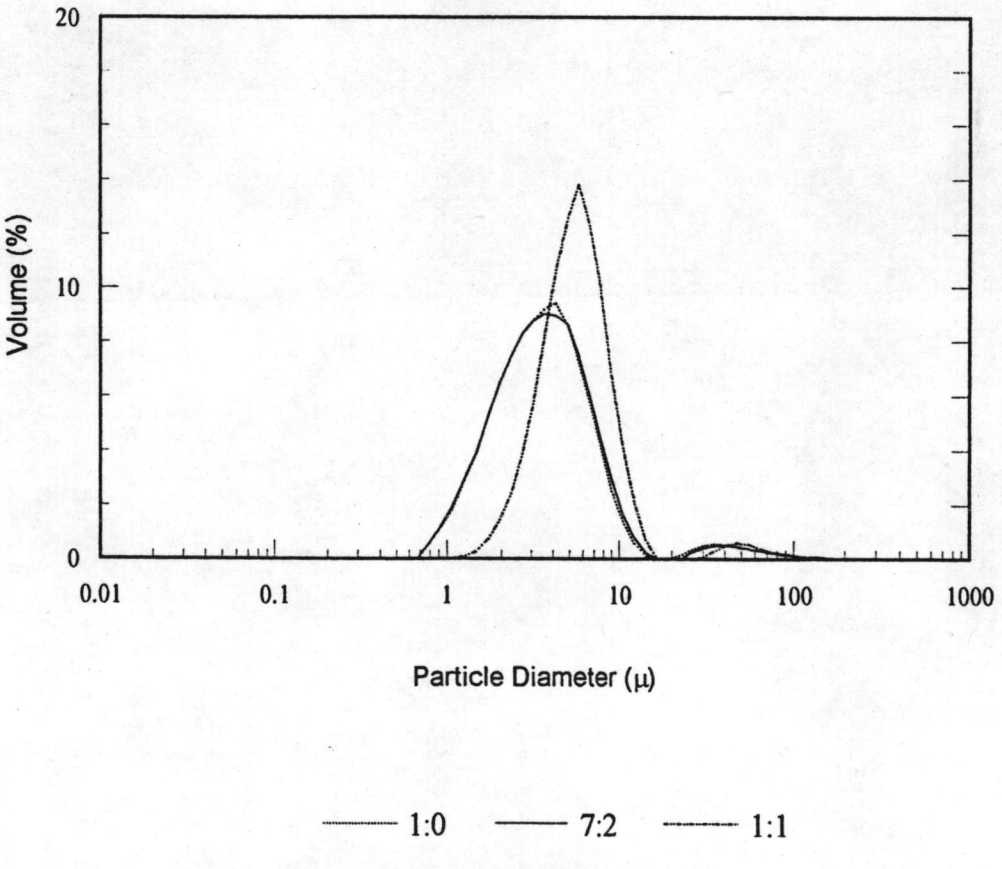
The effect of cholesterol on the liposome particle size could be explained on the basis of the influence of cholesterol uptake on the fluidity of the phosphatidyl choline bilayer, this resulted in an increased expansion of lipid bilayer that induces the formation of large vesicles.

Figure 19 showed the difference in particle size distributions of freshly prepared of liposomes with 1:0, 7:2 and 1:1 molar ratio of egg yolk lecithin to cholesterol by plotting between particle diameter versus percent volume of particle. The curves of 1:0 and 7:2 molar ratio were coincided with corresponding to the Table 6 which showed the median diameter of 3.81 and 3.83 microns. The curve of 1:1 molar ratio was differentiated, it showed a larger median diameter with corresponding to the Table 6.

Table 6 Effect of cholesterol content on the particle size of liposomes containing protein extract from *P. multocida*, prepared by double emulsion technique.

| Lipid composition (Lecithin : Chol) | Median diameter (micron) | |
|---|---|---|
| | Freshly Prepared | 3 Months Storaged |
| 1 : 0 | 3.81 | 6.50 |
| 7 : 2 | 3.83 | 6.39 |
| 1 : 1 | 5.92 | 5.95 |

Figure 19 Particle size distribution of freshly prepared liposomes with 1:0 , 7:2 , 1:1 molar ratio of egg yolk lecithin to cholesterol.



The particle size distribution of prepared liposomes affected by method of preparation. In this study, double emulsion technique was used and obtained the multilamellar liposomes which comfirmed by the transmission electron micrograph. Liposomes which prepared by this techniques had normal particle size distribution with narrow range. The cholesterol content had no effect on the particle size distribution. So that all of preparations had normal distribution and the distributions were narrow range.

The cumulative undersize frequency curve of 1:0, 7:2 and 1:1 molar ratio of egg yolk lecithin to cholesterol liposomes were shown in Figure 60, 61, 62, respectively (see Appendix II )

(d) Transmission electron microscopy

Figure 20 showed the negative staining of 1:1 molar ratio of egg yolk lecithin to cholesterol liposomes containing protein extract from *P. multocida*. They showed clearly concentric lamellae of multilamellar liposomes.

Figure 20 Transmission electron micrograph of liposome prepared by double emulsion technique with 1 : 1 molar ratio of lecithin to choloesterol, negative stained with phosphotungstic acid.

Multilamellar membrane was showed clearly. (magnification 1000x)

(e) Scanning electron microscopy (SEM)

Figure 21, 22, 23 showed the shape, size, and aggregation of liposomes of 1:0, 7:2 and 1:1 molar ratio of egg yolk lecithin to cholesterol, respectively. They appeared round and smooth surface. The 1:1 molar ratio of lecithin to cholesterol liposomes appeared more round and smooth surface than 7:2 and 1:0 molar ratio. These indicated that the addition of cholesterol increased the packing of phospholipid molecules. The 1:1 molar ratio of lecithin to cholesterol or 50% molar of cholesterol in egg yolk lecithin are high ratio that cholesterol will be incorporated in the lecithin bilayer without precipitation of cholesterol and their arrangement were completed. The 1:0 and 7:2 molar ratio showed more aggregate than 1:1 molar ratio.

Figure 24 showed the surface characteristic of 1:1 molar ratio of egg yolk lecithin to cholesterol liposomes. There were no defect of rupture of membrane.

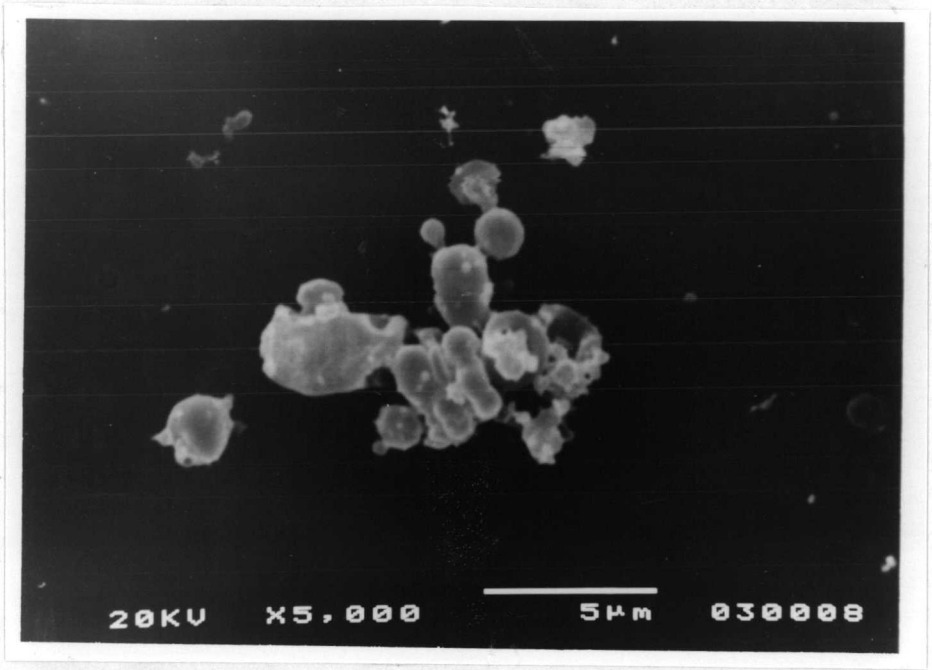



Figure 21 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 1: 0 molar ratio of egg yolk lecithin to cholesterol (magnification : 5000X)

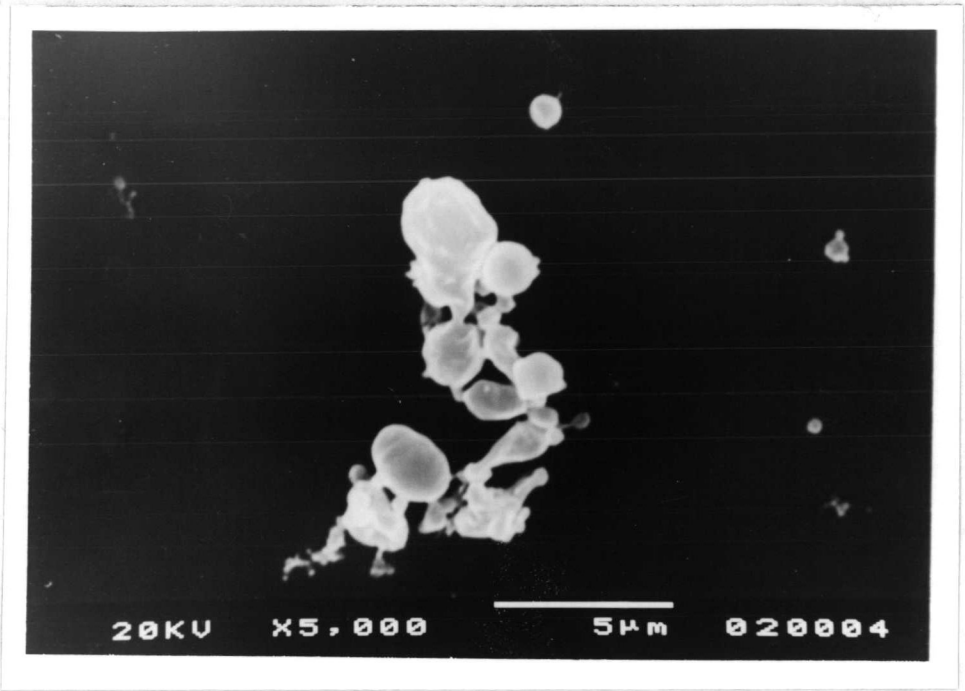



Figure 22 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 7: 2 molar ratio of egg yolk lecithin to cholesterol (magnification : 5000X)

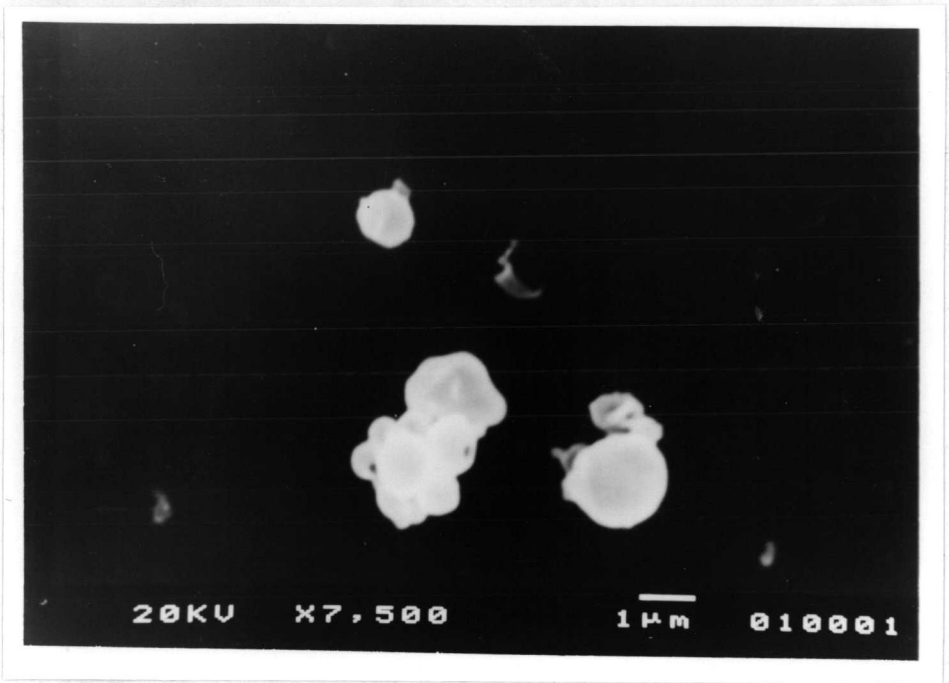



Figure 23 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 1: 1 molar ratio of egg yolk lecithin to cholesterol (magnification : 5000X)

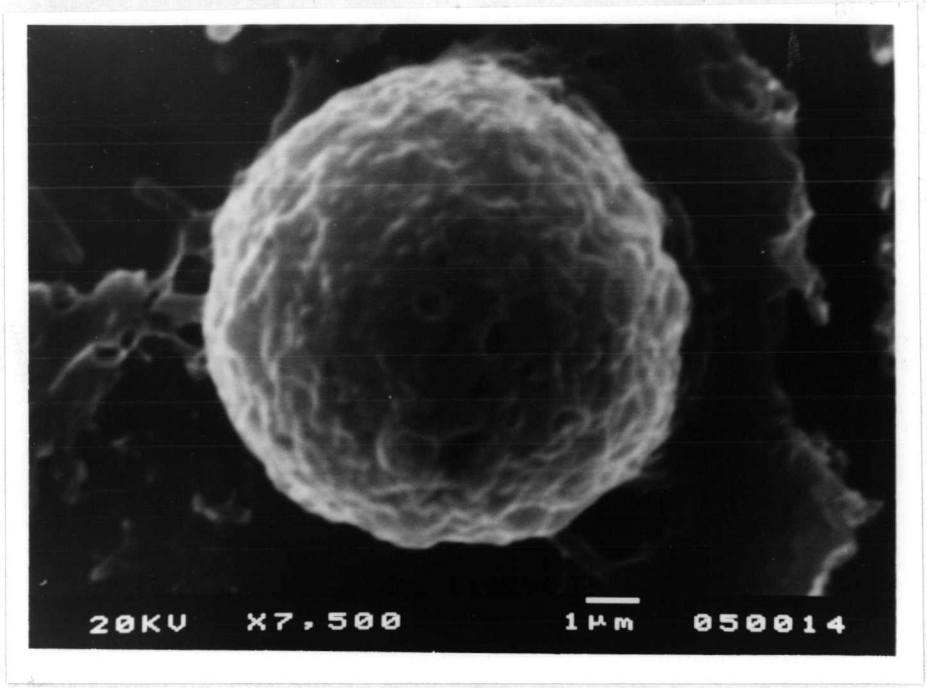



Figure 24 Surface characteristic of polymer coated liposomes containing protein extract from *P. multocida* with 1: 1 molar ratio of egg yolk lecithin to cholesterol (magnification : 7500X)

(f) Assessment of protein leakage rate from liposomes

For study of the stability of liposomes in vivo, assessment of protein leakage from liposomes in PBS pH 7.4 at 37°C were investigated. The effect of cholesterol content on protein leakage of liposomes was represented in the term of percent of released protein which shown in Table 7 and the graph of releasing profile was shown in Figure 25.

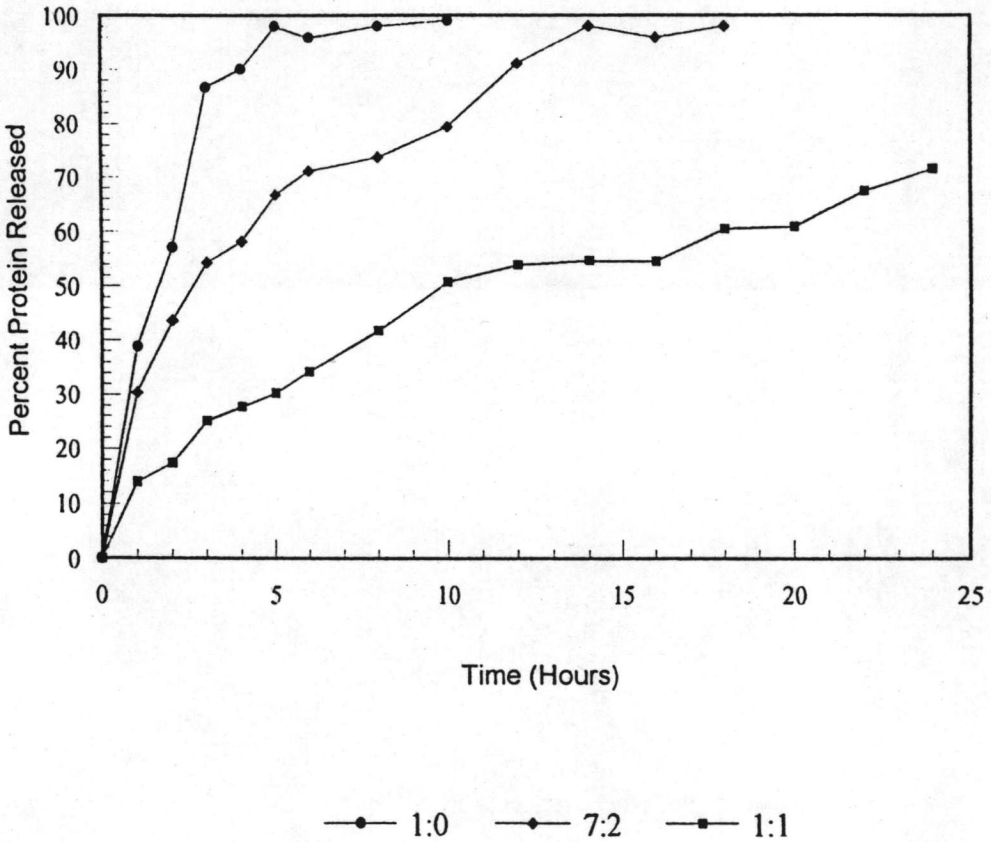
The molar ratio of 1:1 lecithin to cholesterol showed prolong release of protein, 71.5% of protein was released at 24 hours while 100% of protein from 7:2 and 1:0 was released within 18 and 10 hours, respectively. In general, the membrane of liposomes are permeable to water and small ions. The initial rate of permeability of liposomes derived from saturated and unsaturated phosphatidylcholines above their crystalline to liquid-crystalline transition temperature decreased with increasing cholesterol concentration. The decrease in permeability is generally proportional to the concentration of cholesterol. The reduced permeability is explained to be due to the increased packing and decreased mobility of the hydrocarbon chains.

Since protein are large molecules, the leakage occured by rupture of membrane. These concluded that 1:1 molar ratio of lecithin to cholesterol are the most stable in PBS pH 7.4 at 37°C. Because the high cholesterol content effect on phase transition temperature of phospholipid resulted in intermediate transition temperature and decreased temperature sensitivity of phospholipid, this comfirmed by study of differential scanning calorimetry.

Table 7 Percent of released protein from liposomes with various molar ratios of egg yolk lecithin to cholesterol in PBS pH 7.4 at 37°C

| Time (Day) | Percent of released protein from liposomes | | |
|---|---|---|---|
| | 1:1 | 7:2 | 1:0 |
| 0 | 0.00 | 0.00 | 0.00 |
| 1 | 13.84 | 30.29 | 38.78 |
| 2 | 17.25 | 43.56 | 56.99 |
| 3 | 24.96 | 54.23 | 86.60 |
| 4 | 27.59 | 58.09 | 89.96 |
| 5 | 30.17 | 66.64 | 97.99 |
| 6 | 34.16 | 71.09 | 95.88 |
| 8 | 41.69 | 73.75 | 98.00 |
| 10 | 50.71 | 79.33 | 99.05 |
| 12 | 53.82 | 91.03 | - |
| 14 | 54.64 | 98.00 | - |
| 16 | 54.55 | 95.93 | - |
| 18 | 60.48 | 98.00 | - |
| 20 | 60.88 | - | - |
| 22 | 67.31 | - | - |
| 24 | 71.51 | - | - |

Figure 25 Releasing profile of protein from liposomes with various lipid compositions in PBS pH 7.4 at 37 °C



## 5.2 Study of the effect of polymer coated liposomes

5.2.1 In this study, the three different concentrations of 0.02, 0.2 and 0.5% w/v of carboxymethylcellulose and carboxymethylchitosan were used for stabilization of liposomes containing protein extract from *P. multocida*. The double emulsion technique was used in preparation. In this way, the polymer could condensed at the surface of every bilayer membrane of multilamellar liposomes, happened protection of membranes and stabilization of liposomes. Liposomes of 1 : 1 molar ratio of lecithin to cholesterol with polymer appeared white colliodal vesicle. The higher concentration of polymer, the more viscosity of the preparation.

5.2.2 The physicochemical properties of liposomes, containing protein extract from *P. multocida*, which prepared by double emulsion techinque using 1:1 molar ratio of lecithin to cholesterol as membrane and various concentration of carboxymethylcellulose and carboxymethylchitosan as stabilizer were investigated and shown as following.

(a) Entrapping efficiency

The average values from three entrapping efficiency of the prepared liposomes with 1:1 molar ratio of lecithin to cholesterol stabilized with various concentrations of CM-Cellulose or CM-Chitosan, were shown in Table 8 and found to be between 48.45 and 50.30%. The 0.02% w/v of CM-Chitosan gave the higher entrapment of 50.30 ± 1.85%.

Table 8 Entrapping efficiency of liposomes prepared by double emulsion technique with 1 : 1 molar ratio of lecithin to cholesterol stabilized with various concentration of CM-Cellulose or CM-Chitosan.

| Membrane compositions | | Percent of entrapment |
|---|---|---|
| Lecithin : Chol | Stabilizer | |
| 1 : 1 | 0.02% w/v CM-Cellulose | 49.53 ± 2.05 |
| 1 : 1 | 0.02% w/v CM-Chitosan | 50.30 ± 1.852 |
| 1 : 1 | 0.2% w/v CM-Cellulose | 48.51 ± 2.45 |
| 1 : 1 | 0.2% w/v CM-Chitosan | 49.01 ± 1.50 |
| 1 : 1 | 0.5% w/v CM-Cellulose | 48.45 ± 2.95 |
| 1 : 1 | 0.5% w/v CM-Chitosan | 49.22 ± 2.25 |

It can be seen that the entrapping efficiency of prepared liposomes was non significantly different, when 0.02%, 0.2% and 0.5% w/v of CM-Cellulose or CM-Chitosan were used to stabilize liposomes. These may be explained that the coating liposomes with CM-Cellulose or CM-Chitosan do not have any effect on structure of bilayer membrane of liposomes.

(b) Particle size analysis

The effect of polymer coating on the particle size of liposomes were shown in Table 9. The particle size distribution of liposomes stabilized with CM-Cellulose and CM-Chitosan were shown in Figure 26, 27. Liposomes stabilized with CM-Cellulose showed aggregation which detected in median sizes of 50 microns. The vesicles were aggregated but not fused. These were confirmed with scanning electron micrograph and microscopic appearances.

Table 9 Particle size of liposomes containing protein extract from *P. multocida*, prepared by double emulsion technique with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with various concentration of CM-Cellulose or CM-Chitosan.

| Membrane compositions | | Median diameters (micron) | |
|---|---|---|---|
| Lecithin : Chol | Stabilizer | Freshly Prepared | 3 Months Storaged* |
| 1 : 1 | 0.02% w/v CM-Cellulose | 4.77 | 4.87 |
| 1 : 1 | 0.02% w/v CM-Chitosan | 4.77 | 4.81 |
| 1 : 1 | 0.2% w/v CM-Cellulose | 6.17 | 6.14 |
| 1 : 1 | 0.2% w/v CM-Chitosan | 3.63 | 3.72 |
| 1 : 1 | 0.5% w/v CM-Cellulose | 2.54 | 2.67 |
| 1 : 1 | 0.5% w/v CM-Chitosan | 3.33 | 3.43 |

* Stored in phosphate buffer pH 7.4 at 4°C for 3 months

Figure 26 Particle size distribution of freshly prepared 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with various concentration of CM-Cellulose.

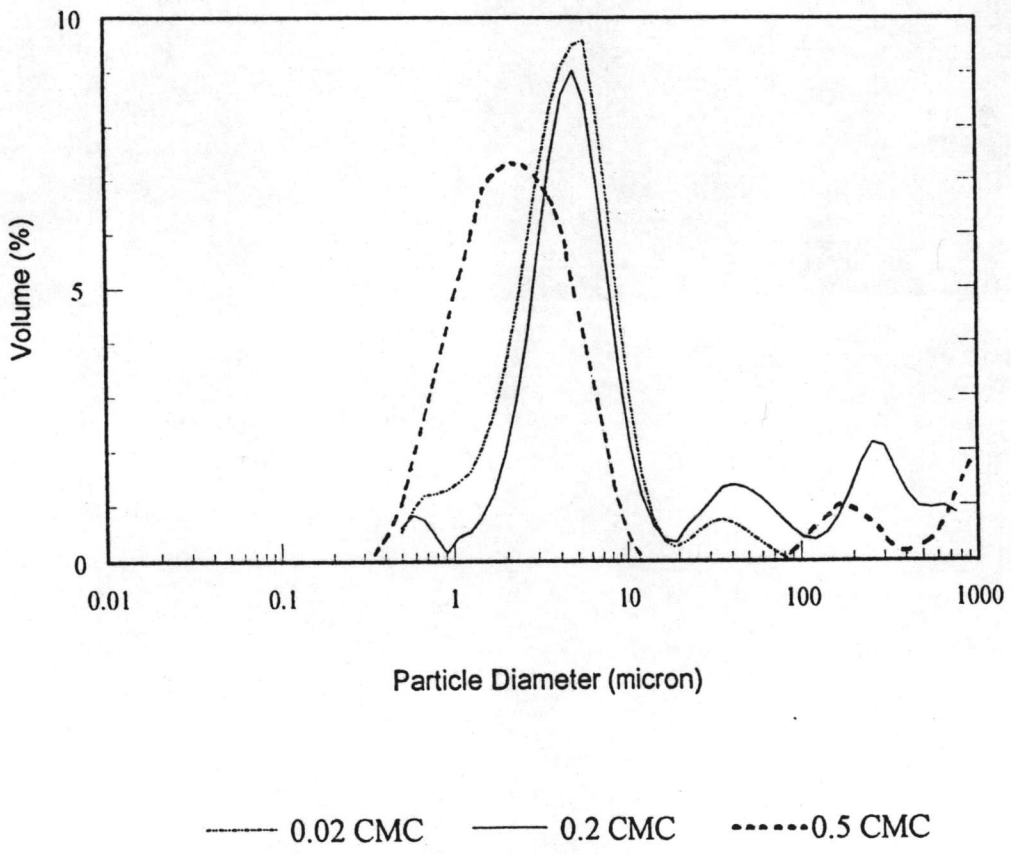
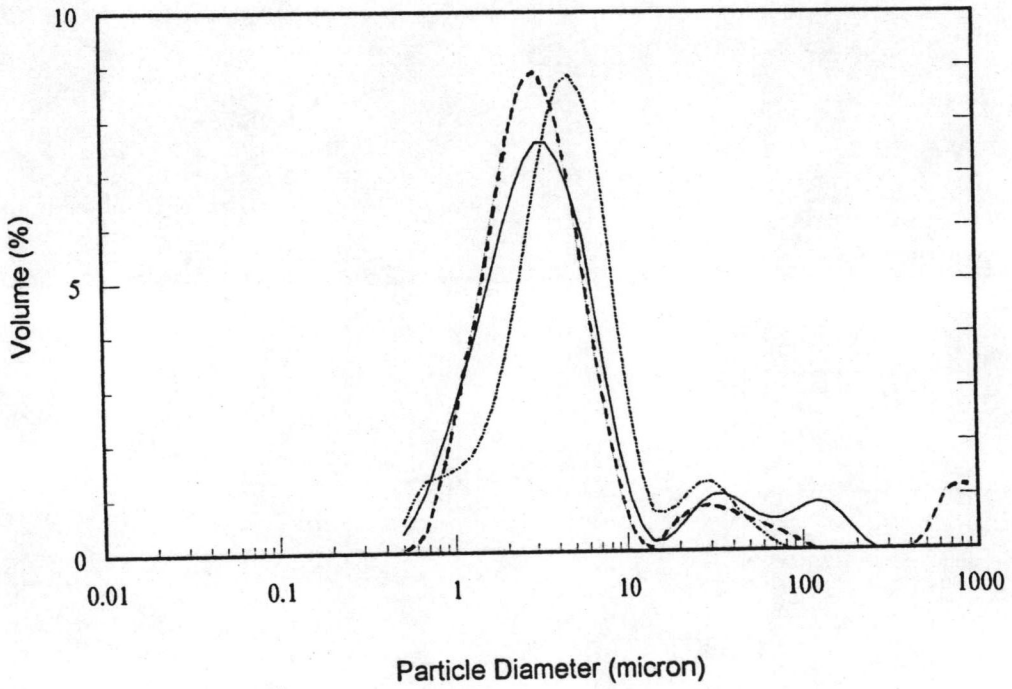


Figure 27 Particle size distribution of freshly prepared 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with various concentration of CM-Chitosan.



Cumulative undersize frequency curve of liposomes stabilized with 0.02%, 0.2%, 0.5% w/v CM-Cellulose and 0.02%, 0.2%, 0.5% w/v CM-Chitosan were shown in Figure 63-68 (see appendix II).

(c) Microscopic appearance

Micrograph of liposomes with 1:1 molar ratio of lecithin to cholesterol stabilized with various concentration of CM-Cellulose or CM-Chitosan were shown in Figure 28, 29, 30, 31, 32, 33. Increasing of polymer concentration appeared condensing of liposome vesicles but no differences between the same concentration of CM-Cellulose and CM-Chitosan.

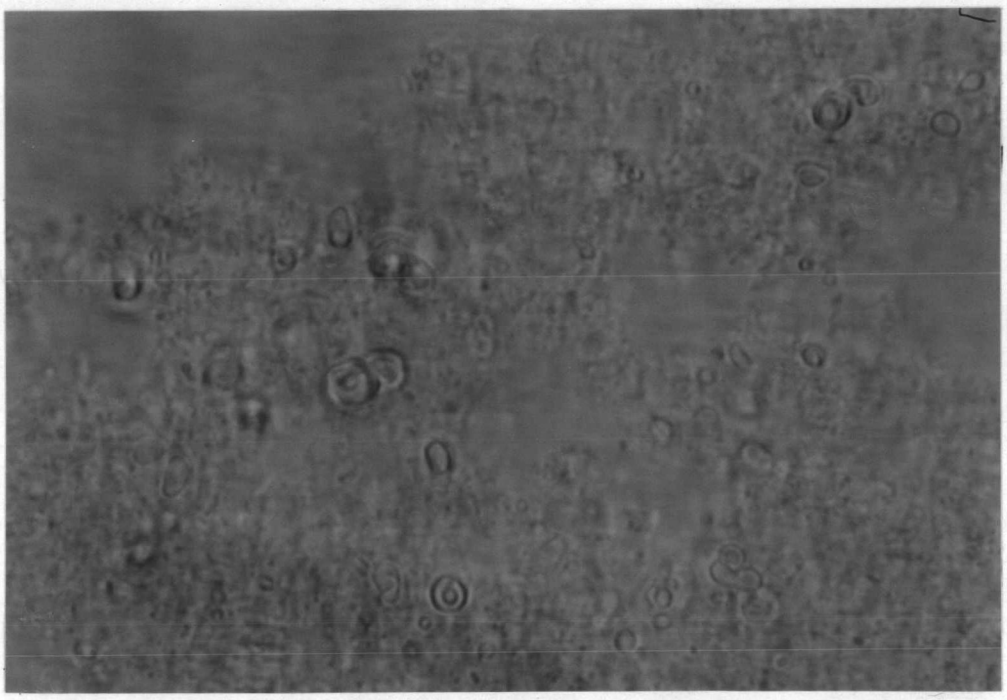


Figure 28 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.02% w/v CM-Cellulose (magnification 1000x)

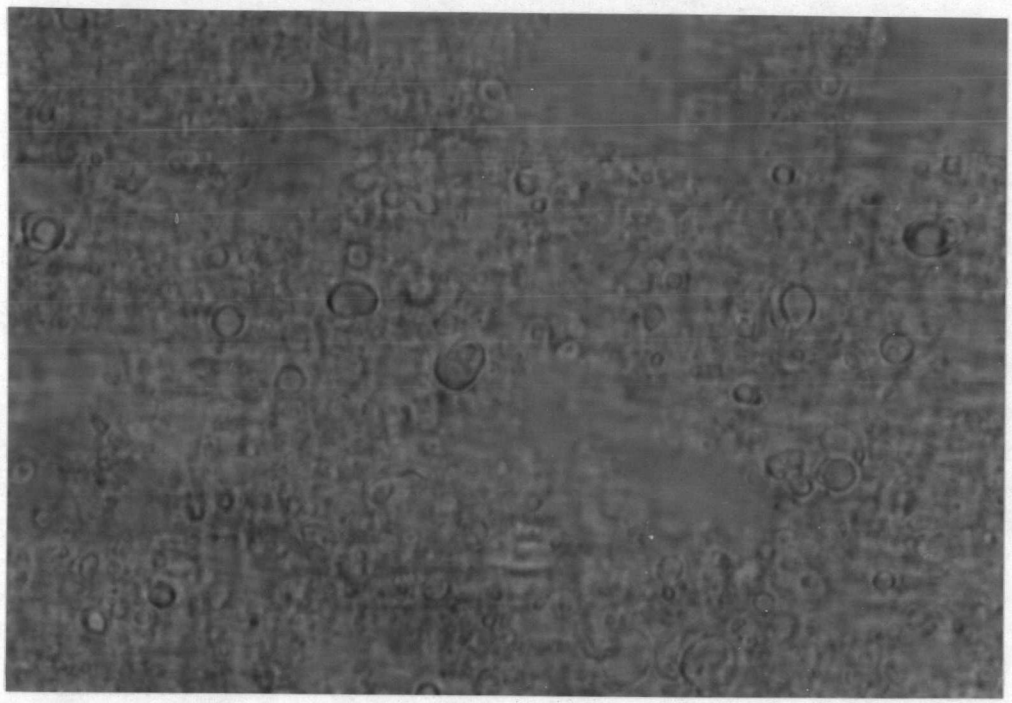


Figure 29 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.2% w/v CM-Cellulose (magnification 1000x)

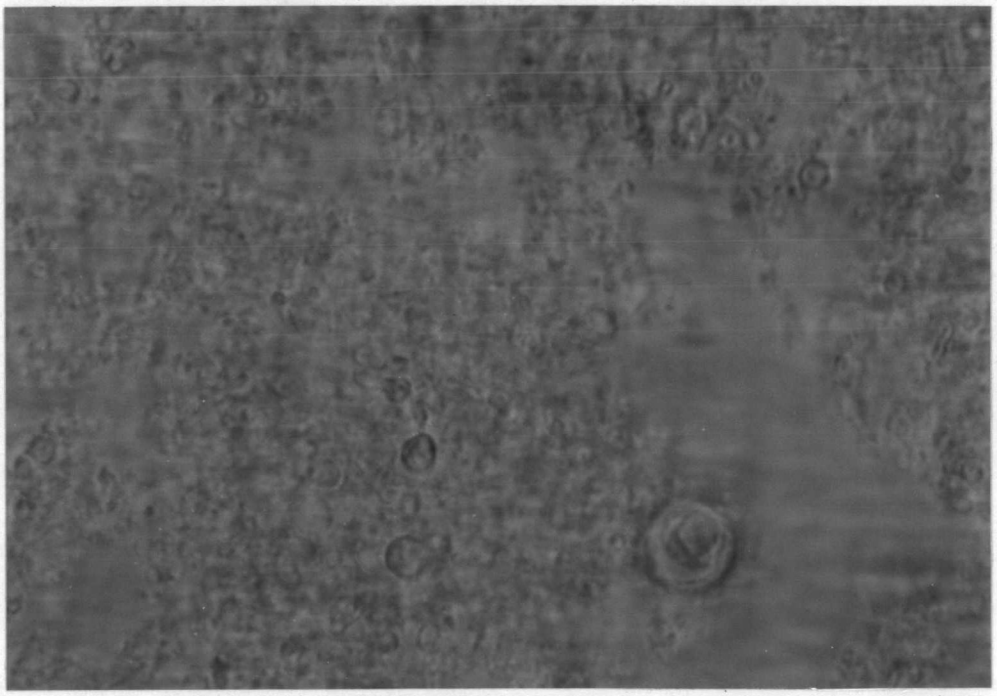


Figure 30 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.5% w/v CM-Cellulose (magnification 1000x)

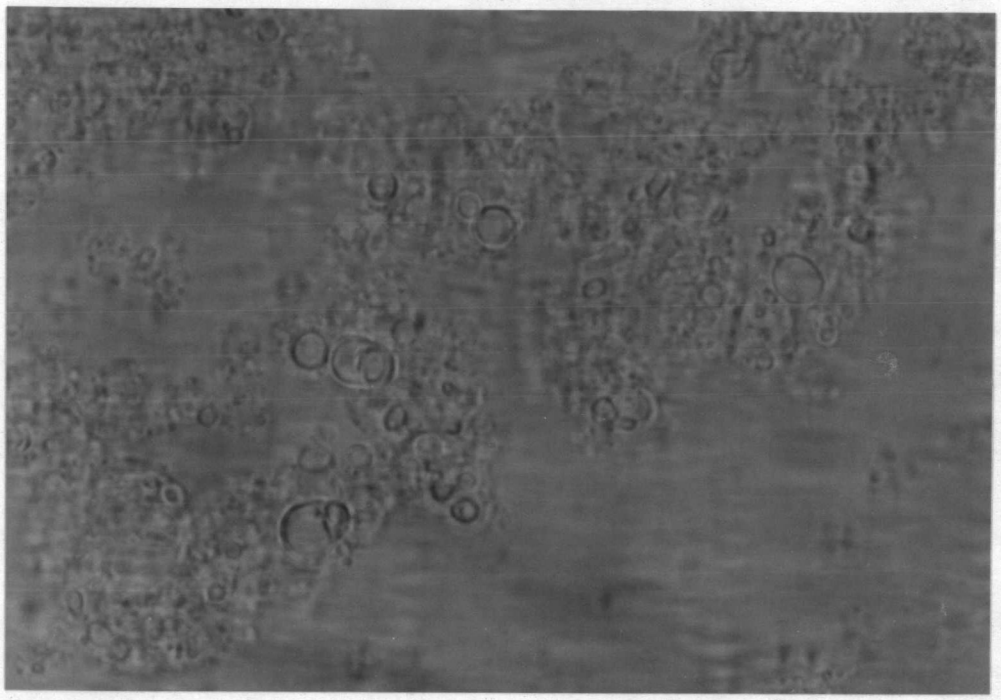


Figure 31 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.02% w/v CM-Chitosan (magnification 1000x)

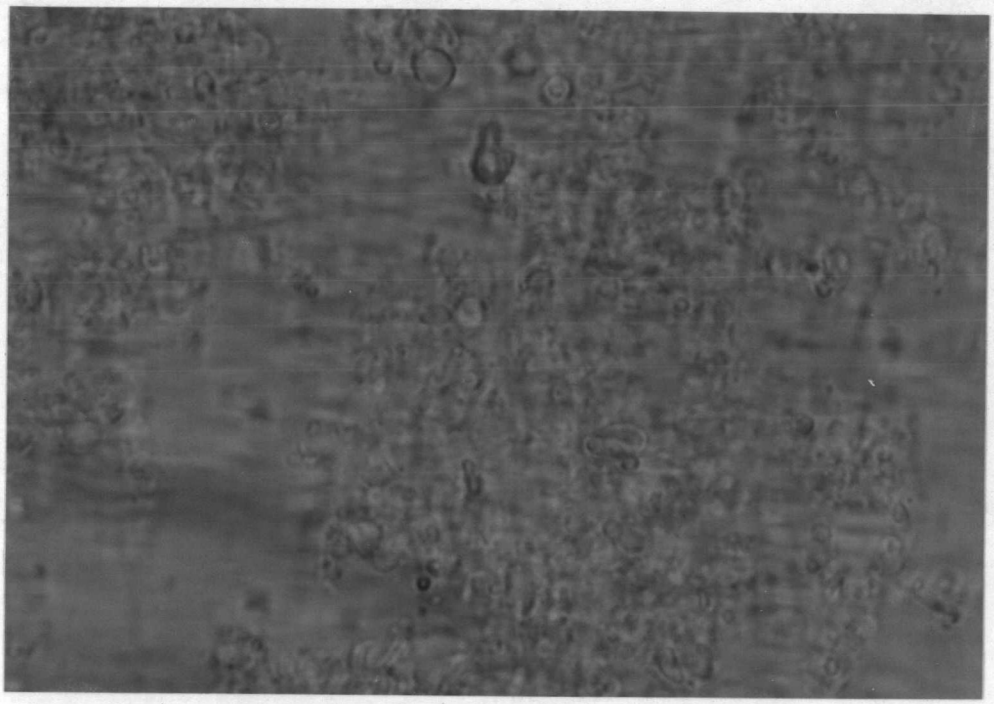


Figure 32 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.2% w/v CM-Chitosan (magnification 1000x)

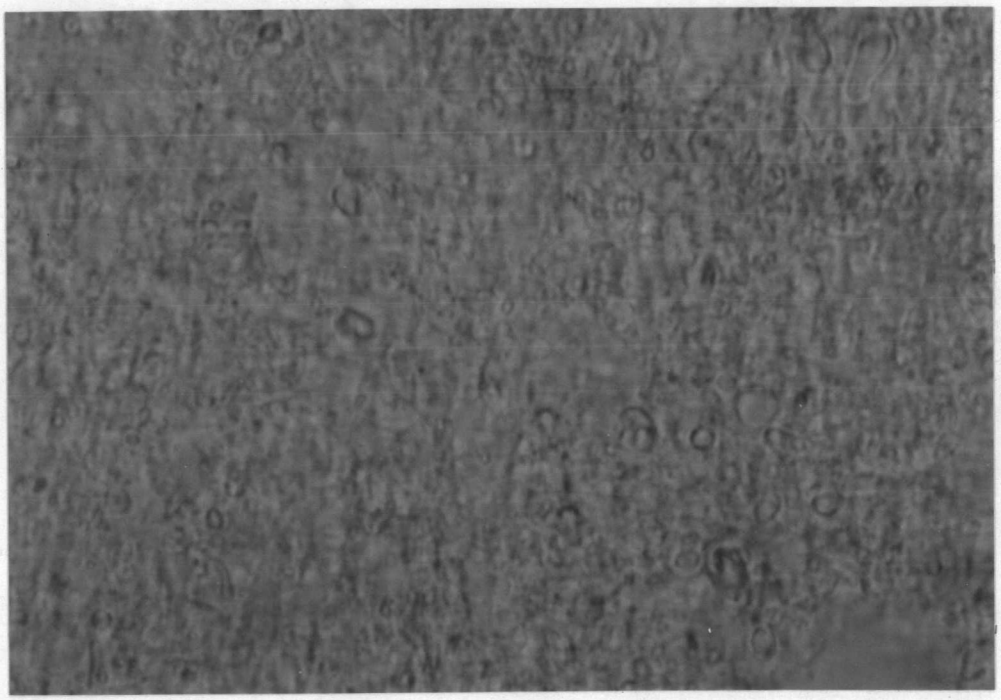


Figure 33 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.5% w/v CM-Chitosan (magnification 1000x)

(d) Scanning electron microscopy (SEM)

Scanning electron micrograph of liposomes with 1:1 molar ratio of egg yolk lecithin to cholesterol stabilized with various concentrations of CM-Cellulose and CM-Chitosan were shown in Figure 34, 35, 36.

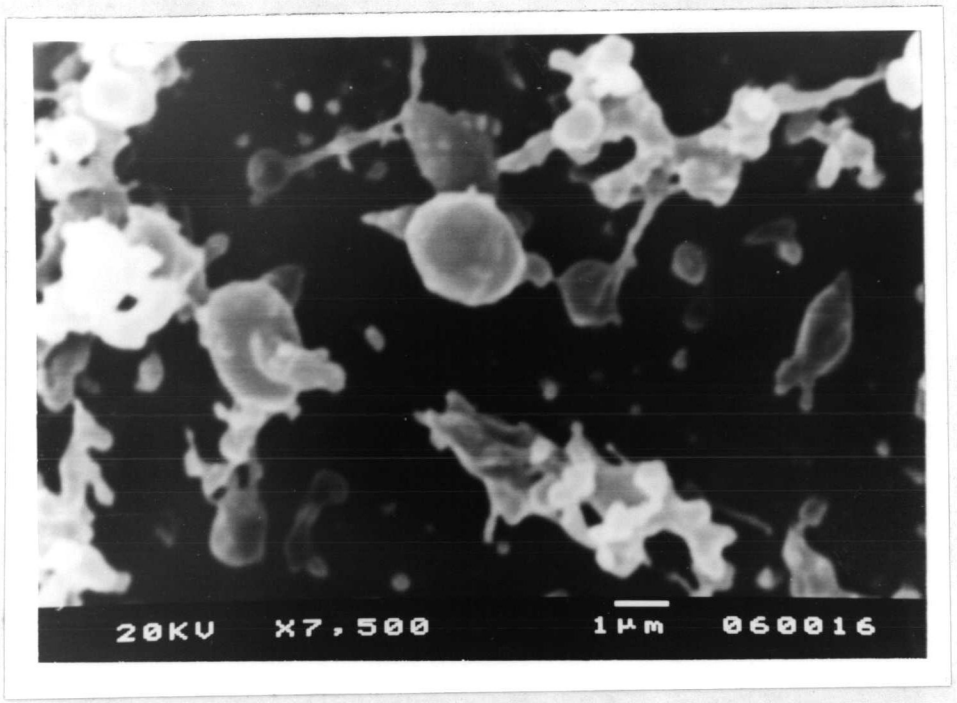
(e) Assessment of protein leakage from liposomes in PBS pH 7.4 at 37°C

Effect of polymer coating on protein leakage of liposomes were represented in the term of percent of released protein which shown in Table 10, 11, and the graphs of releasing profile were shown in Figure 37, 38. The 0.5% w/v of CM-Cellulore and CM-Chitosan gave the more sustained releases than 0.2% w/v and 0.02% w/v, respectively.

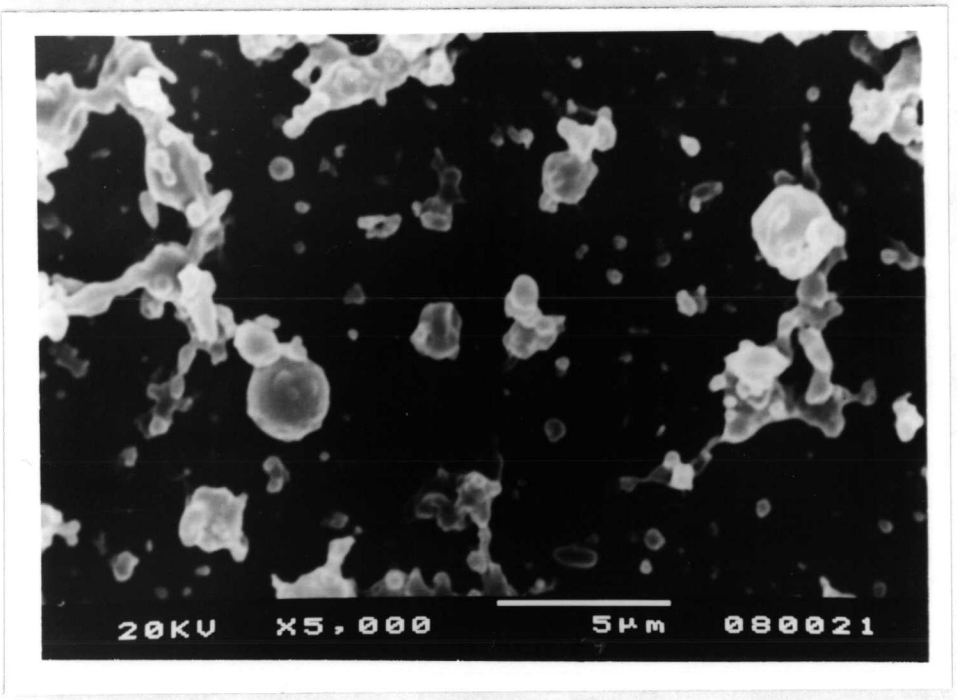
**6. Stability Study of Liposomes in PBS pH7.4 at 4°C for 3 Months**

6.1 Effect of cholesterol content on physical stability of liposomes

(a) Percent of remained protein in liposomes, when they were storaged in PBS pH 7.4 at 4°C, were shown in Table 12 and the graph of percent of remained protein was shown in Figure 39. The 1:1 molar ratio of egg yolk lecithin to cholestnol showed the highest stability. The protein centent was stilled 90% while the 7:2 and 1:0 were 76.32% and 55.16% respectively.

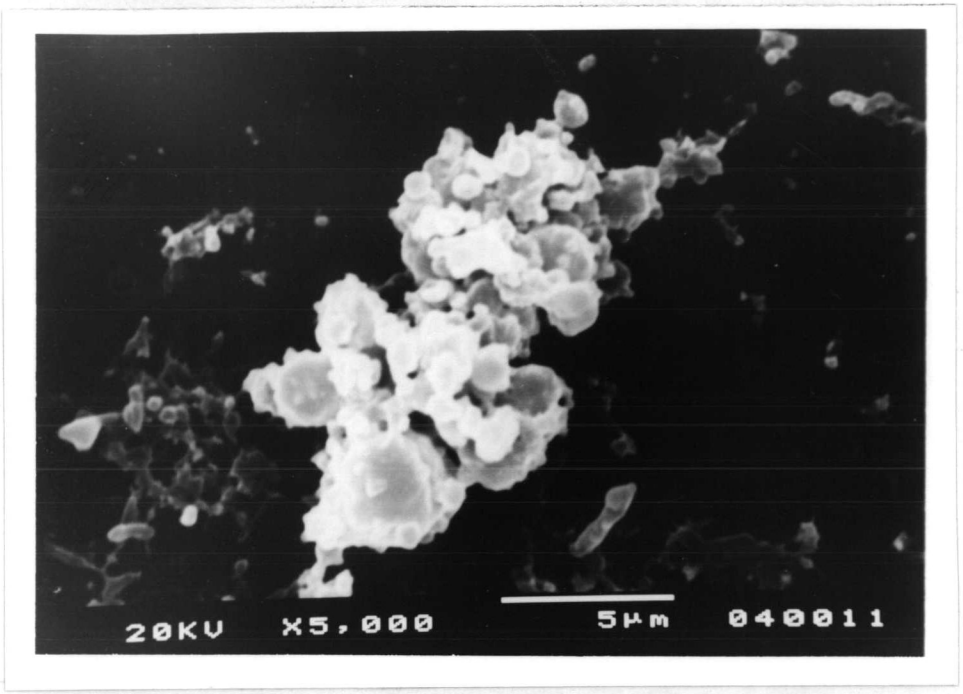



(a)

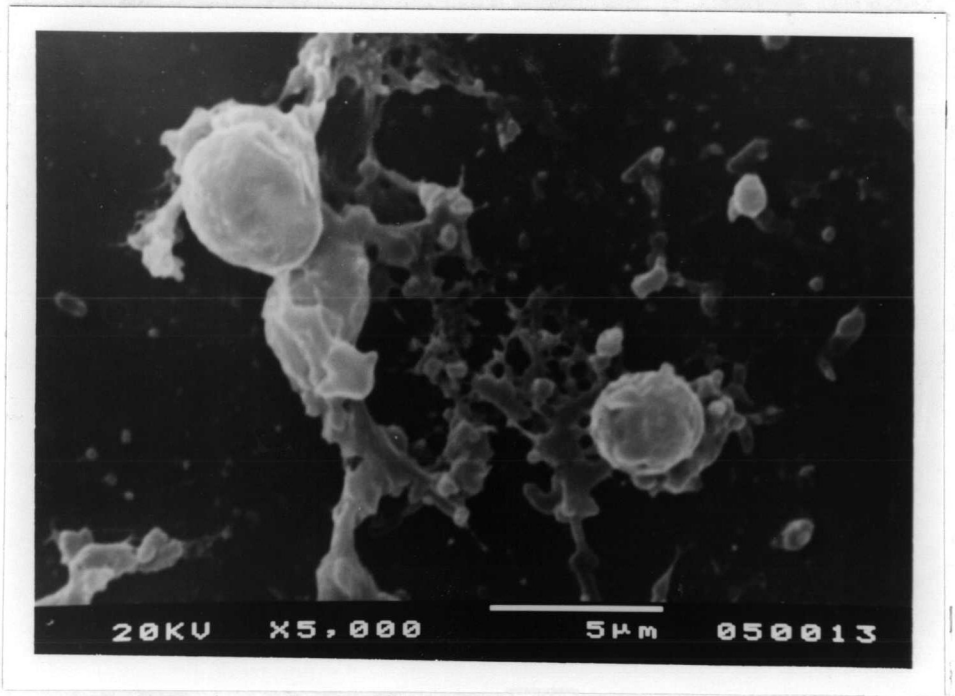



(b)

Figure 34 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 1: 1 molar ratio of egg yolk lecithin to cholesterol (a) stabilized with 0.02%w/v Carboxymethylcellulose (magnification 5000x) (b) stabilized with 0.02%w/v Carboxymethylchitosan (magnification 5000x)

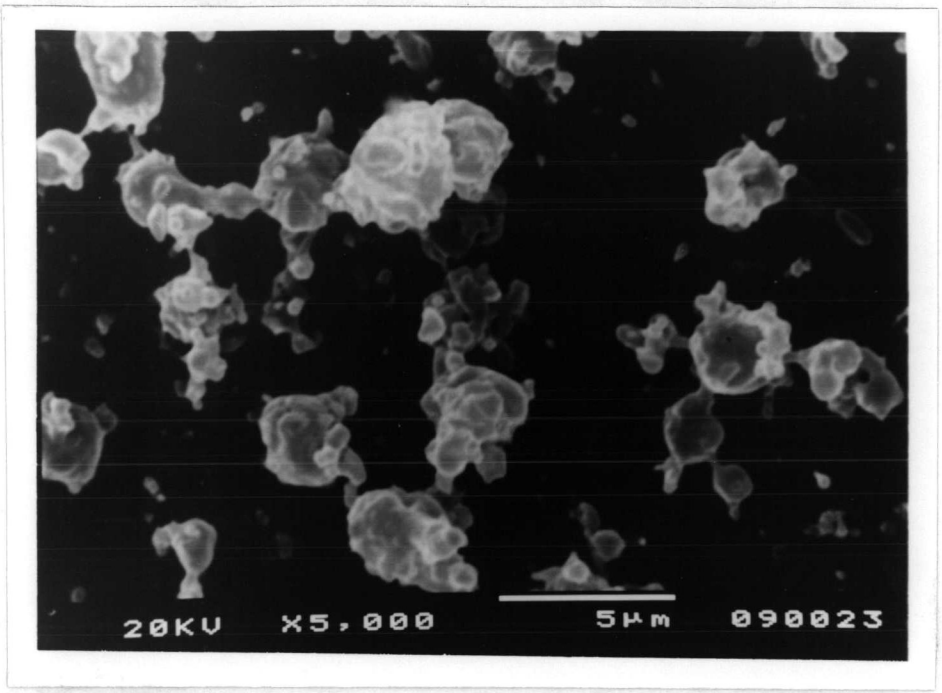



(a)

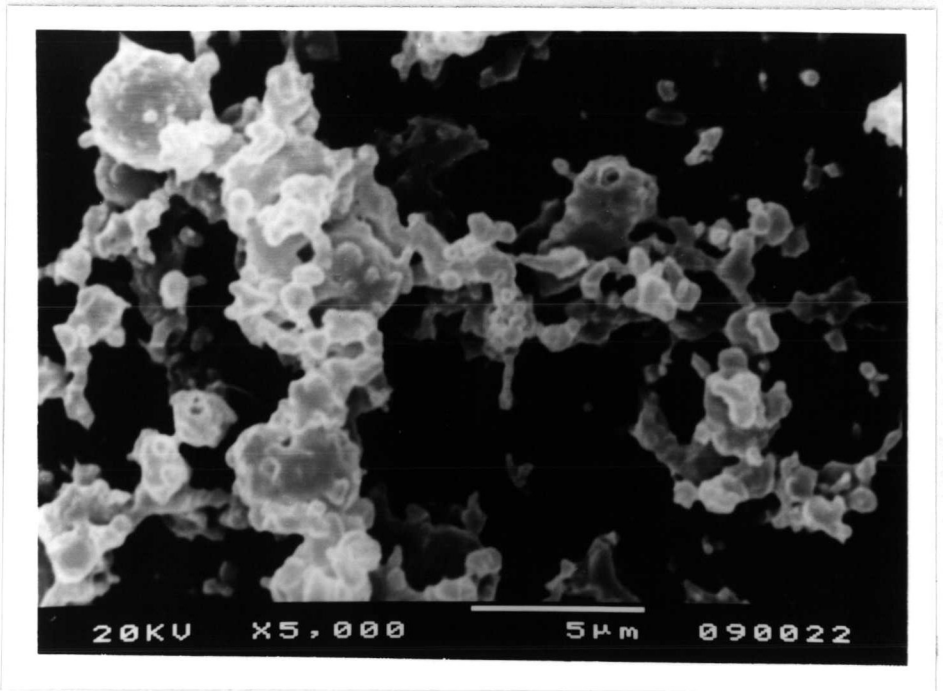



(b)

Figure 35 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 1: 1 molar ratio of egg yolk lecithin to cholesterol (a) stabilized with 0.2% w/v Carboxymethylcellulose (magnification 5000x) (b) stabilized with 0.2% w/v Carboxymethylchitosan (magnification 5000x)




(a)




(b)

Figure 36 Scanning electron micrograph of liposomes containing protein extract from *P. multocida* with 1: 1 molar ratio of egg yolk lecithin to cholesterol (a) stabilized with 0.5% w/v Carboxymethylcellulose (magnification 5000x) (b) stabilized with 0.5% w/v Carboxymethylchitosan (magnification 5000x)

Table 10 Percent of released protein from 1:1 molar ratio of egg yolk lecithin to cholesterol liposomes stabilized with various concentrations of CM-Cellulose in PBS pH 7.4 at 37 °C

| Time (hr) | Percent of released protein from liposomes | | |
|---|---|---|---|
| | 0.02% w/v CM-Cellulose | 0.2% w/v CM-Cellulose | 0.5% w/v CM-Cellulose |
| 0 | 0.00 | 0.00 | 0.00 |
| 1 | 9.88 | 8.05 | 2.02 |
| 2 | 14.97 | 13.50 | 10.16 |
| 3 | 22.30 | 16.07 | 12.02 |
| 4 | 26.12 | 24.24 | 21.79 |
| 5 | 27.25 | 26.11 | 23.29 |
| 6 | 32.49 | 30.01 | 27.84 |
| 8 | 37.49 | 34.49 | 32.54 |
| 10 | 48.00 | 42.36 | 37.56 |
| 12 | 50.71 | 48.64 | 43.85 |
| 14 | 51.78 | 50.78 | 44.03 |
| 16 | 53.59 | 49.34 | 45.09 |
| 18 | 54.87 | 52.26 | 46.35 |
| 20 | 54.94 | 50.61 | 47.56 |
| 22 | 55.63 | 51.09 | 48.51 |
| 24 | 55.25 | 52.28 | 49.69 |

Figure 37 Releasing profile of protein from liposomes containing protein extract from *P.multocida* with 1:1 molar ratio of lecithin to cholesterol stabilized with CM-Cellulose in PBS pH 7.4 at 37° C

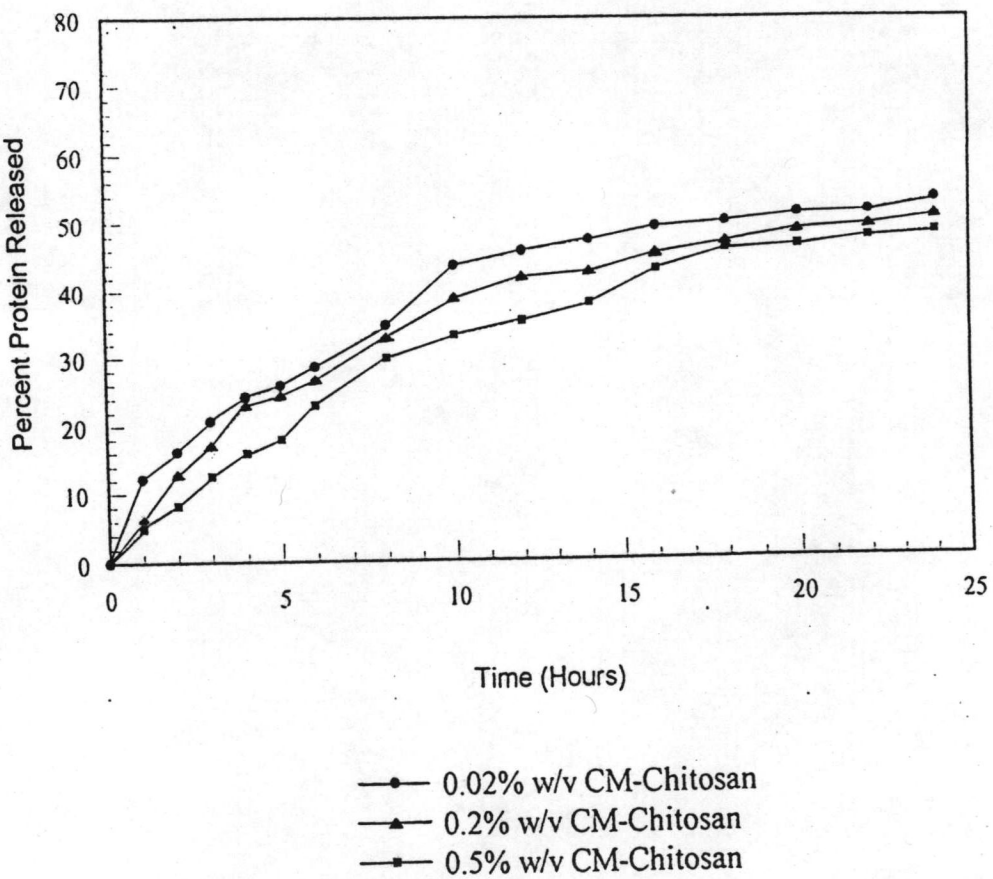


Table 11 Percent of released protein from 1:1 molar ratio of egg yolk lecithin to cholesterol liposomes stabilized with various concentrations of CM-Chitosan in PBS pH 7.4 at 37 °C

| Time (hr) | Percent of released protein from liposomes | | |
|---|---|---|---|
| | 0.02% w/v CM-Chitosan | 0.2% w/v CM-Chitosan | 0.5% w/v CM-Chitosan |
| 0 | 0.00 | 0.00 | 0.00 |
| 1 | 12.11 | 6.11 | 4.91 |
| 2 | 16.04 | 12.78 | 8.25 |
| 3 | 20.64 | 16.94 | 12.46 |
| 4 | 24.17 | 22.89 | 15.91 |
| 5 | 25.72 | 24.33 | 17.91 |
| 6 | 28.46 | 26.54 | 22.90 |
| 8 | 34.60 | 32.74 | 29.71 |
| 10 | 43.49 | 38.65 | 33.02 |
| 12 | 45.51 | 41.72 | 35.17 |
| 14 | 47.04 | 42.45 | 37.72 |
| 16 | 49.00 | 45.13 | 42.75 |
| 18 | 49.73 | 46.75 | 45.72 |
| 20 | 50.95 | 48.39 | 46.16 |
| 22 | 51.19 | 49.09 | 47.29 |
| 24 | 52.95 | 50.45 | 47.97 |

Figure 38 Releasing profile of protein from liposomes containing protein extract from *P.multocida* with 1:1 molar ratio of lecithin to cholesterol stabilized with CM-Chitosan in PBS pH 7.4 at 37 ℃

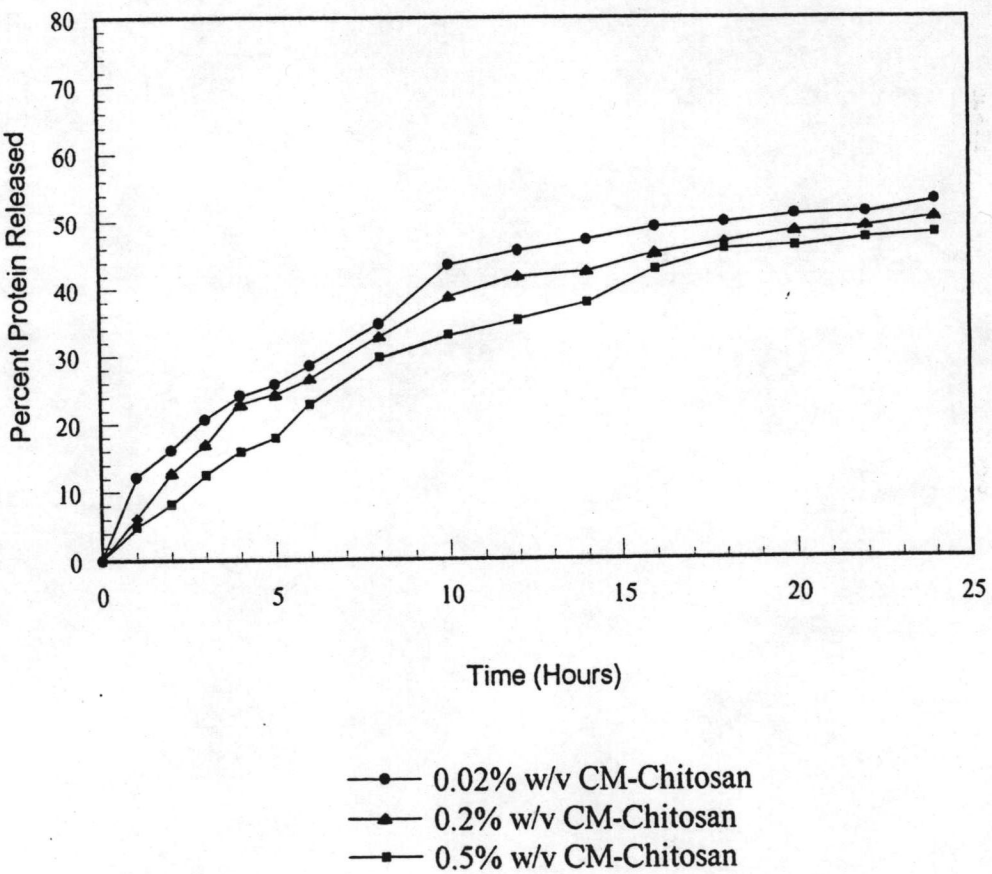
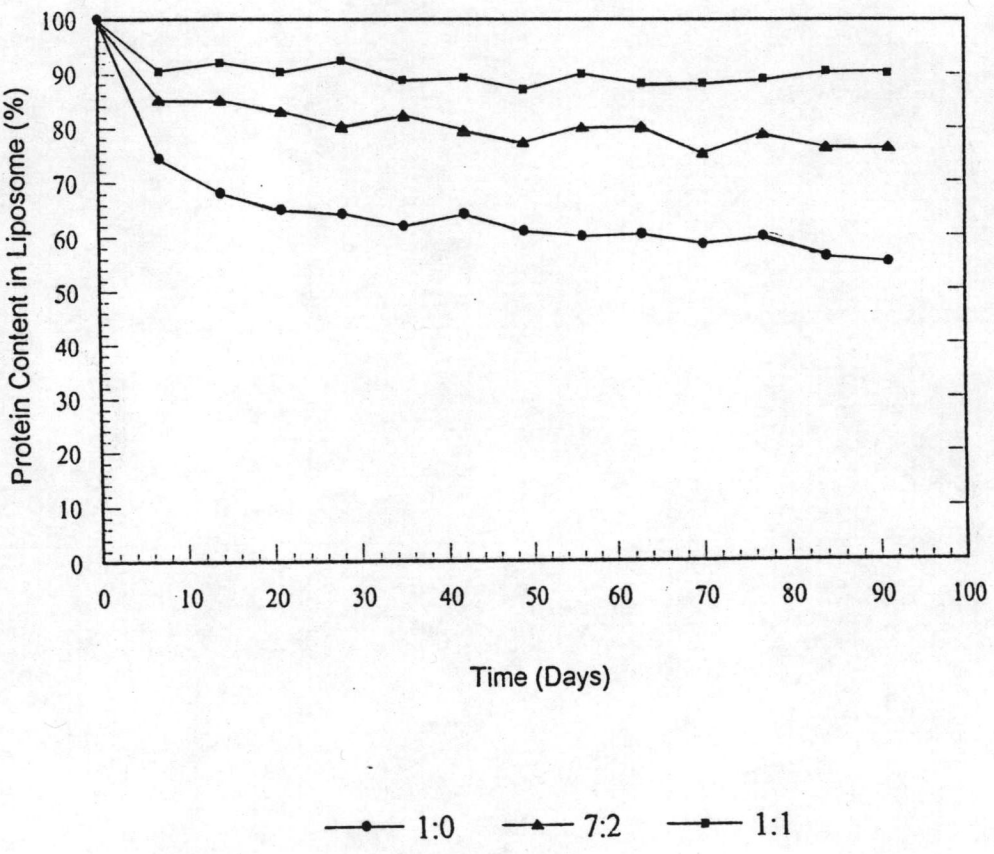


Table 12 Percent of remained protein in liposomes with various molar ratios of egg yolk lecithin to cholesterol, Stored at 4°C for 3 months.

| Time (day) | Percent of protein in liposomes | | |
|---|---|---|---|
| | lecithin : chol 1:1 | lecithin : chol 7:2 | lecithin : chol 1:0 |
| 0 | 100.0 | 100.0 | 100.0 |
| 7 | 90.48 | 85.08 | 74.46 |
| 14 | 92.16 | 85.16 | 68.23 |
| 21 | 90.39 | 83.06 | 65.21 |
| 28 | 92.50 | 80.50 | 64.36 |
| 35 | 88.95 | 82.45 | 62.06 |
| 42 | 89.33 | 79.55 | 64.23 |
| 49 | 87.07 | 77.32 | 61.03 |
| 56 | 90.02 | 80.10 | 59.99 |
| 63 | 88.16 | 80.25 | 60.46 |
| 70 | 88.15 | 75.35 | 58.50 |
| 77 | 88.98 | 78.90 | 59.88 |
| 84 | 90.50 | 76.50 | 56.17 |
| 91 | 90.14 | 76.32 | 55.16 |

Figure 39 Percent of remained protein in liposomes with various molar ratios of egg yolk lecithin to cholesterol, stored at 4°C for 3 months.



(b) Micrograph of liposomes with various molar ratios of lecithin to cholesterol were compared between freshly prepared and 3 months storage liposomes,

1 : 0 molar ratio liposome was shown in Figure 40

7 : 2 molar ratio liposome was shown in Figure 41

1 : 1 molar ratio liposome were shown in Figure 42

(c) The median diameter of liposome vesicles with various molar ratios of lecithin to cholesterol were compared between freshly prepared and 3 months storage liposomes that was shown in Table 6.

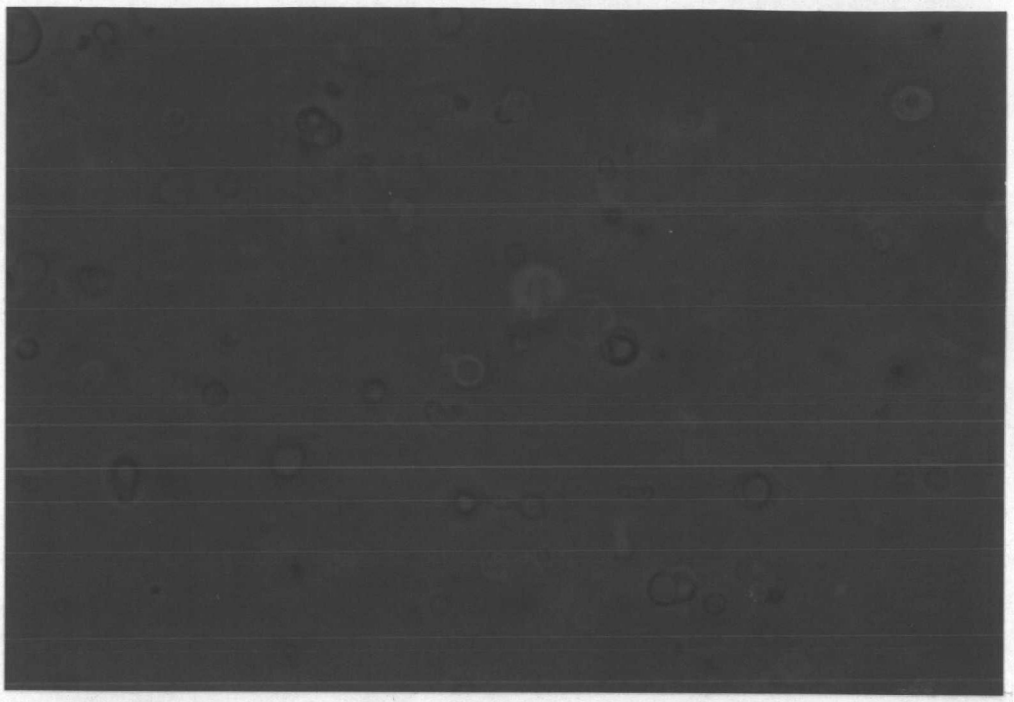
After 3 months storaged, the median diameters of 1:0 and 7:2 of egg yolk lecithin to cholesterol liposomes were increased from 3.81 to 6.50 and 3.83 to 6.39 micron, respectively. They showed the aggregation of liposomes to form the larger particle size, The median diameter of liposomes prepared from 1:1 molar ratio of egg yolk lecithin and cholesterol showed the stable performance of 5.95 micron on freshly prepared and 3 month storage.

The particle size distribution of liposomes with various ratios of lecithin to cholesterol were compared between freshly prepared and 3 months storage liposomes,

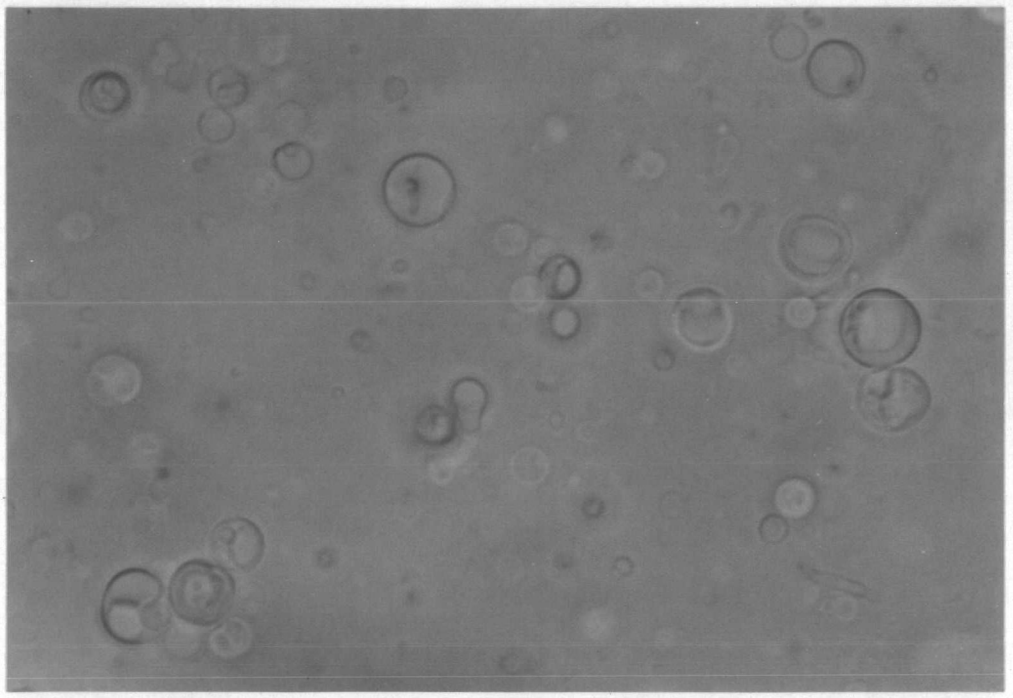
1 : 0 molar ratio liposom was shown in Figure 43

7 : 2 molar ratio liposome was shown in Figure 44

1 : 1 molar ratio liposome was shown in Figure 45



(a)

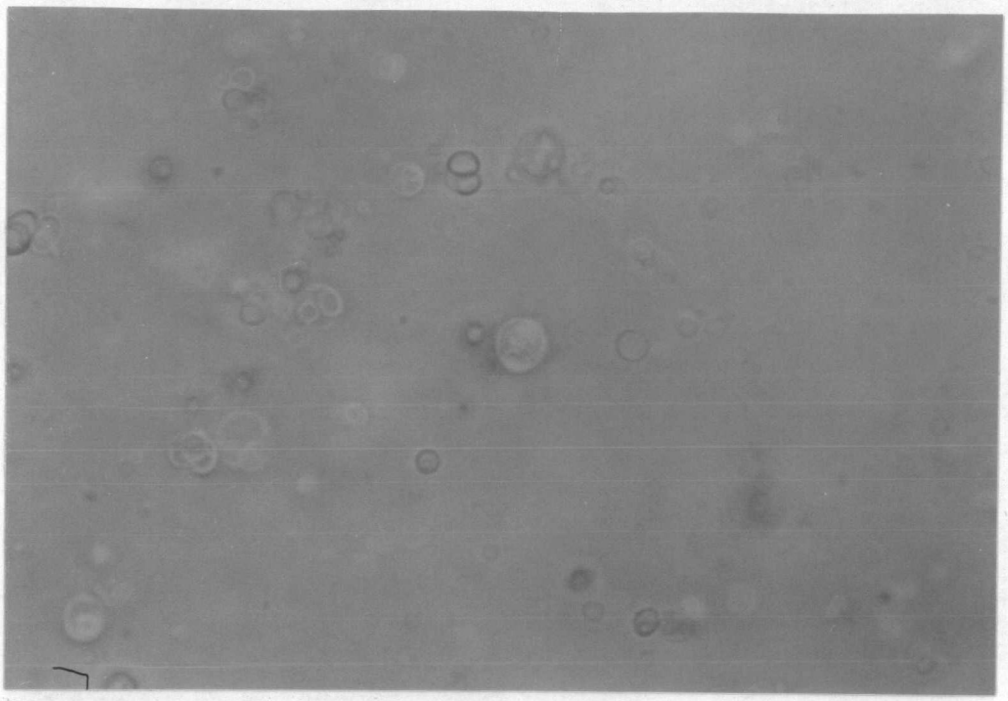


(b)

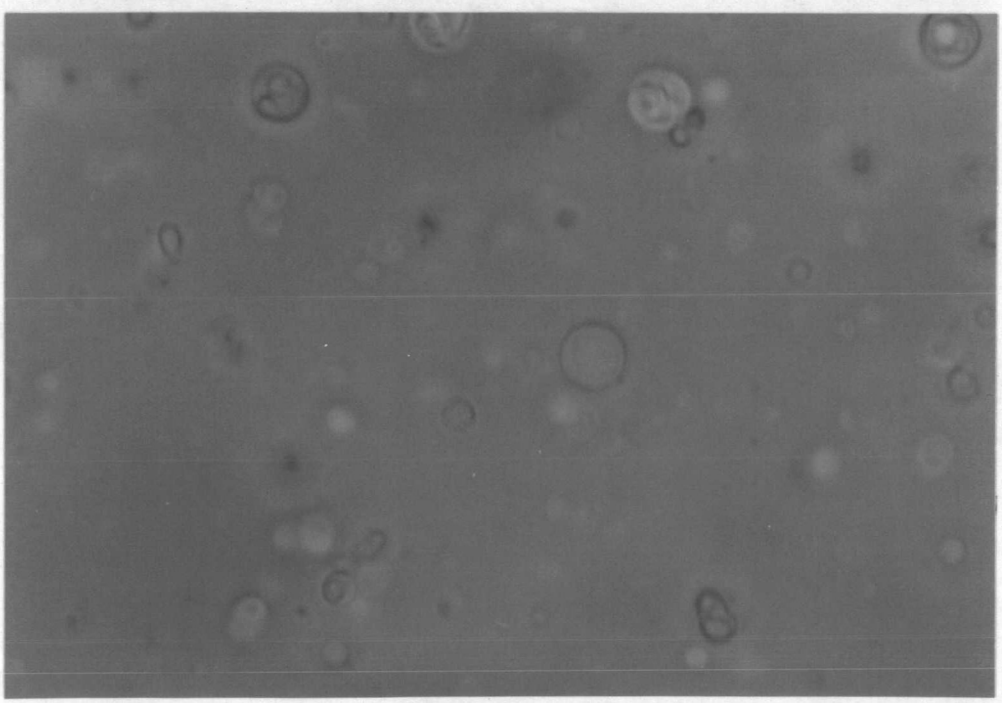
Figure 40 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 0 molar ratio of lecithin to cholesterol (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



(a)

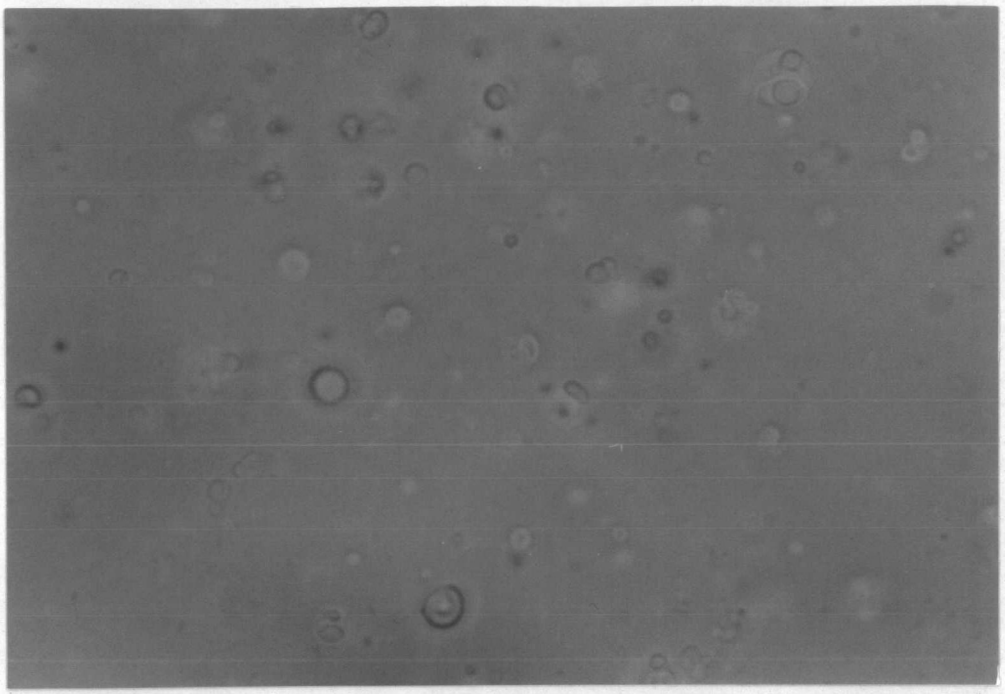


(b)

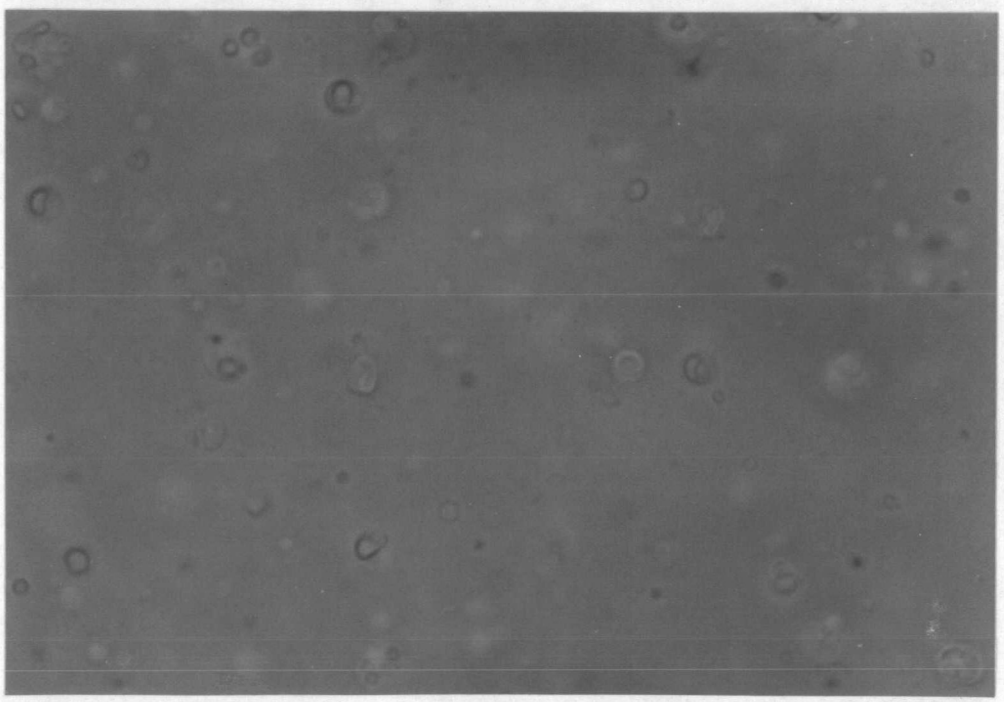
Figure 41 Micrograph of liposomes containing protein extract from *P. multocida* with 7 : 2 molar ratio of lecithin to cholesterol (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



(a)



(b)

Figure 42 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of lecithin to choloesterol (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at $4^{\circ}C$

Figure 43 Particle size distribution of 1:0 molar ratio of lecithin to cholesterol liposomes, comparison between freshly prepared and 3 months storage.

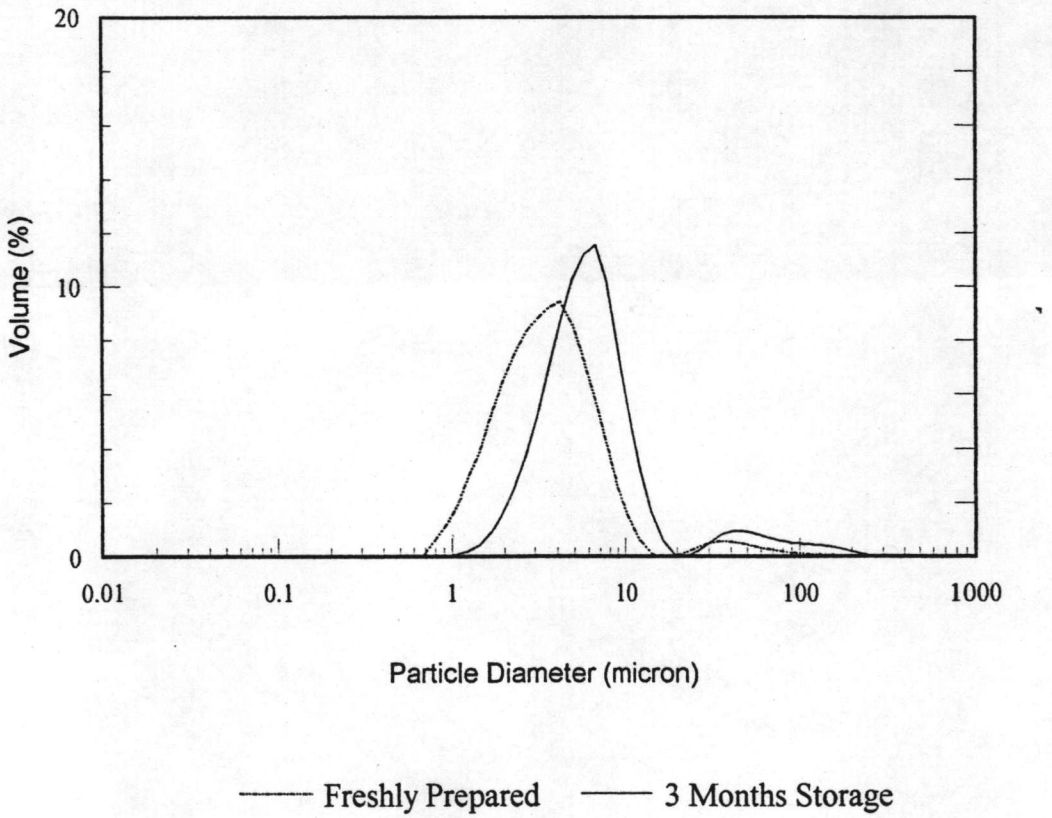


Figure 44 Particle size distribution of 7:2 molar ratio of lecithin to cholesterol liposomes, comparison between freshly prepared and 3 months storage.

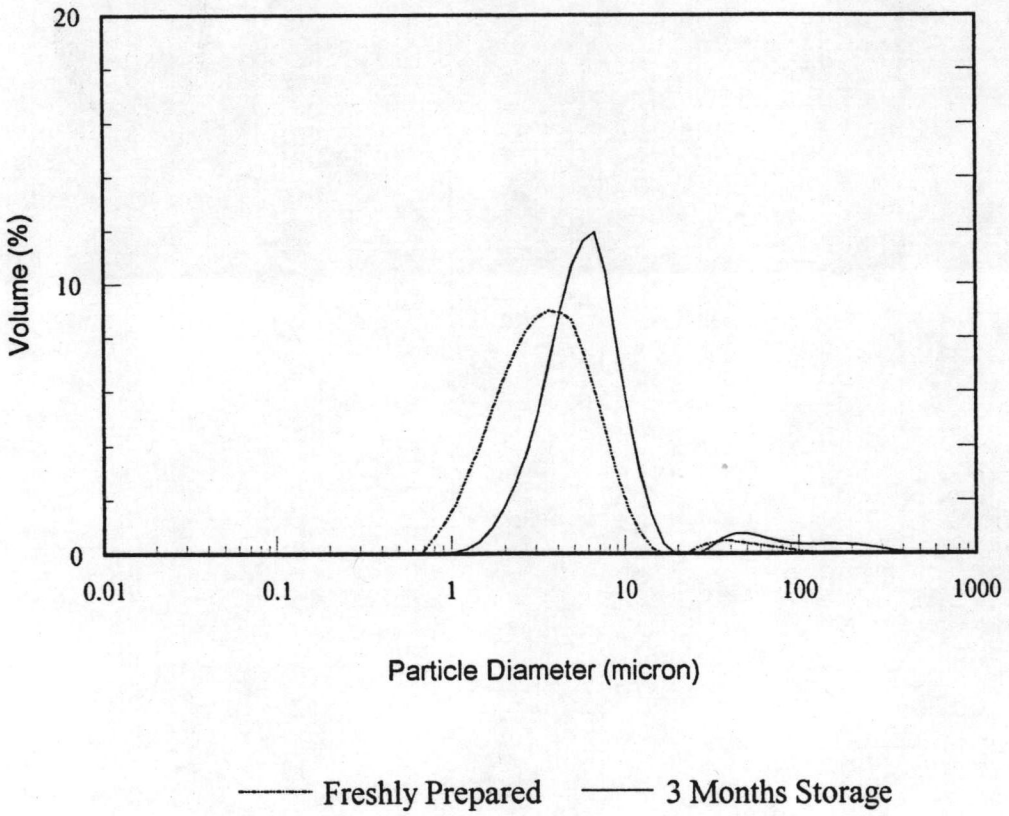
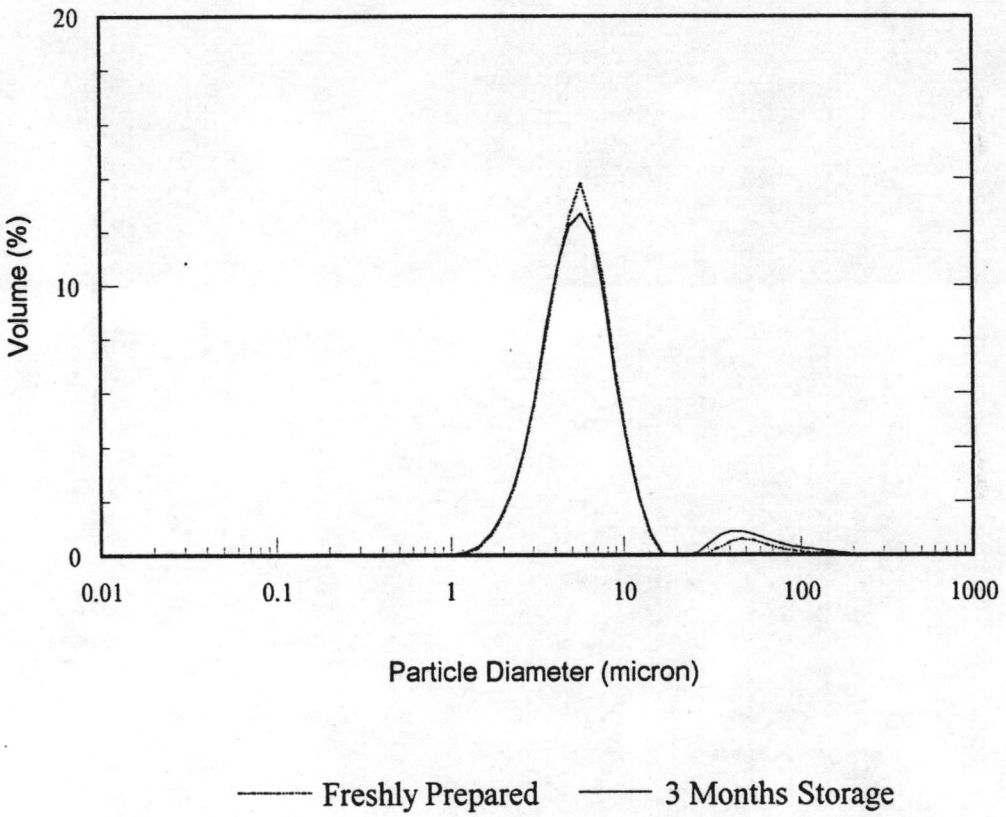


Figure 45 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes, comparison between freshly prepared and 3 months storage.



The cumulative undersize frequency curves of 3 months storage liposomes with various molar ratios of lecithin to cholesterol,

1 : 0 molar ratio liposome was shown in Figure 60

7 : 2 molar ratio liposome was shown in Figure 61

1 : 1 molar ratio liposome was shown in Figure 62

(see Appendix II)

6.2 Effect of polymer coated on physical stability of liposomes

(a) Percent of remained protein in liposomes stabilized with various concentration of CM-Cellulose and CM-Chitosan, when they were storaged in PBS pH 7.4 at 4°C, was shown in Table 13, 14 and the graph of percent of remained protein were shown in Figure 46, 47.

(b) Micrograph of 3 months storage of 1 : 1 molar ratio of egg yolk lecithin to cholesterol liposomes stabilized with various concentration of CM-Cellulose and CM-Chitosan were compared to the freshly prepared liposomes were shown in Figure 48, 49, 50, 51, 52, 53. All of them showed no change between freshly prepared and 3 months storage.

Table 13 Percent of remained protein in liposomes with 1:1 molar ratio of lecithin to cholesterol stabilized with various concentrations of CM-cellulose, Stored at 4°C for 3 months.

| Time (day) | Percent of remained protein in liposomes | | |
|---|---|---|---|
| | 0.02% w/v CM-Cellulose | 0.2% w/v CM-Cellulose | 0.5% w/v CM-Cellulose |
| 0 | 100.0 | 100.0 | 100.0 |
| 7 | 93.5 | 94.24 | 96.00 |
| 14 | 93.03 | 93.77 | 95.48 |
| 21 | 92.99 | 93.06 | 95.08 |
| 28 | 91.23 | 92.80 | 94.82 |
| 35 | 91.10 | 92.16 | 93.85 |
| 42 | 90.88 | 92.17 | 93.00 |
| 49 | 91.17 | 91.99 | 92.93 |
| 56 | 90.01 | 90.98 | 92.59 |
| 63 | 89.21 | 90.48 | 92.23 |
| 70 | 89.54 | 91.00 | 92.16 |
| 77 | 89.07 | 90.99 | 91.99 |
| 84 | 89.40 | 90.00 | 91.48 |
| 91 | 89.00 | 90.48 | 91.21 |

Figure 46 Percent of remained protein in liposomes with 1: 1 molar ratio of egg yolk lecithin to cholesterol stabilized with various concentrations of CM-Cellulose, stored at 4°C for 3 months.

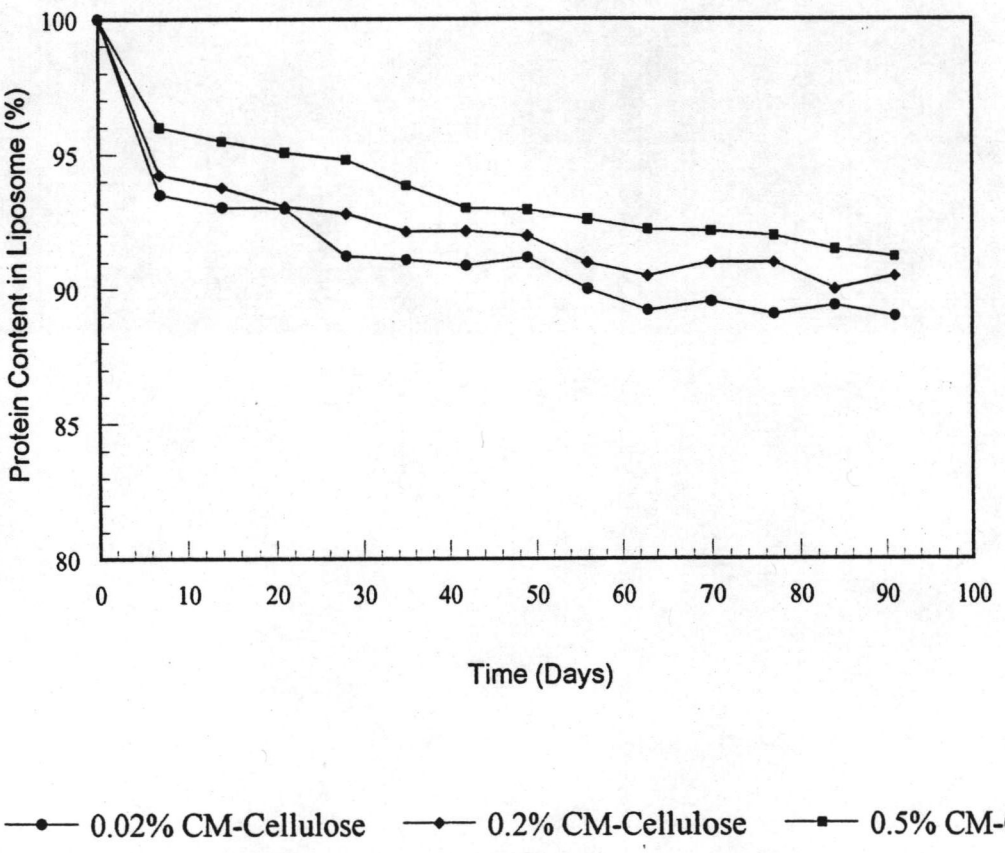
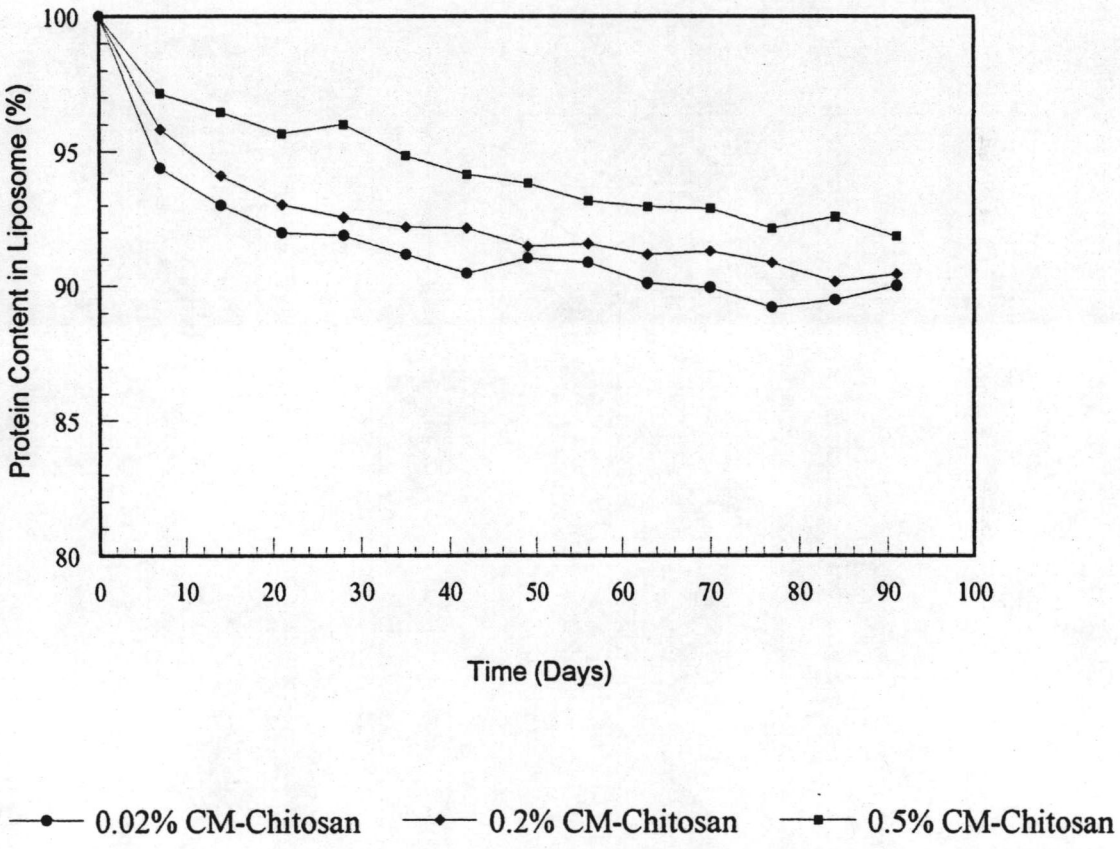
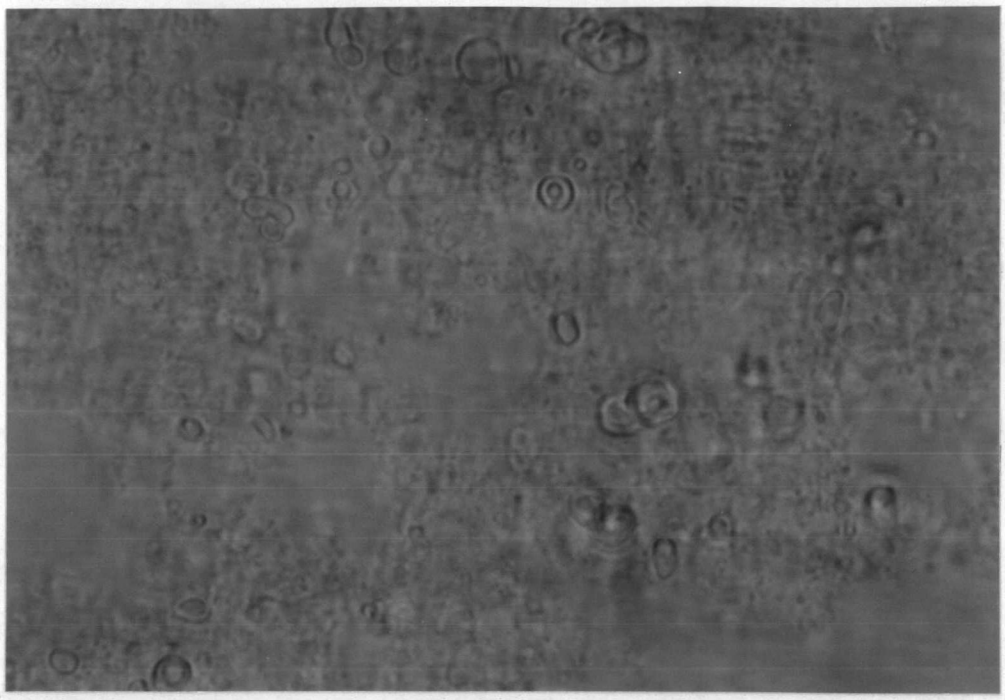


Table 14 Percent of remained protein in liposomes with 1:1 molar ratio of lecithin to cholesterol stabilized with various concentrations of CM-Chitosan, stored at 4 °C for 3 months.

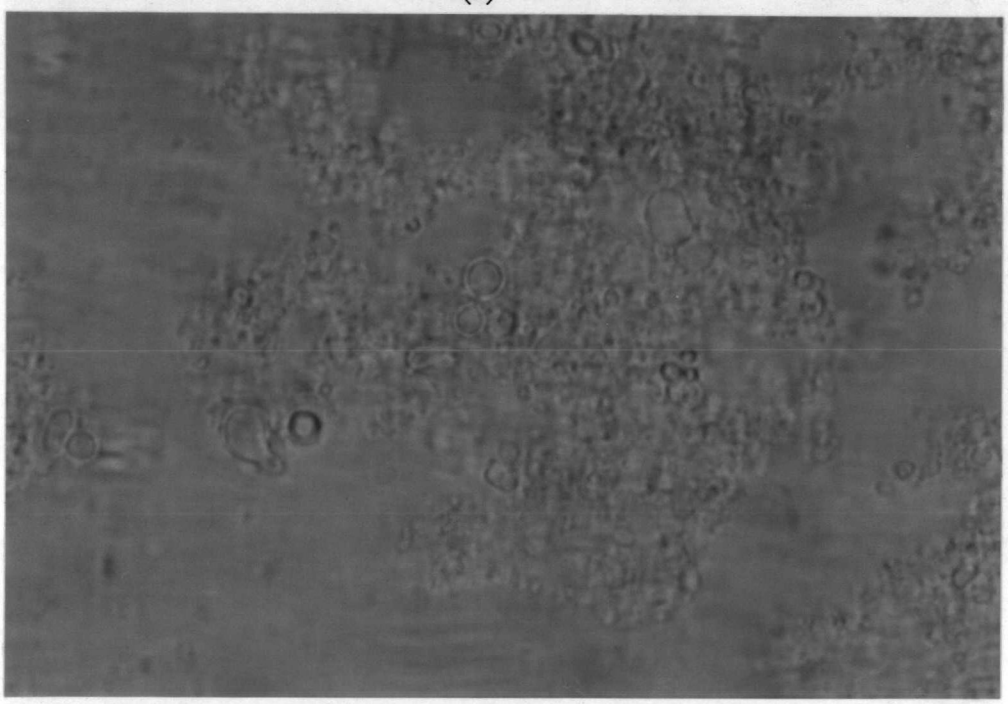
| Time (day) | Percent of remained protein in liposomes | | |
|---|---|---|---|
| | 0.02% w/v CM-Chitosan | 0.2% w/v CM-Chitosan | 0.5% w/v CM-Chitosan |
| 0 | 100.0 | 100.0 | 100.0 |
| 7 | 94.39 | 95.82 | 97.15 |
| 14 | 92.99 | 94.11 | 96.44 |
| 21 | 91.99 | 93.02 | 95.65 |
| 28 | 91.88 | 92.54 | 95.99 |
| 35 | 91.17 | 92.21 | 94.85 |
| 42 | 90.48 | 92.16 | 94.16 |
| 49 | 91.04 | 91.48 | 93.82 |
| 56 | 90.88 | 91.59 | 93.16 |
| 63 | 90.12 | 91.17 | 92.95 |
| 70 | 89.95 | 91.29 | 92.88 |
| 77 | 89.21 | 90.88 | 92.16 |
| 84 | 89.50 | 90.17 | 92.59 |
| 91 | 90.04 | 90.46 | 91.87 |

Figure 47 Percent of remained protein in liposomes with 1: 1 molar ratio of egg yolk lecithin to cholesterol stabilized with various concentrations of CM-Chitosan, stored at 4°C for 3 months.





(a)

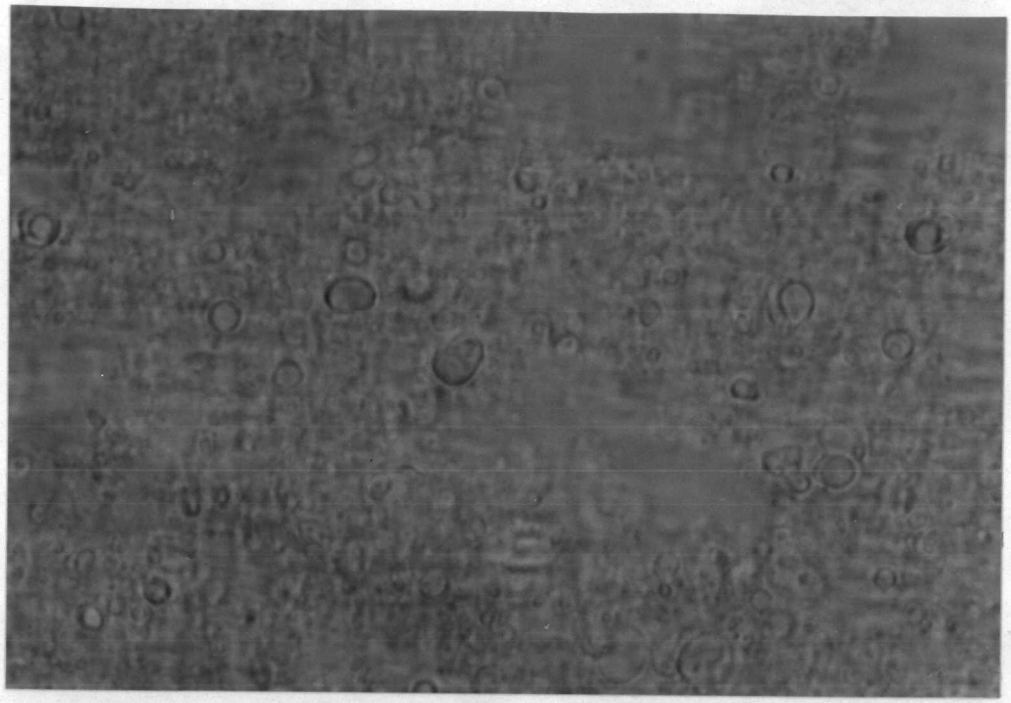


(b)

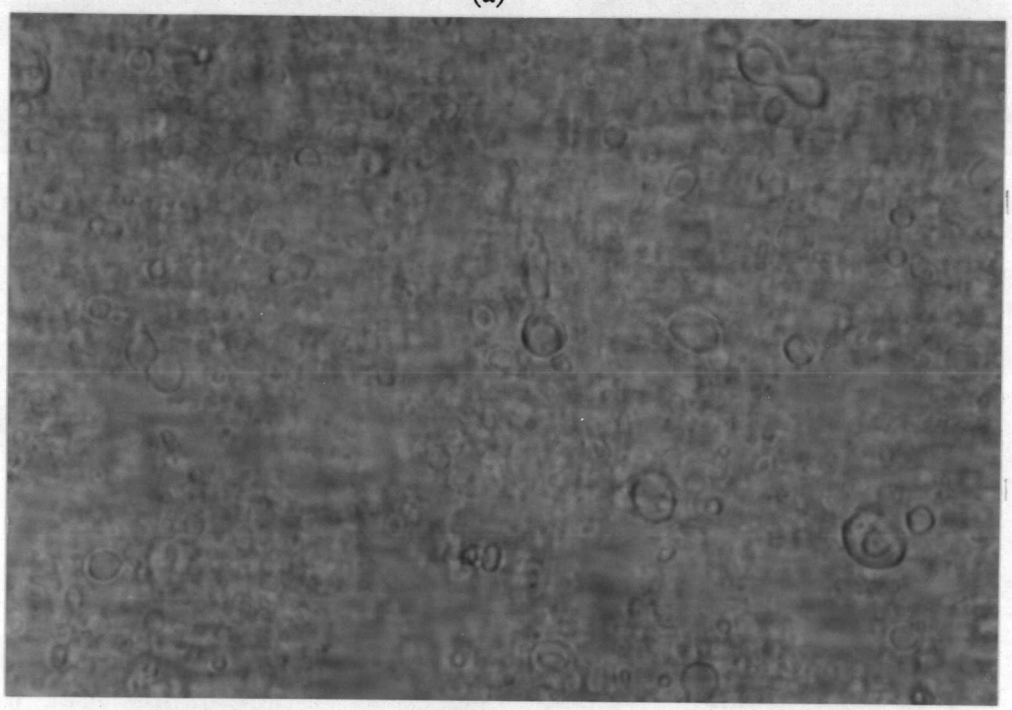
Figure 48 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.02% CM-cellulose (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



(a)

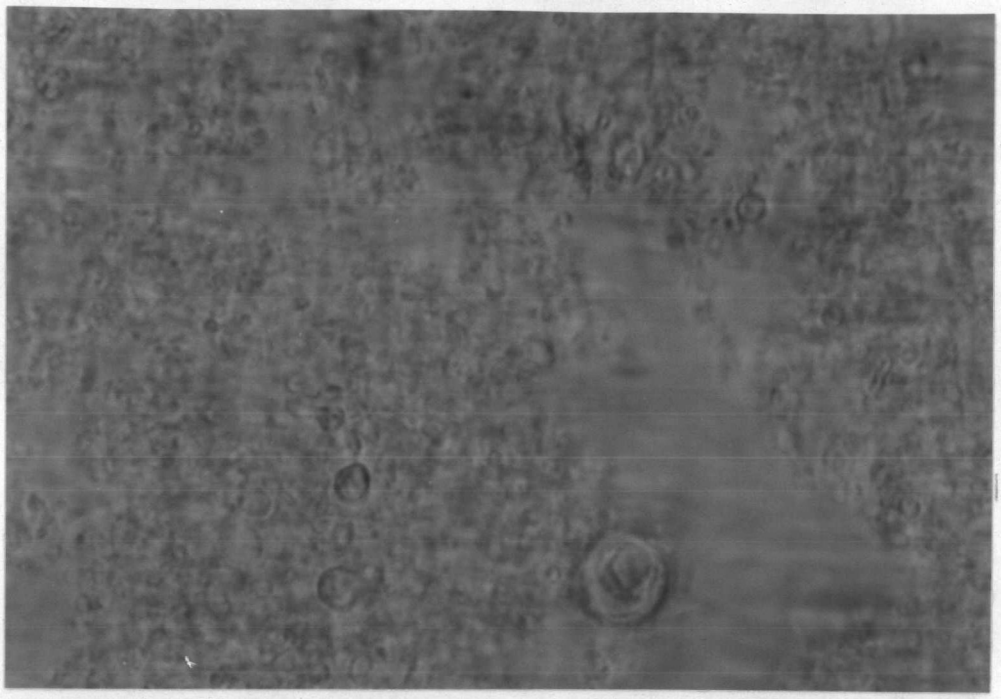


(b)

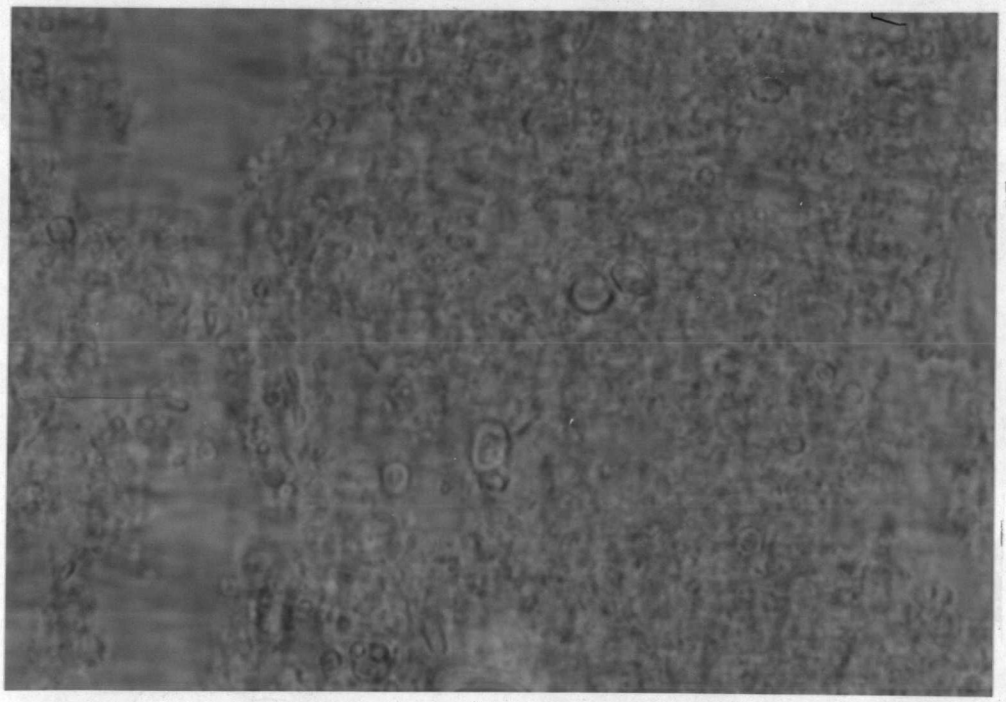
Figure 49 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.2% CM-cellulose (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



(a)

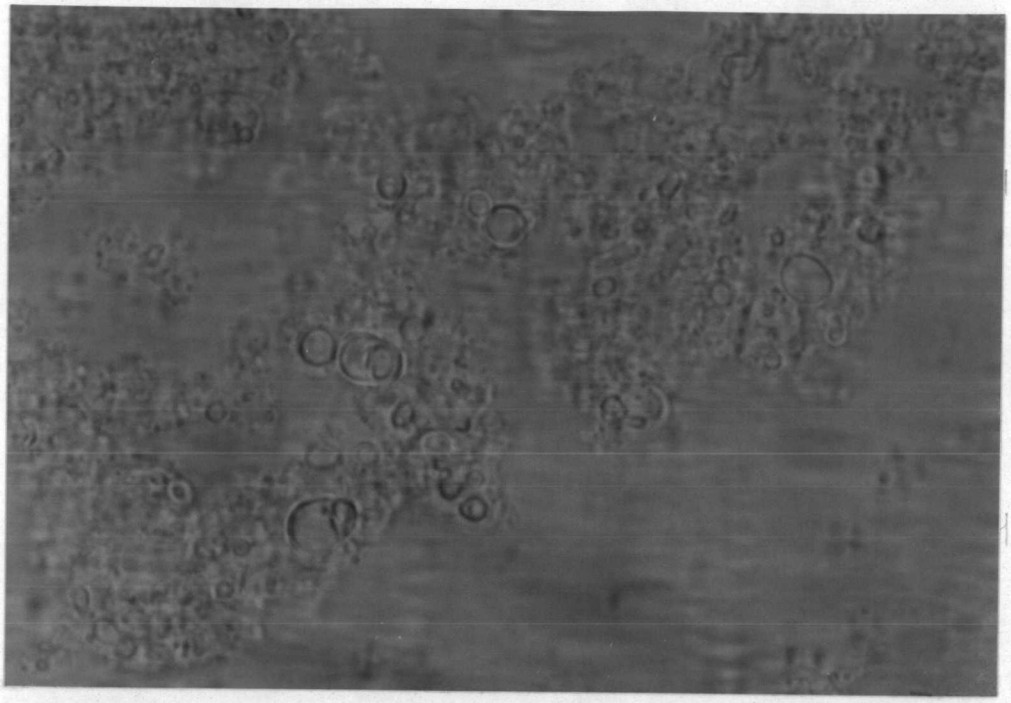


(b)

Figure 50 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.5% CM-cellulose (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



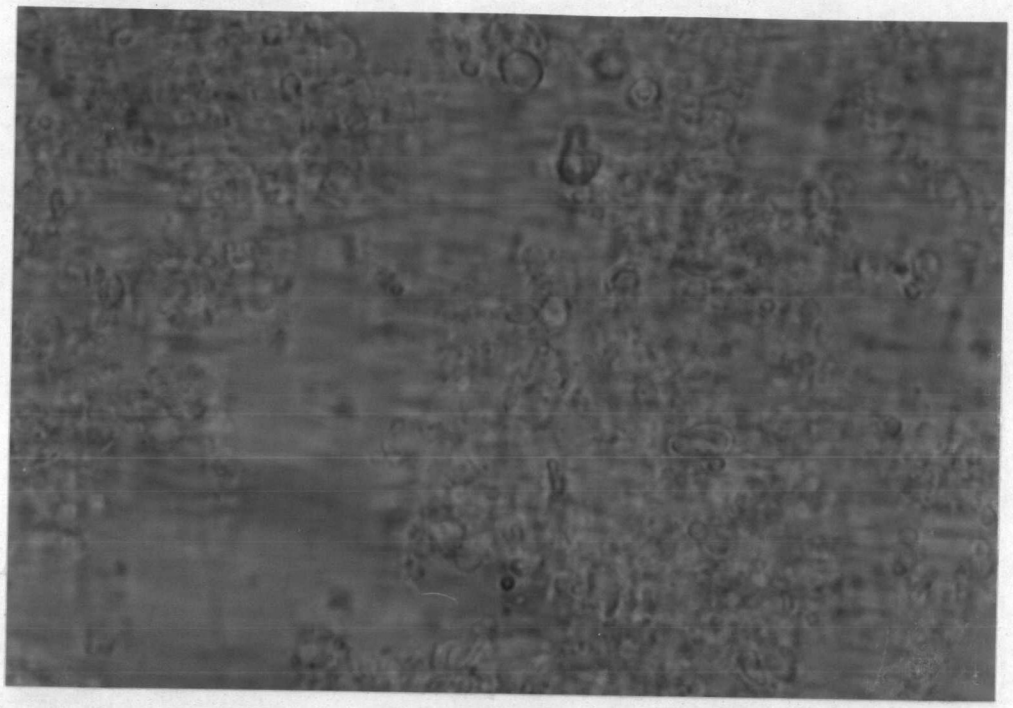
(a)

(b)

Figure 51 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.02% CM-Chitosan (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



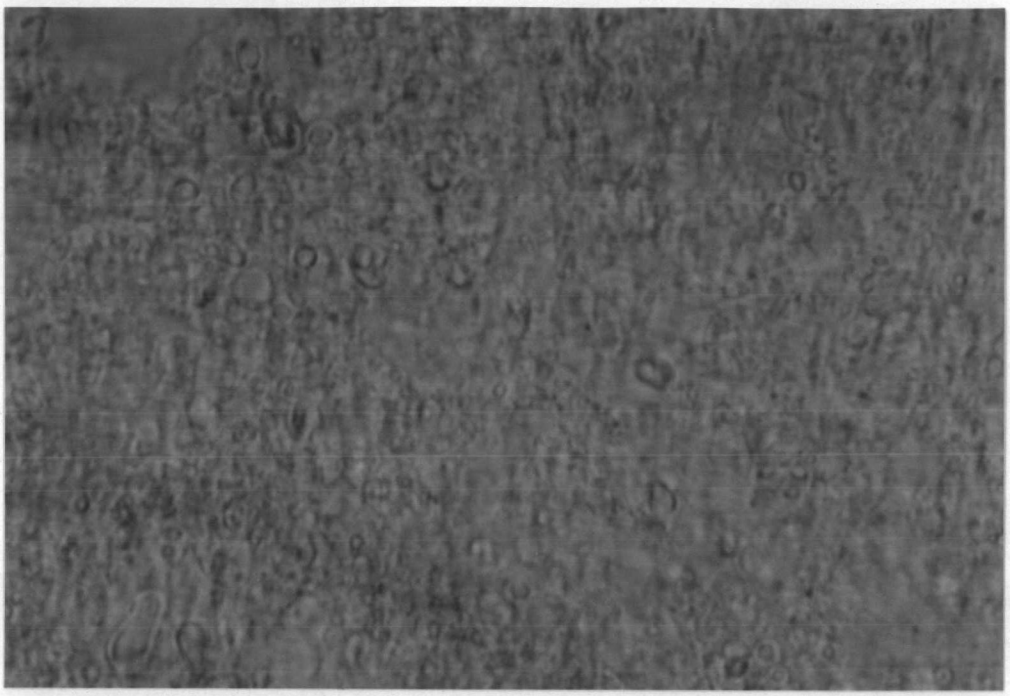
(a)

(b)

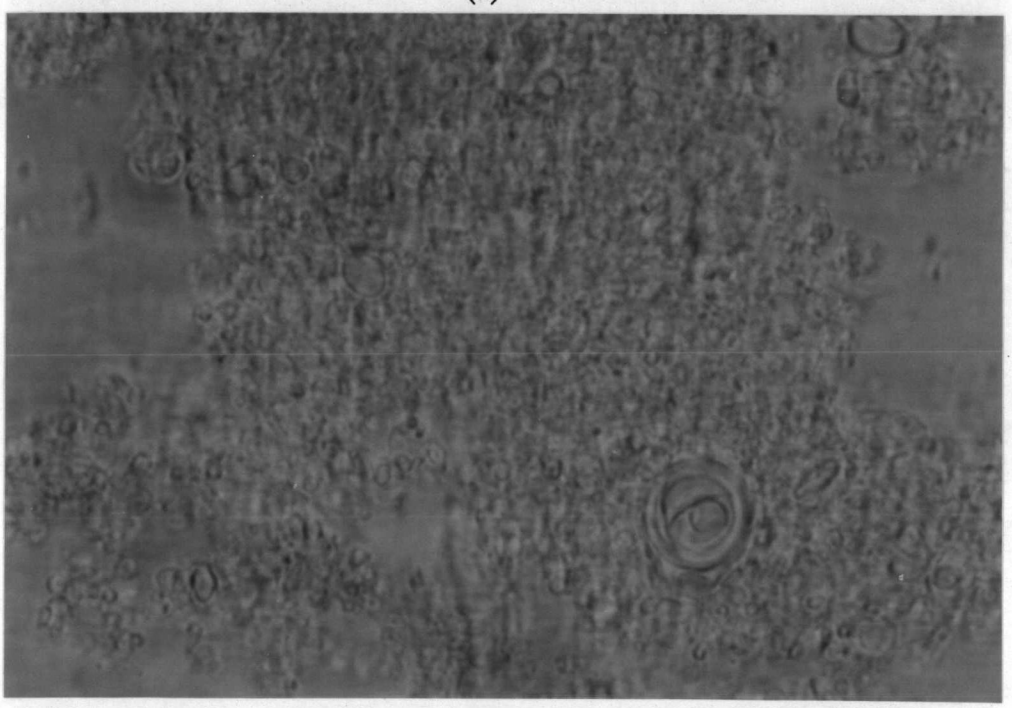
Figure 52 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.2% CM-Chitosan (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C



(a)



(b)

Figure 53 Micrograph of liposomes containing protein extract from *P. multocida* with 1 : 1 molar ratio of egg yolk lecithin to cholesterol stabilized with 0.5% CM-chitosan (magnification 1000x)

(a) freshly prepared

(b) 3 months storage at 4°C

(c) Particle size analysis.

The changing in size of liposomes stabilized with various concentration of CM-Cellulose and CM-Chitosan, when stored in PBS pH 7.4 at $4^{\circ}C$ for 3 months, were shown in Table 9. There were no change in median diameter when stored for 3 months.

The particle size distribution of liposomes stabilized with various concentration of CM-Cellulose and CM-Chitosan were compared between freshly prepared and 3 months storage and were shown in Figure 54-59. All of them showed no change in median diameter.

The cumulative undersize frequency curve of 3 months storage liposomes stabilized with various concentrations of CM-Cellulose and CM-Chitosan were shown in Figure 72-77 (see Appendix II).

Figure 54 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.02% w/v CM-Cellulose, comparison between freshly prepared and 3 months storage.

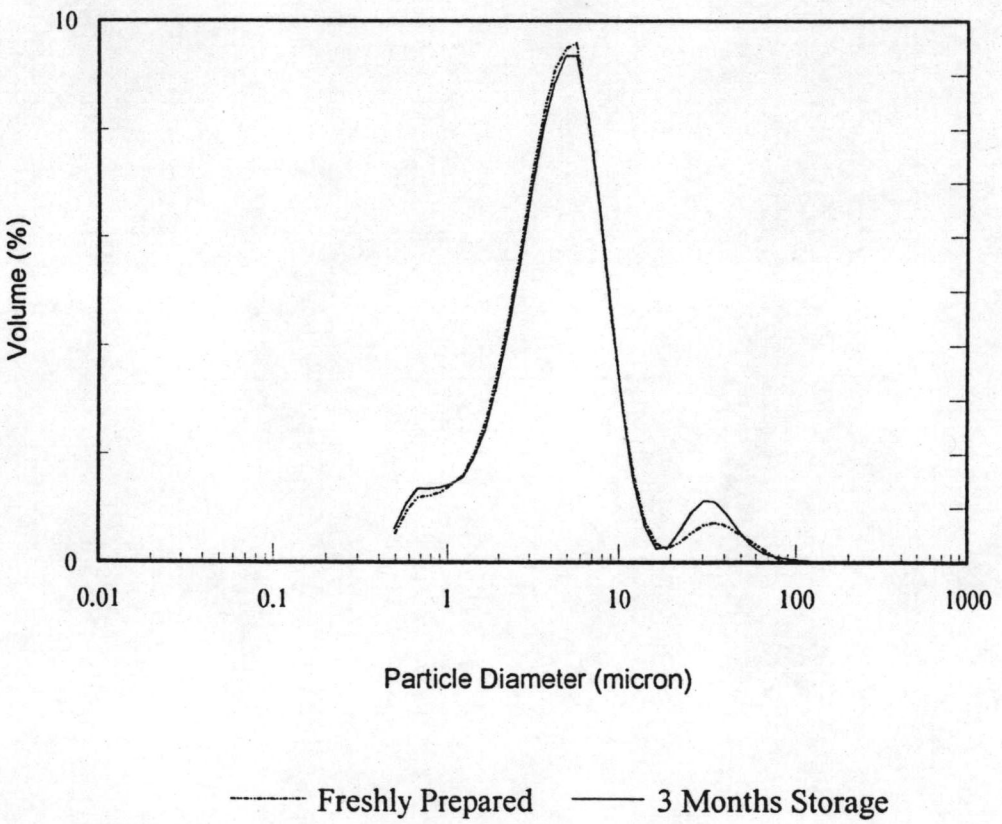


Figure 55 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.2% w/v CM-Cellulose, comparison between freshly prepared and 3 months storage.

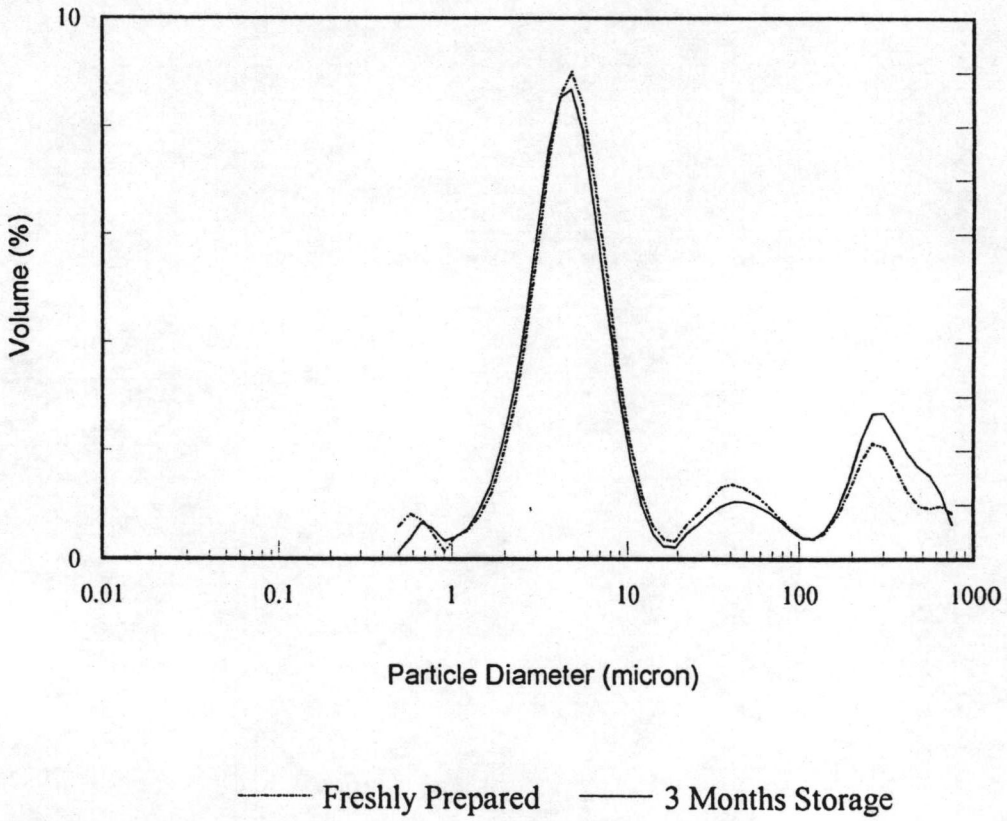


Figure 56 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.5% w/v CM-Cellulose, comparison between freshly prepared and 3 months storage.

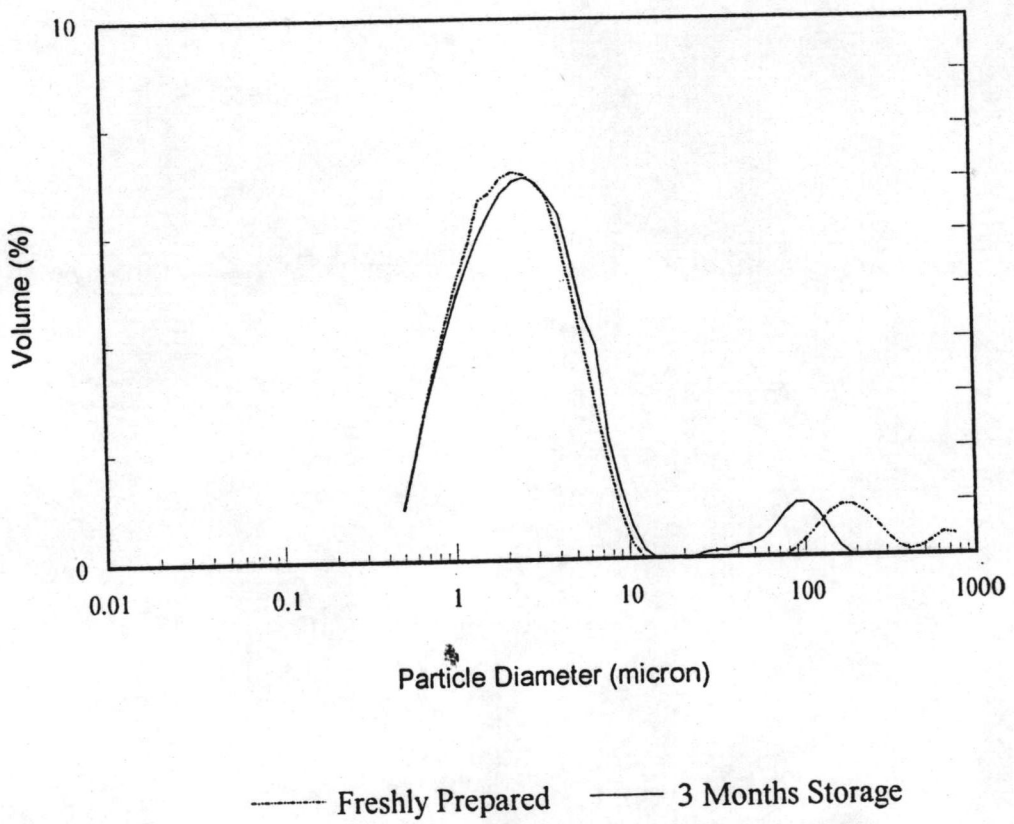


Figure 57 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.02% w/v CM-Chitosan, comparison between freshly prepared and 3 months storage.

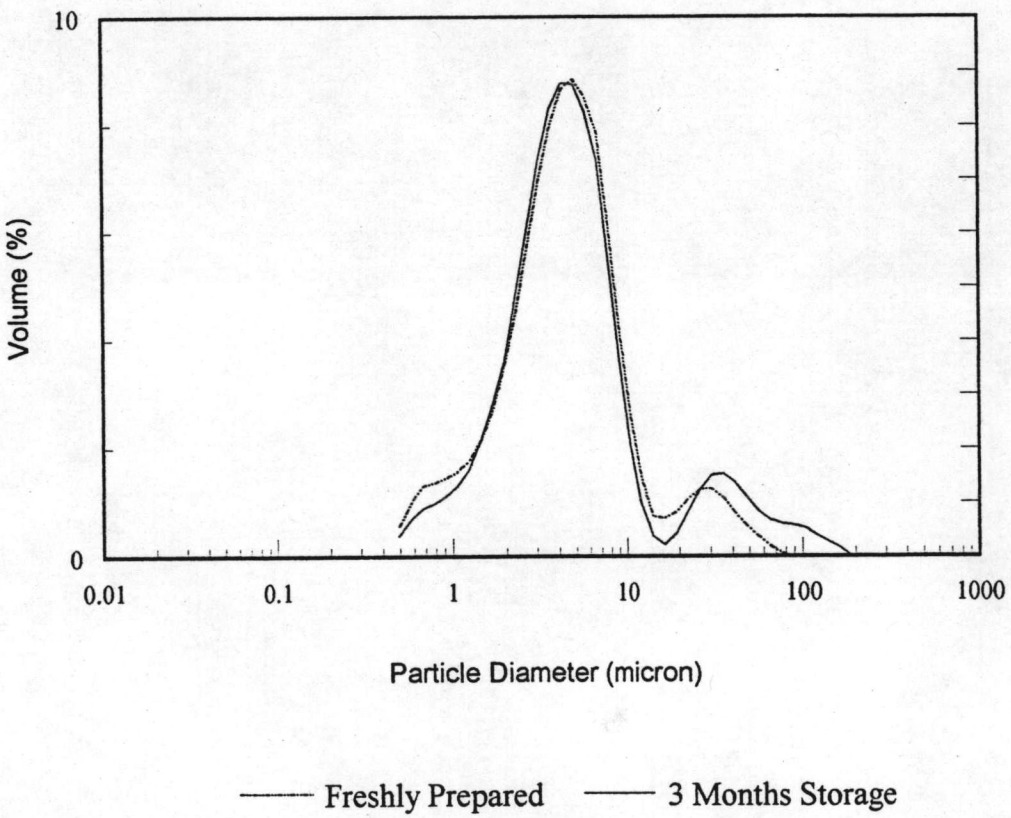


Figure 58 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.2% w/v CM-Chitosan, comparison between freshly prepared and 3 months storage.

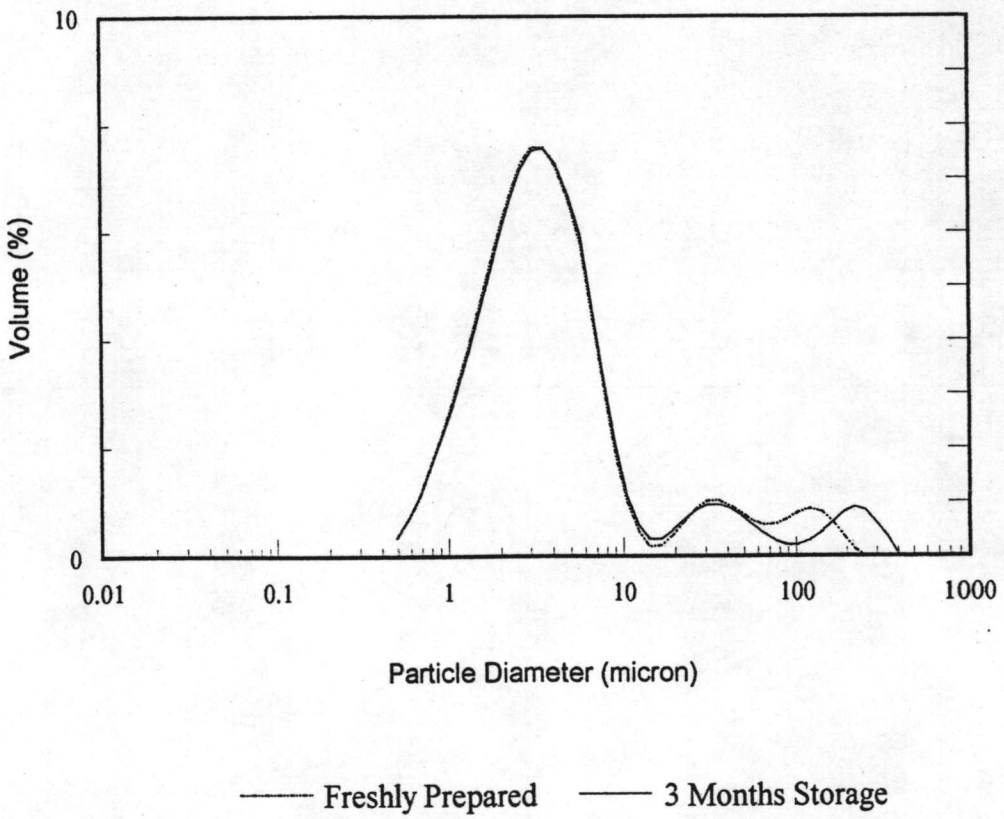


Figure 59 Particle size distribution of 1:1 molar ratio of lecithin to cholesterol liposomes stabilized with 0.5% w/v CM-Chitosan, comparison between freshly prepared and 3 months storage.