CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

It has been reported that planting mulberries and raising silkworms are flourished over 5,000 years. China was the first country that knew and spread of these knowledges to the general public via the "Silk Road". It was not known when the silkworms were raised, but we knew it was promoted since the fifth reign up to now. Thai Government has provided a supporting budget to improve silkworm varieties until Thai silk is well known to all over the world. It can be seen from the festival of Bangkok, a Fashion City, that has made Thai cloth business expanded, and persuaded Thai people to support Thai silk. As a result, the export sales have not only increased from the previous quarter, but also the Thai people have turned to use more goods and products produced domestically (The Customs Department and Department of Export Promotion,2003).

At present, Thailand has three Thai native silkworm varieties which are the followings (Department of Agriculture, 2000):

1. Thai native silkworm variety. It is homegrown and has the high ability to withstand diseases and can polyvoitine throughout the year. The silk farmers can produce silk eggs by a simple method. They produce yellow cocoons that give low production. The cocoons are not dense, but with more fibers. About 20,000 cocoons can produce 1 kg of silk yarns. The examples are Nangnoi and Nang Luang varieties.

2. Thai hybrid silkworms. They are crossed between Thai natives and the foreign varieties. The silk colors are yellow and white. They have been constantly improved to suit the domestic market and can polyvoitine throughout the year. About 10,000 cocoons can spin 1kg of yarn. The examples are Nakhon Ratchasima 1 x Nor Khorg 4 and UB 1 x Nang Luang varieties.

3. Foreign hybrid varieties. They are the type that the farmers can not breed or expand. But, they have to receive from the government or private companies only. Most of them are white and bivoltine to substitute the shortages of the native yellow varieties.

Thai native silkworms are indigenous of Thailand that needs to be conserve against extinction. Each variety has its own characteristics according to its sources. From the survey, Thai native varieties have been found most in the northeast, followed by the north and south. Since there is now more free trade, several countries have legislated their own silk breeds. However, Thailand has no protection for its silkworms, which are important insects for the Thai economy. In several countries, e.g. Japan and India have patented their silkworms as their natural resources, including using silkworms in numerous researches. In Thailand, there is no patent registration on Thai silkworm, in any aspects.

Although there is considerable information concerning the properties and the use of silk, the utilization of silk as raw materials for cosmetic in Thailand has not been extensively researched and studied. There is under utilization of Thai silk. Raw materials from the silk weaving were in low price, with the selling price of cocoons at 80-120 baht per kilogram. Therefore, creating guidelines and the business viability and adding value to raw materials from Thai silk, especially the preparation of protein

and oil from silk to be used in cosmetics should be performed. This study has added the value to the Thai native silkworms. Protein was extracted from the silkworms and the oil will be obtained from many Thai silk pupa varieties and entrapped in niosomes for cosmetic uses. Nanoparticles can increase stability and absorption through the skin of many substances and expect to add value to the silk yarn. After adding the value by applying nanotechnology to silk amino acids, sericin powder and fibroin, the price is expected to be increased to about 3,000-15,000 baht, which is about 300- 400 times higher than the silk extract.

In this study, protein from silk cocoons and oil from silk pupa of at least two varieties from the Thai native silkworms were prepared by alkaline and high pressure in suitable conditions. Sericin and fibroin as well as some essential fatty acids will be obtained. Physicochemical characteristics of the extracts from the Thai native silkworm entrapped and not entrapped in niosome will be investigated. Formulation development of the products containing the developed silk extracts by nanotechnology will be evaluated for cosmetic uses.

1.2 Objective

The object of this study was to develop the extracts from native Thai Silk *(Bombyx mori)* and entrapped in niosomes to be used as anti-wrinkle cosmetic products.

1.3 Scope of the study

This study is divided into 10 steps which are the followings:

1.3.1 Extraction of the five varieties of Thai native silkworm pupa oil by Soxhlet and maceration extraction method

1.3.2 Study of the characteristics and biological activities of the five

varieties of Thai native silkworm pupa oils, in order to select the optimized silkworm strains and methods

1.3.3 Preparation of the five varieties of Thai native silkworm cocoon (sericin) by alkaline and autoclave hydrolysis

1.3.4 Study of the characteristics and biological activities of the five varieties of sericin, in order to select the optimized silkworm strains and methods

1.3.5 Preparation of niosome entrapped with sericin and oil extracted from the None Ruesee silkworm as the following:

1.3.5.1) Preparation of blank niosomes formulations in different compositions and molar ratio by chloroform film method with sonication (CFS)

1.3.5.2) Entrapment of the sericin and oil native Thai silk extracts in niosomes formulations

1.3.6 Characterization of the developed niosomes formulations as the followings:

1.3.6.1) Determination of vesicular sizes by Zetasizer and lamellarity by TEM technique and entrapment efficiency by ultracentrifugation

1.3.6.2) Determination of physical and chemical stability of the entrapped and not entrapped sericin and oil in niosomes

1.3.6.3) Selection of the best niosomes

1.3.7 Development of anti-wrinkle serum containing niosomes entrapped with oil and sericin extracted from silk as the following processes:

1.3.7.1) Five serum base formulations were developed and one formulation was selected, in order to determine physical, chemical and sensory stability of the serum formulations for 3 months

1.3.7.2) Optimizations of the best anti-wrinkle serum formulations

1.3.7.3) Development of serum containing niosomes entrapped with the silk oil and sericin, in order to select of the best serum formulation

1.3.7.4) Selection of color and fragrance of the serum formulations

1.3.7.5) Quality assessment on the developed serum containing niosomes entrapped with the silk oil and sericin

1.38 Stability of the develop serum containing niosomes entrapped with the silk oil and sericin at different storage temperatures

1.3.9 Consumer acceptance study

1.3.10 Cost calculation for the developed serum containing niosomes entrapped with oil and sericin silk protein

1.4 Literature reviews

1.4.1 Silk worm

Silkworms are important economic insects of Thailand. They are night moths feeding on mulberry leaves. There are many different species of silkworms and their thread is used for commercial purpose (Nagaraju et al., 1995), especially thread produced from silk pupa that is woven into silk fabric called "Thai Silk". With its distinctive characteristics of having uneven surface with small knots thoroughly that come from the hand-reeling, Thai silk is well-known worldwide.

Silkworm, or scientific term '*Bombyx mori'* (Linn aeus.), is a kind of moths in Bombyxidae species that has complete metamorphosis involving a conspicuous and relatively abrupt change in its body (Hisao, 1994; Borror et al., 1989) according to scientific classification of silkworms. *Kingdom, Metazoa,Phylum, ArThropoda,Class,*

Hexaopda orInsecta, Sub–class, Pterygota,Divison, Endopterygota, Order, Lepidoptera,Family, Bombycidae,Genus, Bombyx,Species, mori

1.4.1.1 Biology of Silk Worm

A. Silkworm life cycles

Silkworm has the scientific name of *Bombyx mori (*Linn aeus.). It is classified as a butterfly type in the *Bombyxidae* with the complete metamorphosis, which is a metamorphic growth by the stages distinctively (Hisao, 1994; Sawai, 2001; Chaiya, 1996). Silkworm has completed the changes with growth and development in four stages, which are egg, larva, pupa, and butterfly. Its life cycles completely changed in 4 states as follows (**Figure 1)**

(i) The egg stage. The eggs shall be hatched into silkworm. This stage will take about 9-12 days.

(ii) The larvae stage. This is the longest time consumption in its life cycle with the change of size and weight that can be divided into 5 ages as follows.

At the first stage, the newly hatched silkworm bodies will be black or dark brown color covered with dense hair.

At the second stage, the silkworms will eat mulberry leaves for 2-3 days. Their bodies can not be clearly seen, however, this stage of their life cycles will be very short.

At the third stage, the silkworms will eat mulberry leaves for 3-4 days and their bodies are growing bigger with dark spots that can be clearly seen on their white bodies. The silkworms shall alternately be molted according to their ages for the

total of 5 times until they reach the age of maturity. The molt span at 1-3 stages is known as “The young age silkworm”.

Figure 1 Life cycle of silkworm

(http://mulberrysilkworm.blogspot.com/2008/06/life-cycle-of-silkworm.htm)

Source Picture: Documentation of PT Indo Jado Sutera Pratama, Indonesia

The fourth stage, the silkworms will eat mulberry leaves for about 3-4 days. Their bodies are growing which spots and organs can be seen more clearly. Then they begin to molt for 4-5 times which is known as “The old age silkworm”.

The fifth stage the silkworms will eat mulberry leaves for as long as 6-8 days. This stage is known as “The mounting stage”. They will stop eating and their bodies can be seen transparent and in yellow color.

(iii) The pupa stage, The pupa short to crawl out of their feeding cocoons and squirt the webs to cover their bodies as known as “cocooning frame”. The silkworms will take about 2-4 days to spin their webs as silk cocoons, then molt into pupa and lying still for 8-14 days waiting for the right time to be molded into the moth.

(iv) The moth stage (butterfly). After being molted into moths,they cannot fly but flap their wings as the sign of mating. They then lay their eggs and die.

Silkworm cultivation for silk yarn needs 5 days. It shall spin the yarn to make a cocoon to wrap itself. After building the cocoon for 4-7 days, the yarn can be spun, in a boiling process. Silk glue or sericin glue, which is the part that wraps the yarn, is then washed by sodium hydroxide and soap water before dying. The silk glue water is thrown away, and it is expected to have a large quantity. If producing 1-million tons of cocoons, one can get 400,000 tons of silk yarn and 50,000 tons of glue water. Thus, if these wastes is recycled. It can give economic and social benefits. For pharmaceutical benefits, sericin or fibroin is an important protein that can be recycled. Now, there were numerous silk researches have indicated that sericin or fibroin is a natural protein that has no toxic effects and can be used especially in cosmetics. In Thailand, there has been few research works on the extracted proteins from Thai native silkworms for application in the industrial level. These proteins do not have the quality and standard matching with the foreign standards. Thailand has imported these protein extracts in a quite large amount.

A.1 Silk cocoon

Silk cocoon is characterized as the web squirted by the silkworms to cover their bodies as the shields to protect themselves. The silkworm raisers shall sell the silk cocoons to the factory. But, the pupa in the cocoons must be first killed to prevent them from becoming the moths and crawl out of the cocoons. The silk cocoons in general consist of the followings: (**Figure 2)**.

(i) Floss, the outside layer covering the silk cocoon with fluffy look.

(ii) Cocoon layer, the silk web held tightly in order to cover the pupa inside and the thin layer part next to the outside layer.

(iii) The most inner layer is the pupa waiting for the time to be hatched as the moths.

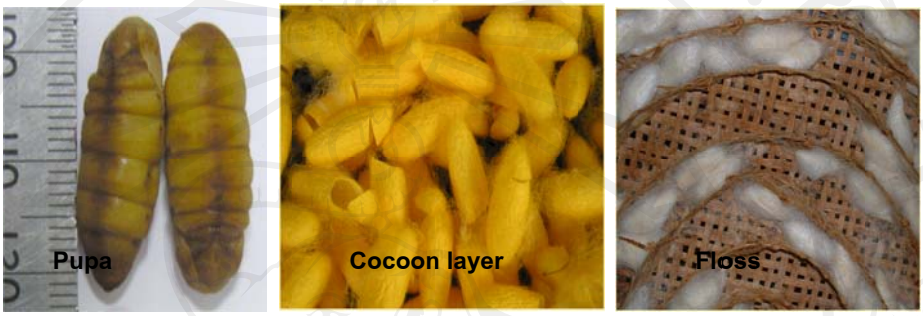


Figure 2 Silk cocoon

Silk cocoons *(Bombyx mori*) is a natural protein with the main ingredient of sericin, a protein of about 20-30% and fibroin protein of about 70-80% of the total cocoon weight. Sericin wraps fibroin by sericin. It is a substance to act as glue or wax that a silk worm builds with fibroin silk yarn to wrap itself and becomes a pupa.

The tough glue becomes silk yarn and held together as a cocoon, which at present is thrown away in wastewater in silk production. It is found around the world. The silk glue was thrown away about 1 million tons compared with the dry weight of about 400,000 tons. In the process of raw silk production, it has the sericin production of about 50,000 tons. The pure sericins sold in the Japanese market at 50,000 baht per kilogram. If we can recycle this sericin, more economic benefits can be obtained. The value of the silk cocoons can be used in various industries especially the cosmetic industry.

B. Species of silkworm

There are more than 400-500 species, but only a few species can be used for the commercial purpose. They are mostly found in Asia and Africa and can be classified as follow (Jolly et al., 1979) **(Table 1).**

(i) Cultivated Silk (Bombycidae)

Cultivated silkworms are divided into three species; Japanese, Chinese, and European. Its reproduction is F1 hybrids depending on type, size, appearance, and color. Its cocoon is in round, oval, or reel shape. Its color can be white, yellow, beige, yellowish green, brilliant yellow, or pink.

(ii) Wild silk (Saturniidae)

Wild silk cocoons can be collected 2-8 times a year. The cocoons are in ovary shape and can be found in different color such as yellowish brown, brown, or green depending on species as the followings:

iiA. Tasar silk yarn (Tussah or Tusser)

Most Tasar silk is found in hot and humid high temperature zone in Indochina region. The silkworms feed on the foliage of trees and shrub. Their cocoons are gray and yellowish brown as shown in **Table 3** in the category of Genus Antheraea. Tasar silk can be produced in commercial scale. Tasar silk is from silkworms *A.mylitta D.* (India) that are grown in tropical zone and from silkworms A. *yamamai M*. (Japan) that are grown at the controlled temperature.

iiB. Muga silk (or Antheraea assama)

Muga silk yarn has natural golden color. It is the product of the silkworm *Antheraea assamensis* endemic to Assam, India. Its cocoon is in oval shape and has brilliant yellow color. It is also called Muga, Moonga, or Mounga.

iiC. Fagara silk

Fagara silkworm likes feeding on clothes. It produces large brown cocoons and is mostly found in Indonesia, Australia, China, and Sudan.

iiD. Coan silkworm

Coan feeds on pine leaves and is mostly found in Mediterranean, south of Greek, Romania, and Turkey.

iiE. Mussel silk

Mussel silk is mainly found in shallow-water zone near Italian coast, Camareon through Atlantic coast. The cocoons are hard and have irregular rough surface in dark brown color.

iiF. Spider silk

Spider silk is fine and soft, but strong and elastic.

iiG. Eri silk (*Samia Cynthai ricini*)

Eri silk is produced by native India *Samia cynthia ricini* silkworms which feed on cassava leaves. The cocoon has oval shape and white, yellow, brown, green, or pink color. It is also known as Eria, Eria, or Era silk.

iiH. Anaphe silk

Anaphe silk is produced by silkworm *A.moloneyi* D., A. reticulate W. which is mostly found in southern and central Africa. The cocoon is white and t gives soft thread when it is wound. Anaphe silk yarn is used for weaving velvet and luxurious fabric.

C. Thai native silkworm

There are three main species of silkworm cultivated in Thailand as shown in **Tables 2 and 6** (Department of Agriculture, 2000).

(i) Native Thai Silkworm, polyvoltine, has high resistance to

disease and can reproduce all year round. Silk farmers can hatch the eggs by themselves. Cocoons of native Thai silkworm, such as Nangnoi and Nanglueng, have yellow color and give low yield of thread. 20,000 cocoons yield about 1 kilogram of silk yarn.

(ii) Thai Hybrid species, such as Nakornratchasima 1 x NorKor. and UB 1xNanglueng, is the hybrid between native Thai silkworm and foreign silkworm. The cocoon has white or yellow color. It is continuously developed in order to be suitable for cultivating in Thailand. 10,000 cocoons yield about 1 kilogram of silk yarn.

(iii) Non-native Hybrid species, bivoltine, cannot be hatched and reproduced by the growers. It has white color and will be used as an alternative in shortage period.

1.4.1.2 Thai Silk Production

The Thai silk moth is adapted to tropical conditions and is polyvoltine, producing at least ten batches of eggs each year. Silk from the Thai moth is hand reeled from green cocoons. These are cocoons that still contain the live pupa. These small cocoons do not have the pupa 'stifled' or killed before the thread is unwound as it would make it difficult to reel. The green cocoons are placed in hot, nearly boiling water, which loosens the end of the thread. With less than 10 days available before the moths emerge and ruin the cocoon, the Thai workers may run out of time limiting the scale of the industry. Even experienced workers rarely produce more than 300kg/day. The emerging pupa may be eaten by local workers.

Table 1 The classification of varieties of silk culture in Thailand (Boonman, S. 2000.Department of Agriculture. Thailand)

Identification Classification	Polyvoltine pure race						Bivoltine	Polyvoltine hybrid		Polyvoltine-Bivoltine hybrid	
	Nang noi Srisaket-1 NNl	Nong Kao	Roi-Et.	Nang Lai	Pakchong 21	Nang Leung	Ubon Ratchathani 60. UR60	Ubon Ratchathani 60-35		Sakhonnakorn	Udorntanee
1) Variety	Thai	Thai	Thai	Thai	Thai	Thai	Japanese	UB60 x NNl	SR5 x NNl	SPl x SB2	SKNI N KSN
2) Silkworm body color	White	Plain white	Plain white	Zebra like in cross way	Plain white	Yellow	White	Yellow	Plain white	Zebra x White	Light – yellow
3) Cocoon color	Yellow	Yellow	Shell egg Co lour of Dark yellow	Yellow	Yellow	Yellow	White	White	Yellow	Yellow	Deep - yellow
4) Cocoon shape	Blunt head. Sharp tip	Blunt head. Sharp tip	Ellipse	Blunt head. Sharp tip	Blunt head. Sharp tip	Blunt head. Sharp tip	Oval and Drum bell	Blunt head. Sharp tip	Ellipse	Ellipse	Oval sharp
Characteristic race	-	-	-	-	-	-	-	-	-	-	-
1) No. of eggs per batch	278	362	412	395	381	364	354	388	460	540	492
2) Rearing period from 1-5 stage date.hr	19.2	22.0	19.2	18.0	19.0	18.0	19.0	18.0	-	19.1	19.0
3) Hatchability. %	86.13	94.07	78.62	92.54	84.20	83.64	85.80	94.90	-	92.07	-
4) Young stage survival. %	76.77	89.90	94.14	88.36	86.0	79.40		-	-	90.55	91.18
5) 10 Adult weight. %	17.77	18.81	21.24	16.02	17.32	17.92	23.80	-	23.76	-	-
6) Cocooning. %	92.63	91.66	94.16	95.14	93.68	92.95	-	-	-	--	-
7) Sound pupa. %	85.40	82.09	81.56	80.82	87.30	79.30	-	-	-	-	91.12
8) Average fresh cocoon weight.(g)	0.960	0.929	0.860	0.960	0.096	0.842	1.100	1.400	1.090	1.750	1.47
9) Cocoon shell layer %	13.30	11.00	12.02	12.50	12.40	11.28	17.80	16.10	14.87	16.01	17.12
10) Average cocoon shell weight (g)	0.130	0.102	0.980	0.132	0.120	0.110	0.196	0.225	16.34	0.283	0.251
11) Filament length.(m)	370-410	263	313	258	253	250	-	519	-	865	677
Characteristic of yarn		-	-	-	-	-	-	-	-	-	-
1) Physical properties	Tough and Soft. glossy	-	-	-	-	-	-	-	-	-	-
2) Size (denier)	-	-	-	-	-	-	-	2.40	-	2.45	2.27
3) Reliability. %	-	-	-	-	-	-	-	63.00	-	71.50	66.00

Table 2 Characteristics of native Thai silkworm of Nangnoi Srisaket, Nang Lai and Nang Lueng (Department of Agriculture, 2000).

Characteristics	Species		
	Nangnoi Srisaket	Nang Lai	Nang Leung
Major characteristics	1. Healthy and easy for cultivation	1. Healthy and easy for cultivating and	1. Easy for cultivation in all seasons
	2. High egg production rate 3. Can be cultivated in high temperature	2. High reeling yield	2. High reeling yield
Morphological appearance			
1. Egg production	Native species lays eggs all year round	Native species lays eggs all year round	Native species lays eggs all year round
2. Color of the caterpillar	Bright yellow	White stripe round the body	Lemon yellow without dots
3. Size of the caterpillar	Long and pure white	White stripe round the body	Long and yellow
4. Size of cocoon (w x l (cm.))	3 x 1.5	1.2-1.45 x 3.21-3.67	1.11-1.28 x 2.7-3.3
5. % of spinning cocoon	-	-	-
6. Hatching percentage	-	-	-
7. Color of silk egg	Off-white	Off-white	Off-white
8. Shape of cocoon	Olive shape	Cone shape	Cone shape
9. Color of cocoon	Dark yellow	Dark yellow	yellow
10. Color of filament	yellow	yellow	yellow

Table 2 Characteristics of native Thai silkworm of Nangnoi Srisaket, Nang Lai and Nang Lueng (Department of Agriculture, 2000). (continued)

Characteristics	Species		
	Nangnoi Srisaket	Nang Lai	Nang Leung
Cultivating characteristics			
1. Number of eggs	378	331	358
2. Period of caterpillar (days)	18 – 22	19 – 21	20 – 24
3. Weight of fully grown caterpillar, average weight of 10 worms	22.5	23	25
4. Percentage of complete pupation	85.4	84	88.78
5. Survival percentage of larvae	90-95	90-92	95-98
6. Weight of fresh cocoon (g./cocoon)	0.98	0.68-1.64	0.75-0.80
7. Average weight of cocoon shell	10.3	8.24	9.04
8. Percentage of cocoon shell (%)	12.8 – 13.5	11 – 14	13.5
9. Size of filament (dinear)	2.50	2.9	2.4
10. Length of filament (cm.)	370 – 410	311	229
11. Reelability (%)	80-90	80-90	80-90
12. Yield of fresh cocoon (kg./1 line of eggs)	12-13	12-13	12.5-13
13. Number of cocoons (per 1 kg.)	1200	1300-1400	1300-1400

Note : (-) no data available.

Table 3 Characteristics of native Thai silkworm of Chor. Yoi. 1,Chor. Yoi. 2 and Chor Yoi. 3 (Department of Agriculture, 2000)

Characteristics	Species		
	Chor. Yoi. 1	Chor. Yoi. 2	Chor Yoi. 3
Major characteristics	-	-	-
Morphological appearance			
1. Egg production	Native species lays eggs all year round	Native species lays eggs all year round	Native species lays eggs all year round
2. Color of the caterpillar	white	white	white
3. Size of the caterpillar	Long and pure white	Long and pure white	Long and pure white
4. Size of cocoon(w x l (cm.))			
5. % of spinning cocoon	-	-	-
6. Hatching percentage	-	-	-
7. Color of silk egg	Yellow white	Yellow white	Yellow white
8. Shape of cocoon	Cone shape	Cone shape	Cone shape
9. Color of cocoon	yellow	yellow	yellow
10. Color of filament	yellow	yellow	yellow
Cultivating characteristics			
1. Number of eggs	325	350	341
2. Period of caterpillar (days)	22	23	20
3. Weight of fully grown caterpillar, average weight of 10 worms	-	-	-
4. Percentage of complete pupation	84.4	83.3	89.9
5. Survival percentage of larvae	-	-	-

Note : (-) no data available.

Table 3 Characteristics of native Thai silkworm of Chor. Yoi. 1,Chor. Yoi. 2 and Chor Yoi. 3 (Department of Agriculture, 2000) (continued)

Characteristics	Species		
	Chor .Yoi. 1	Chor .Yoi. 2	Chor. Yoi. 3
6. Weight of fresh cocoon (g./cocoon)	-	-	-
7. Average weight of cocoon shell	-	-	-
8. Percentage of cocoon shell (%)	12	12	14
9. Size of filament (dinear)	-	-	-
10. Length of filament (cm.)	-	-	-
11. Reelability (%)	-	-	-
12. Yield of fresh cocoon (kg./1 line of eggs)	12-13	11-12	12-13
13. Number of cocoons (per 1 kg.)	1100	1300	1000

Note: (-) no data available.

Table 4 Characteristics of native Thai silkworm of Paengphuay, Nangmai and Sam Rong (Department of Agriculture, 2000)

Characteristics	Species		
	Paengphuay	Nangmai	Sam Rong
Major characteristics	-	-	-
Morphological appearance			
1. Egg production	Native species lays eggs all year round	Native species lays eggs all year round	Native species lays eggs all year round
2. Color of the caterpillar	white	White yellow without dots	White stripe round the body
3. Size of the caterpillar	Long and pure white	Long and pure white	Long and pure white

Note : (-) no data available.

Table 4 Characteristics of native Thai silkworm of Paengphuay, Nangmai and Sam Rong (Department of Agriculture, 2000) (continued)

Characteristics	Species		
	Paengphuay	Nangnoi Srisaket	Sam Rong
4. Size of cocoon (w x l (cm.))	-	-	-
5. % of spinning cocoon	-	-	-
6. Hatching percentage	-	-	-
7. Color of silk egg	White yellow	White yellow	White yellow
8. Shape of cocoon	Obtuse head, rear sharp	Obtuse head, rear sharp	Obtuse head, rear sharp
9. Color of cocoon	yellow	yellow	yellow
10. Color of filament	yellow	yellow	yellow
Cultivating characteristics			
1. Number of eggs	302	317	309
2. Period of caterpillar (days)	20	20	24
3. Weight of fully grown caterpillar, average weight of 10 worms	-	-	-
4. Percentage of complete pupation	94	95.8	91.2
5. Survival percentage of larvae	-	-	-
6. Weight of fresh cocoon (g./cocoon)	-	-	-
7. Average weight of cocoon shell	-	-	-
8. Percentage of cocoon shell (%)	12	12	11
9. Size of filament (dinear)	-	-	-
10. Length of filament (cm.)	-	-	-

Note : (-) no data available.

Table 4 Characteristics of native Thai silkworm of Paengphuay, Nangmai and Sam Rong (Department of Agriculture, 2000) (continued)

Characteristics	Species		
	Paengphuay	Nangnoi Srisaket	Sam Rong
11. Reelability (%)	-	-	-
12. Yield of fresh cocoon (kg./1 line of eggs)	10	11	13
13. Number of cocoons (per 1 kg.)	1000	1000	1400

Table 5 Characteristics of native Thai silkworm of None Ruesee ,Keaw Sakol and Khaki (Department of Agriculture, 2000)

Characteristics	Species		
	None Ruesee	Keaw Sakol	Khaki
Major characteristics	-	-	-
Morphological appearance			
1. Egg production	Native species lays eggs all year round	Native species lays eggs all year round	Native species lays eggs all year round
2. Color of the caterpillar	White	Green	White
3. Size of the caterpillar	Long and pure white all body	Long and stripes all body	Long and pure yellow all body
4. Size of cocoon (w x l cm.))	-	-	-
5. % of spinning cocoon	-	-	-
6. Hatching percentage	-	-	-
7. Color of silk egg	White yellow	White yellow	White yellow
8. Shape of cocoon	Obtuse head, rear sharp	Obtuse head, rear sharp	Obtuse head, rear sharp
9. Color of cocoon	yellow	yellow	yellow
10. Color of filament	yellow	yellow	yellow
Cultivating characteristics			

Note: (-) no data available

Table 5 Characteristics of native Thai silkworm of Noneruesee ,Keawsakol and khaki (Department of Agriculture, 2000) (continued)

Characteristics	Species		
	None Ruesee	Keaw Sakol	Khaki
1. Number of eggs	382	380	380
2. Period of caterpillar (days)	20	22	20
3. Weight of fully grown caterpillar, average weight of 10 worms	-	-	-
4. Percentage of complete pupation	91.2	95.2	92.3
5. Survival percentage of larvae	-	-	-
6. Weight of fresh cocoon (g./cocoon)	-	-	-
7. Average weight of cocoon shell	-	-	-
8. Percentage of cocoon shell (%)	13	13	13
9. Size of filament (dinear)	-	-	-
10. Length of filament (cm.)	-	-	-
11. Reelability (%)	-	-	-
12. Yield of fresh cocoon (kg./1 line of eggs)	13	12	11
13. Number of cocoons (per 1 kg.)	1400	1300 -1400	1200

Table 6 Characteristics of native Thai silkworm of Neueasrithun and Mor. (Department of Agriculture, 2000)

Characteristics	Species	
	Neueasrithun	Mor.
Major characteristics	-	-
Morphological appearance		
1. Egg production	Native species lays eggs all year round	Native species lays eggs all year round
2. Color of the caterpillar	white	Stripes and blank all body

Table 6 Classification of native Thai silkworm (Department of Agriculture, 2000) (continued)

Characteristics	Species	
	Neueasrithun	Mor.
3. Size of the caterpillar	Long as the white	Long as the white
4. Size of cocoon (w x l (cm.))	-	-
5. % of spinning cocoon	-	-
6. Hatching percentage	-	-
7. Color of silk egg	Yellowish white	Yellowish white
8. Shape of cocoon	Obtuse head, rear sharp	Obtuse head, rear sharp
9. Color of cocoon	champaca	yellow
10. Color of filament	Light champaca	yellow
Cultivating characteristics		
1. Number of eggs	293	331
2. Period of caterpillar (days)	21	21
3. Weight of fully grown caterpillar, average weight of 10 worms	-	-
4. Percentage of complete pupation	89	82.8
5. Survival percentage of larvae	-	-
6. Weight of fresh cocoon (g./cocoon)	-	-
7. Average weight of cocoon shell	11	12
8. Percentage of cocoon shell (%)	-	-
9. Size of filament (dinear)	-	-
10. Length of filament (cm.)	-	-
11. Reelability (%)	-	-
12. Yield of fresh cocoon (kg./1 line of eggs)	-	-
13. Number of cocoons (per 1 kg.)	-	-

Note: (-) no data available.

1.4.1.3 Silk worm products

A. Chemical composition of silk worm

Silk cocoons are slightly different depending on species of silkworm and the way of cultivation. Silk from cultivated silkworm has the composition of sericins and fibroins. Fibroin is double protein fibers joined together by sericins which is considered as silk gum (Montha, 1998). Structure of raw silk contains the major chemical ingredients as shown in **Table 7** (http://www.smiss.cn).

Raw silk worm is also composed of protein or polyamide from α-amino about 97% and the rest is wax, fat, carbohydrate, as shown in **Table 8** (Hyogo and Yoshiko,1967) pigments and inorganics.There are 2 types of protein of raw silk worm. Fibroin protein or silk fibroin or silk filament, **(Figures 3 and 4**) has the chemical structure formula of $C_{15}H_{26}N_6O_6$ (Weerasak, 1999) which is β-fibroin (**Figure 5).** The molecular arrangement is in high degree of crystallinity, and some area is in low degree of crystallinity. Therefore, silk filament is strong and elastic (Montha, 1998).Another protein is sericin or silk gum which coats and joins fibroins

Table 7 Types of chemical ingredients of silk (http://www.smiss.cn)

Type	Percentage
Fibroin	75-80
Sericin	20-30
Wax and Oil	0.4-0.8
Hydrocarbon	1.2-1.6
Color	0.2
Mineral other	0.7

Table 8 Compositions of carbohydrate in silk protein of *Bombyx mori* and Taihei strains. (Hyogo,S. and Yoshiko, A., 1967)

Carbohydrate type	Fibroin (%)	Sericin A (%)	Sericin B (%)
Glucosamine	0.16	0.18	0.65
Galactosamine	0.00	0.62	1.61
Mannose	0.17	0.24	0.66
Galactose	0.00	0.06	0.16

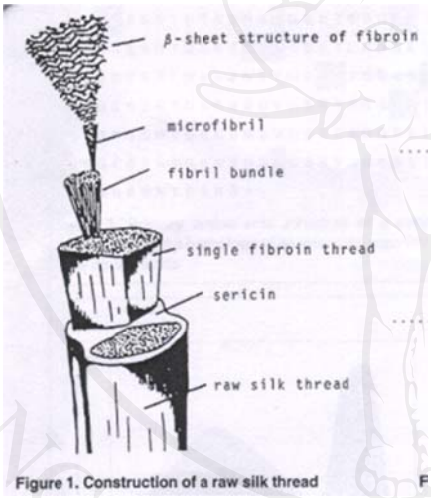


Figure 3 Composition of silk filament (Rainer and et.al.1993)

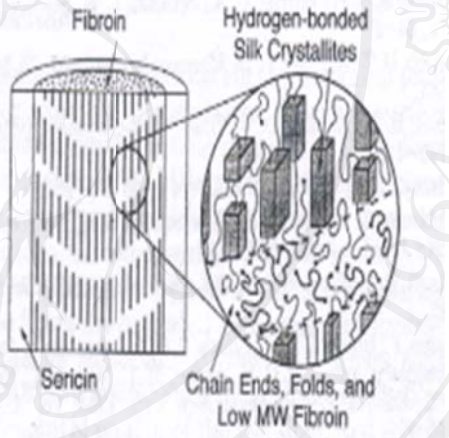


Figure 4 Structure of silk (http://www.biochem.usyd.edu.au)

together. Its chemical formula is $C_{15}H_{23}N_5O_8$, (**Figure 6)** which contains natural pigments of yellow, light green, pink, light brown.

Besides, it is found that fibroin contains several important elements, i.e.carbon 48-49%, hydrogen 6.4-6.51%, nitrogen 17.35-18.89%, and oxygen 26-27.9% (Weerasak, 1999)

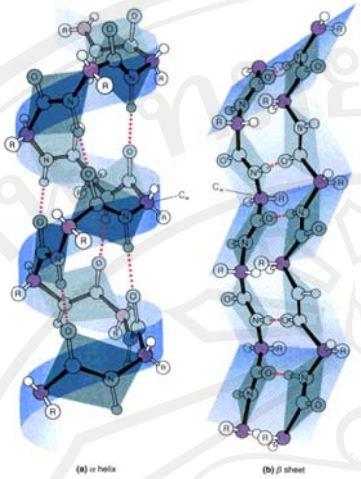


Figure 5 Structure of β-sheet and α-helix of silk (http://www.chembio.ca)

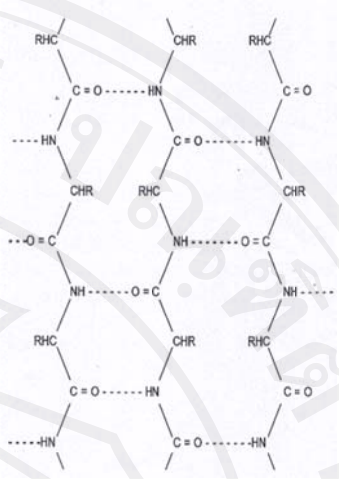


Figure 6 Chain molecule of silk (Rainer et al., 1993)

According to the research of the Sericulture Research Institute, Department of Agriculture in 2001, it is found that both soluble and insoluble silk powder contain different types of amino acids. Silk powder of Thai silk contains 18 types of amino acids and powder of wild silk contain 17 types, whereas powder of non-native silk has only 15 types. The most frequently found amino acids with the amount higher than 10%, **(Table 9)**, are glycine alanine serine and tyrosine. The powder amount found in Thai silk and wild silk is higher than the foreign silk.

In the report from http://www.newsilkroad.com, it was found that the silk is hydrolyzed by acid or alkali or enzyme to be the complex amino acids with nutrition, healthy, medicine and physiological functions. Different molecular weight of the silk degradation have different physiological function to the body, and the

Table 9 Types and amount of amino acids found in Thai silk, wild silk and foreign silk (Prateep,et. al., 2002)

Amino acid	Thai silk powder (%)	Wild silk powder (%)	Foreign silk powder (%)
Total nitrogen	17.24	17.06	-
Glycine	42.77	39.94	41.31
Alanine	33.92	32.21	31.63
Serine	12.12	11.76	8.51
Tyrosine	10.85	9.68	8.52
Valine	2.85	2.73	3.07
Histidine	1.06	ND	0.19
Glutamic	0.90	1.31	1.20
Aspartic	0.88	1.26	1.20
Phenylalanine	0.78	0.83	0.60
Threonine	0.58	0.72	0.63
Isoleusine	0.52	0.54	0.00
Proline	0.41	0.41	0.52
Leusine	0.41	0.40	0.54
Arginine	0.34	0.49	0.29
Lysine	0.30	0.27	0.00
Tryptophan	0.23	0.19	0.00
Methionine	0.09	0.14	0.00
Cystine	0.04	0.09	0.00

function of the basis amino acids, has been deeply known. The people are paying more and more attention to the exploiting of the silk protein physiological function. The silk protein constituents of amino acids, characteristic constant, and the functions are concluded in **Table 10.**

Table 10 Chemical structure, molecular weight and crystal conformation of amino acids found in silk (http://www.newsilkroad.com)

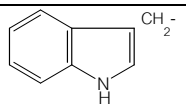
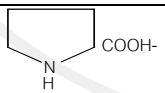
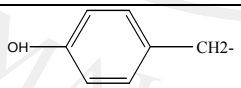
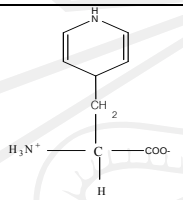
Name	Chemical structure (R-)	Molecular weight	Crystal Conformation
Glycine	H-	75.06	White monoclinic crystal
Alanine	CH_3-	89.09	Orthorhombic crystal
Valine	$(CH_3)_2CH_2$-	117.15	Hexagonal leaflets crystal, columner Crystal
Leucine	$(CH_3)_2CHCH_2$-	131.17	Colourless leaflets crystal
Isoleucine	$CH_3CH_2CH(CH_3)$-	131.17	Orthorhombic leaflets crystal, Tabular crystal
Phenylalanine	$C_6H_5CH_2$-	165.19	Leaflets crystal, needle crystal
Metionine	$CH_3SCH_2CH_2$-	149.21	Hexagonal tabular crystal
Tryptophane		204.23	Colorless hexagonal leaflets crystal
Proline		115.03	Columner crystal, needle crystal
Tyrosine		181.19	Filiform needle crystal
Cystine	SOH_2- SOO_2-	240.30	Hexagonal tabular crystal ,Columner crystal
Serine	HOCH2-	105.09	Hexagonal tabular crystal, columner crystal
Threonine	$CH_3CH(OH)$-	119.12	Orthorhombic crystal
Aspartic acid	$HOOCCH_2$-	133.10	Colourless orthorhombic leaflets crystal

Table 10 Chemical structure, molecular weight and crystal conformation of amino acids found in silk (http://www.newsilkroad.com) (continud)

Name	Chemical structure (R-)	Molecular weight	Crystal Conformation
Glutamic acid	$HOOCCH_2CH_2$-	147.13	Colourless diametric tabular crystal
Histidine		155.16	Leaflets crystal
Lysine	$H_2NCH_2CH_2CH_2CH_2$-	146.19	Hexagonal tabular crystal, needle crystal
Arginine	$H_2NCNHCH_2CH_2CH_2$-NH	174.20	Columner crystal, anhydrous

For the characteristics of silk amino acid, since the amino a amino acid have the ($-NH_2$) and the carboxyl (-COOH) groups, the amino acids have two charactersistics of electrolyte. So, we can divide them into polar amino acid and non-polar amino acid. The number of the amino and the carboxyl group of the amino acid is different. So, they are acidic amino acid and basic amino acid. Due to the different degree in the format faction (-N- -C-), the amino acids have different affinity with water. These characteristics can be summarized in **Table 11.** These characteristics can be used to separate and extract to get the required amino acid.

Table 11 The polarity of the amino acids found in silk (http://www.newsilkroad.com)

Symbol	Name	polarity			Non-polarity
		neutrality	acidity	alkali	
Gly	Glycine	-	-	-	o
Ala	Alanine	-	-	-	o
Val	Valine	-	-	-	o
Leu	Leucine	-	-	-	o

Table 11 The polarity of the amino acids found in silk (http://www.newsilkroad.com) (continued)

Symbol	Name	polarity			Non-polarity
		neutrality	acidity	alkali	
Iso	Isoleucine	-	-	-	o
Phe	Phenylalanine	-	-	-	o
Met	Metionine	-	-	-	o
Try	Tryptophane	-	-	-	o
Pro	Proline	-	-	-	o
Tyr	Tyrosine	o	-	-	-
Cys	Cystine	o	-	-	-
Ser	Serine	o	-	-	-
Thr	Threonine	o	-	-	-
Asp	Aspartic acid	-	o	-	-
Glu	Glutamic acid	-	o	-	-
His	Histidine	-	-	o	-
Lys	Lysine	-	-	o	-
Arg	Arginine	-	-	o	-

For the function of the amino acids function to body, the different molecular weight of the amino acids has different values for body **(Table 12).** For examples, the polypeptide at low molecular weight of 6000, and the small peptide including some compound amino acids, might be used in functional foods and beverage. The polypeptide with the molecular weight between 2000~4000 is the excellent material for the makeup products. The bipeptide, tripeptide and amino acids may be absorbed directly. So, the value in use of the silk protein presently is inestimable.

Table 12 The functions of various amino acids in the body (http://www.newsilkroad.com)

Type	Function
Glycine	Reduce cholesterol levels, prevent and cure high blood pressure, and bloods condense thrombus. Helps trigger the release of oxygen the energy requiring cell making process, important in the manufacturing of bones responsible for a strong immune system.
Alnine	Important source for muscle tissue, the brain and central nerve system, strengthens the immune system by producing antibodies, helps the metabolism of organic acids. Protect liver.
Valine	Promotes mental vigor, muscle coordination and calm emotions.
Leucine	Reduce blood sugar, cure swirl and promote wound healing.
Isolecine	Provide ingredients for the manufacturing, the other essential biochemical components in the body, some of which are utilized for the production of energy, stimulants to the upper brain and helping body to be more alert.
Phenylalaine	Promote the healthy function of the thyroid, adrenal and pituitary glands. Used by the brain to produce norepinephrine, a chemical that transmits signals between nerve cells and the brain, keeps you awake and alert.
Cystine	Function as an antioxidant and is a powerful aid to the body in protecting against radiation and pollution. It can help slow down the aging process, deactivate free radicals, neutralize and aid in protein synthesis and present cellular change. It is necessary for the formation of the skin, which aids in the recovery from burns and surgical operations. Hair and skin are made up of 10-14% cystine.
Serine	Reduce cholesterol levels, a storage source of glucose by the liver and a muscle helps strengthen the immune system by providing antibodies, synthesizes fatty acid sheath around nerve fibers.

Table 12 The functions of various amino acids in the body (http://www.newsilkroad.com)(continued)

Type	Function
Threonine	An important constituent of collagen, elastin, and enamel protein. Help prevents fat build up in the liver, help the digestive and intestinal tracts function more smoothly, assists metabolism and assimilation.
Aspartic	Acid aids in the expulsion of harmful ammonia from the body. When ammonia enters the circulatory system, it acts as highly toxic substance which can be harmful to the nervous system. Recent studies have showed that aspartic acid may increase resistance to fatigue and endurance.
Glutamicacid	Reduce blood ammonia, participate in brain protein and sugar metabolism, consider to be nature's brain food by improving mental capacities, help the healing of ulcers, gives a lift from fatigue, help control alcoholism, schizophrenia and the craving for sugar. Keeping skin water and prevent drying.
Methionine	It is a principle supplier of sulfur which prevents disorders of the hair, skin and nail, help lower cholesterol levels by increasing the liver's production of lecithin, reduces liver fat and protects the kidneys, a natural chelating agent for heavy metals, regulates the formation of ammonia and creates ammonia and creates ammonia free urine which reduces bladder irritation, influences hair follicles and promotes hair growth.
Tryptophan	Prevent, help and reduce the risk of artery and heart spasms, works with lysine in reducing cholesterol levels.
Proline	Cure high blood pressure, is extremely important for the proper functioning of joints and tendons, also helps maintain and strengthen heart muscles.

Table 12 The functions of various amino acids in the body (http://www.newsilkroad.com) (continued)

Type	Function
Lysine	Insure the adequate absorption of calcium, help form collagen, aid in the production of antibodies, hormones and enzymes. Recent studies have shown that lysine may be effective against herpes by improving the balance of nutrients that reduce viral growth. A deficiency may result in tiredness, inability to concentrate irritability, bloodshot eyes, retarded growth, hair loss, anemia and reproductive problems.
Arginine	Studies have shown that it has improved immune responses to bacteria, viruses and tumor cells, promotes wound healing and regeneration of the liver, cause the release of growth hormones, considered crucial for optimal muscle growth and tissue repair.
Tyrosine	Transmits nerve impulses to the brain, helps overcome depression, improves memory, increases mental alertness, and promotes the healthy functioning of the thyroid adrenal and pituitary glands. Prevention and cure senile dementia and promote metabolism.
Histidine	It is found abundantly in hemoglobin, has been used in the treatment of rheumatoid arthritis, allergic, allergic diseases, ulcers and anemia. A deficiency can cause poor hearing. Form histamine, expand blood vessel and enhance osmosis.

B. Chemical composition of sericin

The thread from cultivated silkworm *Bombyx mori* contains two natural protein fibers. One is sericin and the other is fibroin. Sericin content is about 25-30% that coats and joins 2 fibroins which are inside. **Figure 7** show that fibroin fiber is 10-137 microns thick. Sericin that coats fibroin is about 0.5-2 micron thick (Shen et al., 1998). Sericin is composed of carbon 48.8%, hydrogen 6.23%, oxygen 25.97%, nitrogen 19%. The rest is water which composes of oxygen (Siriporn, 1991).

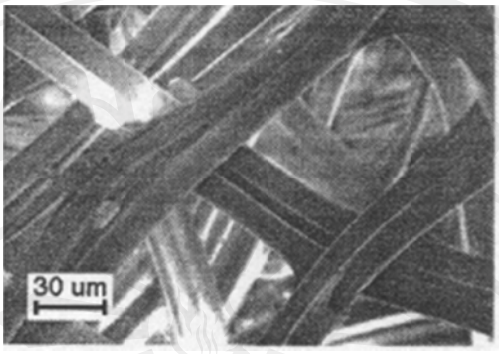
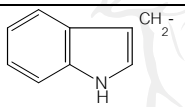
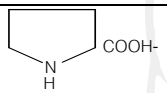
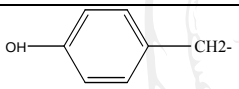
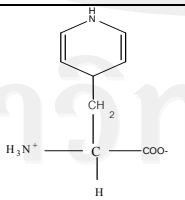


Figure 7 Electron photos of 2 fibroin fibers which are joined together by sericin (Sarovart et al., 2003)

Sericin is normally known as silk gum because of its glutinous properties that joins fibroins together and keeps the cocoon in shape. Therefore, it contains important chemicals that generate binding force between molecules. After synthesizing sericin, polar amino acid, 40 mol % sericin, is found binding by hydrogen bond of Via polar zipper interaction (Huang et al., 2003) such as hydroxyl group, carbonyl group and amino group that can generate strong hydrogen bond. The molecular weight of sericin is about 10-300 kDa. There are about 18 amino acids compose in protein of sericin. The major ones are serine (Hydroxyamino acid), glysine and aspartic acid and each is about 31.0-33, 13.9-19.1and 14.5-17.8% respectively. Other amino acids are threonine, lysine, glutamic acid, alanine, and valine and the composition of each is respectively less. These proteins are hardly found in fibroin. **Table 13** and **Figure 8** show the proportion of amino acid in sericin and a comprehensive protein structure (Freddi et.al., 1993). Sericin is produced from the middle silk gland of fully grown caterpillars (H.O. et al., 1982).

Table 13 Types, volume, and chemical structure of amino acids found in sericin of Non- native silk of ChulThai Silk Co., Ltd (Sarovart et al., 2003)

Name	Chemical structure(R-)	Sericin (Mol %)	
		Silk gland	Cocoon
Glycine	H-	12.27	13.75
Alanine	CH_3-	4.33	4.90
Valine	$(CH_3)_2CH_2$-	2.92	2.02
Leucine	$(CH_3)_2CHCH_2$-	1.32	0.80
Isoleucine	$CH_3CH_2CH(CH_3)$-	1.01	0.91
Phenylalanine	$C_6H_5CH_2$-	1.64	1.07
Metionine	$CH_3SCH_2CH_2$-	0.97	0.87
Tryptophane		0.80	0.50
Proline		1.60	1.40
Tyrosine		3.12	2.97
Cystine	SOH_2- ,SOO_2-	0.20	0.20
Serine	$HOCH_2$-	32.62	33.31
Threonine	$CH_3CH(OH)$-	6.64	8.07
Aspartic acid	$HOOCCH_2$-	18.55	19.62
Glutamic	$HOOCCH_2CH_2$-	4.83	3.25
Histidine		2.60	1.91
Lysine	$H_2NCH_2CH_2CH_2CH_2$-	1.16	0.87
Arginine	$H_2NCNHCH_2CH_2CH_2$- NH	3.52	3.58

Sericin has random coil or amorphous structure as shown in **Figure 8**. Raw silk shows scattering peak and its chemical structure can be examined by infrared ray as shown in **Figure 9**. Wave number 1, 6600 and 1,515 cm-1 illustrates amide bond in random. The absorption wavelength occurs at the lower wave number.

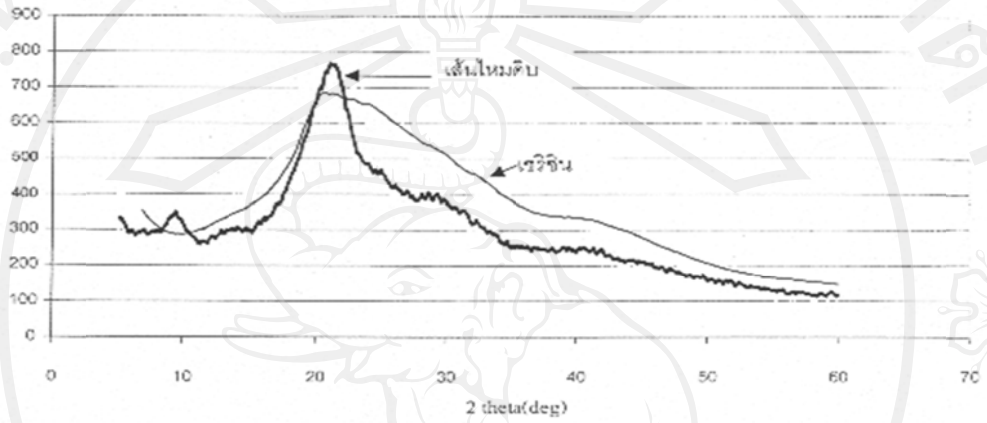


Figure 8 4XRD of sericin, which has amorphous structure in comparing to fibroin. (Freddi et al.,1993)

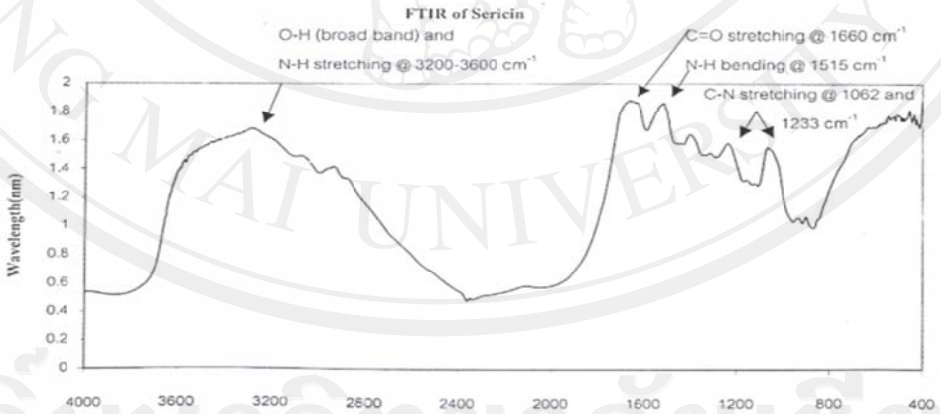


Figure 9 FTIR Spectrum of Secirin shows the random coil, (Freddi et al., 1993)

Silk thread from the cultivated silkworms is normally is composed of 4 major amino acids which are glycine, alanine, serine, and tyrosine of 83% of the total amino

acids. Proportion of glycine is higher than alanine and serine is higher than tyrosine. The silk thread also contains little polar amino acid that the side chain is acid or base.

Sericin that coats fibroin contains serine, threonine of oxyamino, aspartic acid, and glutamic acid in arginine, lysine in base of amino acid as shown in **Table 14.** This composition is different from that in the fibroin fiber. After extracting the 4 major acids of sericin from the raw silk cocoon with 1 normal of potassium hydroxide at 0-5°C, glutamic acid, alanine, leucine, isoleucine, phenylalanine, and lysine, are found in the considerable amount. In the F1, F2 layers, sericin dissolves easily, but in F3, F4, it dissolves more difficult because there are serine, threonine, aspartic acid, and histidine as shown in **Table 15.**

Table 14 Amino acid compositions in sericin and fibroin (g / 100 g) (Minakawa, 1985 [(a)] ; http://www.dongchangchemical.com, [(b)] ; Nutsawan and Watchree, 2002 [(c)])

Amino acid		Sericin [(b)] (g/100g)	Total sericin [(a)]	Fibroin[(c)]
Non-polar Amino acid	Glycine	-	8.66	41.25
	Alanine	6	3.51	28.87
	Valine	2.8	3.14	2.63
	Leucine	-	1.02	0.32
	Isoleucine	0.7	0.77	0.44
	Proline	0.7	0.66	-
	Phenylanine	-	0.50	0.58
Acid amino acid	Aspartic	-	17.03	0.76
	Glutamic	4.4	7.46	0.69

Table 14 Amino acid compositions in sericin and fibroin (g / 100 g) (Minakawa, 1985 [a] ; http://www.dongchangchemical.com, [b] ; Nutsawan and Watchree, 2002 [c]) (continued)

Amino acid		Sericin [b] (g/100g)	Total sericin [a]	Fibroin[c]
Basic amino acid	Arginine	3.1	6.07	0.86
	Histidine	1.3	1.88	-
	Lysine	3.3	4.95	0.17
Oxy amino acid	Serine	33.4	27.32	13.22
	Threonine	9.7	7.48	0.81
	Tyrosine	2.6	4.43	10.96
Sulfur-complex Amino acid	Methionine	0.04	-	-
	Cystine	0.2	0.20	-
	Valine	2.8	-	-
	Tryptophan	0.20	-	-
	Total	101.00	95.08	101.56

Table 15 Amino acid compositions of in sericin extracted from 4 layers (.g / 100 g) (Minakawa, 1985)

Amino acid		Layers of sericin			
		F1	F2	F3	F4
Acid amino acid	Glycine	8.59	8.44	8.91	8.99
	Alanine	3.64	3.56	2.80	2.37
	Valine	3.12	3.01	3.04	2.98
	Leucine	1.27	1.11	1.42	0.78
	Isoleucine	0.87	1.02	0.76	0.59

Table 15 Amino acid compositions of in sericin extracted from 4 layers (g / 100 g) (Minakawa, 1985) (continued)

Amino acid		Layers of sericin			
		F1	F2	F3	F4
Acid amino acid	Proline	0.98	0.43	0.12	0.25
	Phenylanine	0.65	0.52	0.29	0.19
	Aspartic acid	16.50	16.94	17.72	18.94
	Glutamic acid	7.61	7.40	6.84	5.82
Basic amino acid	Arginine	5.82	6.08	5.71	5.7
	Histidine	1.91	2.07	2.39	2.39
	Lysine	4.78	4.81	4.15	3.91
Oxy amino acid	Serine	26.31	26.95	27.97	29.68
	Threonine	6.94	7.29	8.18	8.41
	Tyrosine	3.60	4.53	4.50	4.59
Sulfur-complex Amino acid	Methionine	0.34	-	-	-
	Cystine	0.66	0.12	-	-
	Total	93.69	94.28	94.80	95.59

C. Chemical compositions of fibroin

Fibroin of cultivated silk is composed of 4 major amino acids which are glycine, alanine, serine, and tyrosine of 85% of the total amino acids. Proportion of glycine is higher than alanine and serine is higher than tyrosine. It also contains a little of polar amino acid (Motoii et al., 1987).

Fibroin is a silk protein fiber that the gum is removed. Normally, fibroin is coated with sericin as shown **Figure 10 and 11.** Fibroin is formed polypeptide of α-amino acids. The molecular weight of fibroin is 350 kDa (Sarovart et al., 2003) and

25 kDa. The fibroin molecule contains 2 polypeptides that have the molecular weight of $2.8x10^5$ and $2.6x10^4$ respectively. The polypeptides are joined together with the disulfide bonds as in **Figure 12**. The proteins found in fibroin are in small amount. The major amino acids found in fibroin are glycine 44.5%, alanine 29.3%, serine 12.1%, tyrosine 5.2%, valine 2%, aspartic acid 1.3 respectively shown in **Table 16.**

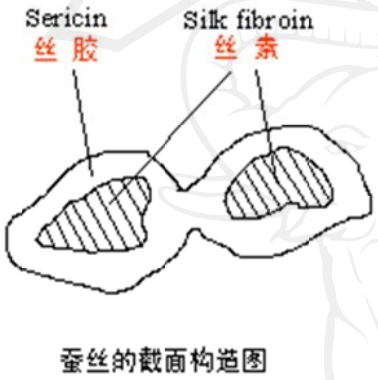


Figure 10 Composition of fibroin cocoon (http://www.smiss.cn)

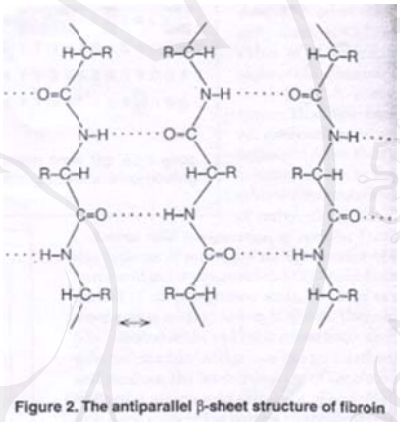


Figure 11 Structure of β-sheet silk of in fibroin (Rainer et al., 1993)

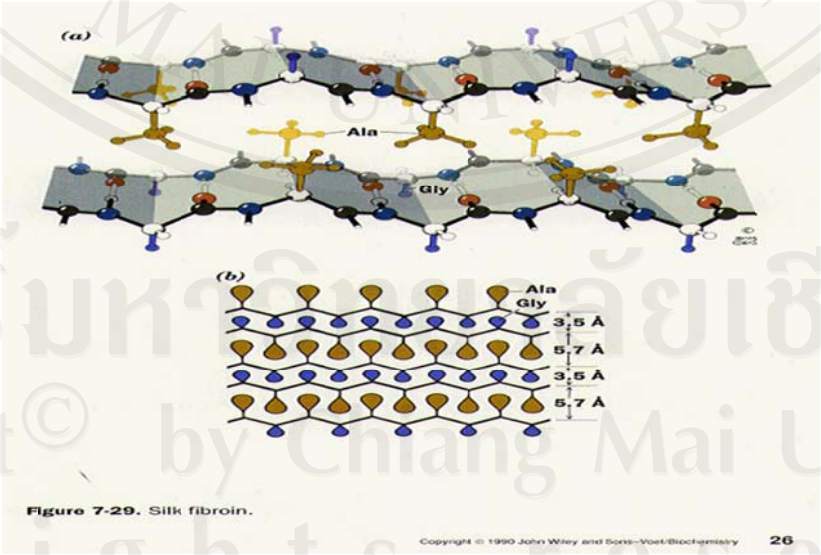


Figure 12 3D structure of β-form of fibroin in silk, (http://www.biochem.usyd.edu.au)

Table 16 Types and amount of fibroin in *Bombyx mori* silk in comparing to *G.rufobrunnae* ([a] http://www.biochem.usyd.edu.au [b] Freddi et al., 1993)

Amino acid	Fibroin *Bombyx mori* (Mol. %) [a]	Fibroin *G.rufobrunnae* (Mol. %) [b]
Glycine	44.6	35.95
Alanine	29.4	23.75
Serine	12.2	12.88
Glutamic +Gln	1.0	1.84
Cystine	0	0.06
Proline	0.3	2.07
Arginine	0.5	6.07
Leucine	0.5	1.04
Threonine	0.9	1.08
Aspartic	1.3	7.06
Valine	2.2	1.12
Tyrosine	5.2	5.11
Isoleucine	0.7	0.74
Phenylalanine	0.5	0.53
Lysine	0.3	0.49
Tryptophane	0.2	0
Histidine	0.2	0.13
Metionine	0	0.12

Fibroin fiber is produced at the outer silk gland and transferred to store in the middle silk gland where sericin is produced for coating and joining fibroin together.

Fibroin structure of the cultivated silk normally is - Gly – Ala – Gly – Ala – Gly – Ser – Gly – Ala – Ala – Gly – (Ser – Gly – Ala – Gly – Ala – Gly) (http://www.biochem.usyd.edu.au) Fibroin structure of the wild silk is – Ala – Ala – Ala – Ala – which is similar to polyalanine. Normally, G means glycine and A means

alanine. However, A in fibroin sometimes changes to Serine. Residues of most amino acid in fibroin are serine and glycine. Molecule of fibroin is orderly arranged as it has β-pleated structure (Freddi et al., 1993). Also, there is board peak in fibroin structure at the frequency of 3200–3600 cm^{-1} that presents amine and hydroxyl group.

The crystalline regions of fibroin that come from the small side chains of amino acid are, for example, glycine, alanine, and serine. The one that comes from the large side chains of amino acid is, for example, tyrosine. The fibroin atom arrangement is formed by the amino acid composition and the molecular bonds that lean forward in antiparallel line and forms multi layer structure.

In comparing to sericin, the fibrils of fibroin fiber contain different amino acids including glycine, alanine, serine, tyrosine, and lysine as primary substance, but there is no protein fiber (1.5 – 3.0%). The crystalline ratio in fibroin is quite high in comparing to sericin that dissolves in strong base solution. These amino acids play an important role in joining fibrils of fibroin fiber and affecting the thin fiber and hair (Minakawa, M., 1985).

Fibroin is different from sericin because fibroin does not dissolve in water while sericin dissolves in boiling water. Fiber coated with sericine looks untidy. So, degumming process is required to remove the sericin gum in order to get lustrous fibroin (Gulrajani et al., 1997).

The process to purify fibroin can be done by extracting using 8 M ether solution. From this method, fibroin containing glycoprotein and fibroin containing equimolar are received with total molecular weight 350 and 25 kDa respectively (Tanaka et al., 1993). These two fibroins are joined together the with bonds (Michaille et al., 1986). The structure is similar to those of other protein contained.

From the study, the molecular weight of fibroin is about 300 – 420 kDa (Tashiro et al., 1972; Tashiro et al., 1970 and Lizardi, 1979).

The fibroin structure is like other natural fiber structure which is crystalline and amorphous. From the X-ray photograph, Fibroin protein consists of layers of antiparellel β-sheets. Because of the arrangement of the polymer layers of the large protein amount and the bond of Van der Waals force in each layer, fibroin fiber is very elastic, strong, lustrous, and soft (Rainer et al.,1993). Hence, fibroin fiber is suitable for clothing (Niranam, 2002).

D. Chemical composition of silk worm oil

According to the study increased of the Sericulture Research Institute in, Thailand, silk production has to the total of 1,581.47 tons since 1992. The weight proportion of silk cocoon to silk pupa is 1,000 g.: to 755 g. or about 1 to 3 accordingly. From the ratio, the total silk pupa is about 4,744.41 tons per year.

After the caterpillar has spun cocoon for 10 days, the cocoons must be dried in order to stop life cycle of worms and to keep the quality of cocoon until the reeling process. Silk pupa is the residues in the reeling process of the industrial production and can be used for the development quality of other products in order to be value added.

Therefore, residues from the silk production is used as a by-product to develop other products, especially silk pupa which is reported to have a high nutrient substance containing important amino acids that are important to human health. Many researchers have been studying, developing, and analyzing the possibilities to develop healthy foods, cosmetic products, food supplements, and medicines by using silk pupa.

Many insects, including silk pupa, have been consumed in Thailand, because they are easily found in the north and the northern east of Thailand all year round. There are also franchise shops selling edible insects. This is because Thai people realize that many nutrients are found in insects. There are different ways to cook insects, i.e. frying, roasting and grinding.

Pupa is the excellent and inexpensive source of protein and is classified as the economic insects that can be made as the occupation for the supplement income and has been preferred to be opened as the franchise business among the group of small business operators. According to the report of Nipha et al. (1997) and Pongsathorn et al. (1990) from the result of the silk pupa analysis per 100 grams in weight, up to 98 kilocarrories can be given as the source of energy. Other sources are protein, fat, carbohydrate and many major minerals (**Table 17).**

Table 17 Nutrients found in 100 grams of silk pupa (Nipa and Uruyakorm, 1997[(a)] ; Pongsathorn et al., 1990[(b)])

Nutrition value	Type	Capacity	Unit
Value [(a)]			
	Energy	98	Kilocalorie
	Protein	9.6	Gram
	Fat	5.6	Gram
	Carbohydrate	2.3	Gram
	Fiber	1	Gram
	Ash	0.9	Gram
	Moisture	80.6	Gram
Mineral[(b),(a)]	Calcium	41.7	Milligram
	Phosphorus	155.4	Milligram
	Iron	1.8	Milligram
	Potassium	138.7	Milligram
	Sodium	13.6	Milligram
Vitamin[(b),(a)]	Vitamin B 1	0.12	Milligram
	Vitamin B 2	1.06	Milligram
	Niacin	0.86	Milligram

According to the research work of Apichart (1994). On the chemical compositions of pupa of Jula breed, Korat breed, Thai hybrid breed and foreign hybrid breed, it was found that the linolenic acid, oleic acid and palmitic acid were at 31.7, 30.6 and 24.5% respectively. Meanwhile, the linolenic acid, oleic acid and palmitic acid were found in Korat hybrid breed at 31.0, 28.2 and 24.9 %respectively, 16.9, 31.5 and 30.4% respectively for Thai hybrid breed, and 14, 24.6 and 14% respectively for the foreign hybrid breed.There are 6 amino acids found as show in **Table 18.**

Boonlerd (1994) reported that silk pupa can be used for animal feed because it contains protein, fat, and some vitamins. Protein content is as high as 60-62%. Amornrat (1976) reported that silk pupa contains 51-63% protein, and 20-29% fats.

Table 18 Types and amounts of amino acids found in silk pupa of Thai Silk Co., Ltd (Apichart, 1994)

Amino acid	Chemical structure	Percent	Carbon atom
Palmitic	$CH_3(CH_2)_{14}COOH$	24.32	18
Palmitoleic	$CH_3(CH_2)_6CH=CH(CH_2)_7COOH$	0.94	16
Stearic	$CH_3(CH_2)_{16}COOH$	6.17	18
Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	33.84	18
Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COH$	5.44	18
Linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	29.29	18

Sompote (1992) reported that silk pupa contains many important amino acids such as phenylalanine, lysine, leucine, isoleucine, and valine. Also, Joachim (2000)

reported that silk pupa contains 49.4-60.9% protein, and 14.2-30.3% fats of its dry weight. Nandeesha et al., (1990) found protein 41 and fats at 18% in silk pupa.

Lakhana (1995) that reported vitamin type β, α, χ and σ are found in essential oil of about 0.013%. Boonlerd (1994) said that silk pupa contains vitamin A, vitamin B1, and vitamin B2. Vitamin A at 100 mg, vitamin B1 at 1-2 mg. and vitamin B2 at 0.05 mg are found in silk pupa powder of 100 grams. It also contains phosphorous and sulfur.

Silk pupa contains 15-18% of fats (Sompote, 1992) and there are many low-melting-point amino acids in this fat. **Tables 19 and 20** show that silk pupa contains free fatty acid as high as 61% (Amornrat, 1976).

Kobkul et al., (2000) studied the chemical composition of amino acid of silk pupa for the development and processing. Silk pupa, hybrid Nakornrachasima1, (K1XK8), (KTXMD4), (KT1XMD6) and (MD1XK8) were used. The amount of 200 g. of each species of the pupa are separated to male and female, dried in oven, and ground by a grinding machine. Then, the samples were processed for extracting oil by dissolving in petroleum ether. The essential oil from the silk pupa was analyzed for chemical compositions, i.e. moisture, fats, protein, fiber, ash, and carbohydrate. The result showed that there was no different composition in different species, except the hybrid Nakornrachasima 1 that has protein as high as 54.54%. It also contains saturated fatty acid and unsaturated fatty acid of about 32.70% and 67.3% respectively as shown in **Table 20.** This study also showed that female silk pupa of all samples had higher protein and carbohydrate than the male pupa, whereas the male pupa had higher fats and fiber than the female pupa.

Table 19 Composition of fatty acids of silk pupa powder from species Jul1, Hybrid Nakornrachasima, and Native Thai ([a] Sompote, 1992, [b] Joachim, 2000, [c] Oratai, K, 1998, [d] Kobkul, S. et al., 2000)

Fatty acid type	Fatty acid[c] Ju variety1 (%)	Fatty acid [d] Korat KTXMD4 (%)	Fatty acid (%)[a] (Fat 10%)	Fatty acid [b] (%)
Saturated fatty acids				20.7
Myristic,C 14:0	(*)	(*)	1.30	(*)
Palmitic, C16:0	24.58	24.96	30.4	(*)
Stearic C18:0	5.70	8.06	7.47	(*)
Unsaturated fatty acids				70.1
Palmitoleic,C16:1	0.96	0.95	1.66	14.0
Oleic,C18:1	30.61	28.27	31.50	9.1
Linoleic,C18:2	6.37	6.73	6.34	24.6
Linolenic,C18:3	31.77	31.03	16.9	14.0
(Arachidonic,C20:4	(*)	(*)	1.52	(*)

Note: (*) Unavailable document

Oratai (1998) and Lakhana et al., (1999) studied the process to extract oil from silk pupa Jul1 and Jul5 to find new sources of important fatty acids, especially Omega-3 type Alfalinolanic which are edible and can be used in food processing on consumable price. The study found that silk pupa Jul1 has higher oil contents than Jul5 and its acidity is higher than the standard Omega-3. It is also found that Jul1 and Jul5 silk pupa can be found during the process of the extraction of oil as shown in **Table 21.** The shelf lives of the extracted oil were also studied. The result shows that the oil of Jul1 and Jul5 kept in perfectly sealed containers at 25°C have the oxide values of 1.42 and 0.46 ppm/1 kg. /day respectively.

Yang et al., (1993) has reported the properties and stability of the pupa oil. The Chinese silk was analyzed for the chemical compositions, the percent of by-

products obtained from the dry pupa extracted with the solvent was found at 30% with triglyceride as the main composition and the group of phospholipids was found at 648 ppm. When the analysis was made to find the fatty acids, oleic acid, linoleic acid and linolenic acid were found at 29, 6 and 36% respectively, including 28% of the saturated fatty acids. The study on the pupa oil deterioration was performed by testing the stability of the Chinese pupa oil in comparison with the oil and the oil from plants and animals stored at the temperature of 60^{O}C. It was found that the Chinese pupa oil deteriorated at the slower rate in comparing to the oil from peanuts and the oil from animals due to the more compositions of phospholipids.

Table 20 Chemical composition and fatty acid contents of the silk pupa both sex separated and non- separated (Kobkul et al., 2000)

species / compositions	Hybrid Nakhonratchasima silk pupa KT1XMD4		
	Sex separate		
	Male	Female	Intercourse
Moisture (%)	2.07	2.46	2.26
Fat (%)	32.85	25.09	28.97
Protein (%)	49.75	57.12	53.43
Ash (%)	5.54	4.74	5.14
Fiber (%)	6.14	4.76	5.46
Carbohydrate (%)	3.65	5.8	4.74
Fatty acid (%)			
C16:0	26.57	23.35	24.96
C18:0	7.65	8.48	8.06
Total Saturated fatty acids	34.22	31.83	33.02
C16:1	0.84	1.06	0.95
C18:1	29.66	26.87	28.27
C18:2	6.26	7.20	6.73
C18:3	29.02	33.04	31.03
Total Unsaturated fatty acids	65.78	68.17	66.98

Table 21 Chemical characteristics of extracted oil from Jul1 and Jul5 silk pupa (Oratai, 1998; Lakhana et al., 1999)

characteristics	Unit	Reference quality	Jul1 species	Jul5 species
Acid value	mg KOH / oil 1 g	≤ 0.6	0.56±0.04	0.51±0.02
Color L*	-	-	74.87± 0.04	72.00 ± 044
a*	-	-	-5.52± 0.04	-5.10 ± 0.22
b*	-	-	53.81± 0.11	50.88± 0.81
Peroxide value	Milliequiv/ oil 1 kg	≤ 10	8.28 ±0.46	4.77 ± 0.27
Water and volatile substances	% by weight	≤ 0.2	0.0070±0.0016	0.0128±0.0040
Contamination cannot soluble in fat	% by weight	≤ 0.05	0.0106±0.0032	0.0237±0.0100
Relative density	-	-	0.8970±0.0001	0.8860±0.0030
Refractive index	-	-	1.5 ± 0.1	1.5 ± 0.1
Iodine number	g iodine/ oil 100g	-	80.08±1.17	82.16± 2.24

Doneanu et al., (1997) have studied the capillary gas-chromatography mass spectrometry characterization of fatty acids from the indigenous silkworm oil by using the cold on-column and split injection system technique and separated the substances by using three polar fused-silica capillary columns, coated with FFAP,

Table 21 Chemical Quality of extracted oil from Jul1 and Jul5 silk pupa (Oratai, 1998 and Lakhana et al., 1999) (continued)

Quality value	Unit	References	Jul1species	Jul5 species
Saponification	mg KOH/ oil 1 g	-	201.11±0.43	218.01± 0.54
Unsaponification	%by weight	-	0.8478±0.0130	0.9021±0.0518
α-Tocopherols in process	IU/100 G	-	20.75±1.20	23.00±1.70
α-Tocopherols in oil	IU/100 G	-	29.96±0.78	41.00±1.84

Carbowax 20M, and SP-2340 stationary phases. The Romanian native pupa oil gave the compositions of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid were found at 21.23, 0.2-0.5, 5-7, 31-34, 6-8 and 30-32% respectively. The rest of them were lauric acid, myristic acid, arachic acid and behenic acid.

Lu Ping et al., (1998) have analyzed the compositions of Chinese pupa oil by gas-chromatography mass spectrometry method. The result of the pupa oil extraction and analysis by the gas-chromatography mass spectrometry, using fatty acids found in the pupa oil to saponified method with KOH-MeOH and modified to Meester by BF3-MeOH, The quality was then checked by way of thin layer chromatography, and compared with 9 fatty acids. The result found that 79.88% of the unsaturated fatty acid was observed. More than 71.5% α-linolenic acids of all unsaturated fatty acid were found. Thus, the Chinese pupa oil is the source of α - linolenic acid with the properties to reduce the fat and blood pressure as well as to defer the causes of cancer.

Pereira et al., (2003) have extracted and analyzed the chemical compositions of the fatty acid from the pupa oil to be used as the guideline and ingredients for adding the nutritional value to the food products. The pupa oil was enriched with protein of more than 51%, fat 34% and with 24% of essential fatty acid or linolenic acids. The pupa can be one of the alternatives using to control the diet and used as the ingredients in the food supplements food for the fatty acid type.

1.4.1.4 Preparations of oil and sericin

A. Method of oil extraction from natural products

A.1 Rendering extraction (Bredeson, D., 1983)

This method is preferred to be used with the animal fat tissue with tender form and high volume of fat. The principal is to put them into high temperature of heat until the cell wall is broken and the fat is transformed into liquid flowing which can be taken out easily from the cell wall. The rendering method can be done in the form of dry or wet.

A.2 Hydraulic pressing extraction (Peter, J.W. et al., 2000)

This method is suitable for plant raw materials. The hydraulic pressing method can be done by batch and continuous pressing and can also be done in the cold and hot pressing, depending on the type of raw materials. For examples, if the raw materials have the high contents of fat, the cold pressing may be chosen due to its low cost and more oil can be obtained.

A.3 Solvent extraction (Kotake, N.E. et al., 2002)

This method is the use of solvent to run through the raw materials in order to extract the fat and oil. Then, the solvent will be evaporated from the extracted fat or oil (the crude oil). According to the theory, the selection of the solvent to be

used for the extraction should depend on the following factors; no oil soluble substances (such as protein and carbohydrate), easy to be evaporate. So, no need to be left over night). For solvent, it had low boiling point, nonflammable, non-toxic, and easy to seep into the molecules. In addition, the ratio of the solvent to the raw materials shall depend on the type of raw materials, method of the extraction, and raw material particle sizes. Importantly, the suitable method should leave the oil in the waste of not exceed 1%.This method appears to be the most effective and has the maximum advantages. The solvents to be used are petroleum ether, hexane, ether, acetone and chloroform. The most use extraction methods is the Soxhlet method which is usually performed for 18-24 hrs of the extraction time, depending on the type of raw materials.

A.4 Microwave digestion or oven extraction (Iva, J. et al., 2003)

This method employs the microwave principle to scatter the cells to obtain the active substances. This method has been used widely in Spain, Mexico, and Romania for extracting oil from animals and plants. The solvents such as hexane, acetone, water and other solvents are used together with microwave. The type of microwave to be used is the domestic microwave oven, such as Samsung Co., Ltd. (859 W, 2450 MHz or model 1250 W. 2450 Hz). For example place about 2-5 grams of the fine ground raw materials and 25 ml of flammable hydrocarbon in the microwave oven for 3 minutes, take them out and leave them to cool down for 10 seconds, then evaporate the solvent and gather the evaporated solvent for further extraction (repeatedly) for 3 times, only using 25 ml. of the solvent. Next, gather the extracted substances for the evaporation to eliminate the solvent and the oil is finally

obtained. This method is very interesting due to its less time consumption in comparing to the solvent extraction, with low cost and energy consumption.

B. Preparations of sericin

Sericin is the part that coats and holds fibroin, being silk glue with viscosity. it is a protein that dissolves well in diluted alkaline solution, because it is an amino acid with high hydrophilic property and reacts to water upon touching with acid-alkali. It can be digested by an enzyme. It is sensitive to pH and temperature, If the heat is higher than 150°C. From the research report of Minamoto Toshiaki and Hiroya Ichiro (1999). They extracted sericin by starting from cutting cocoons into small sizes with the ratio of cocoon 5-g to water 100 g.Then wash the silk pieces in the running water at 10-100 l/kg/min for 10-180 minutes.Next, extract it boiled water temperature at 90-130°C. Tsubouchi Kozo, Yamada Hiroo and Takasu Yoko (1997). Have studied the extraction method of large molecular sericin. They soaked 1 gram of in 20 to 60-ml of urea solution with the concentration of 2 mol/l and extracted at the temperatures between 100-130°C for 10 minutes to 1 hour. After extracting, the molecular weight of sericin about 50,000 was obtained. The product was fractionalized again to get a smaller molecular weight of sericin by sedimentation.

C. Application of sericins

Sericins represent a voluminous waste when the cocoons are processed to the textile threads. It has been assessed that worldwide cocoon processing yields 50,000 tons of sericins annually (Zhang, 2002). No data were published on the amounts of sericins used by the cosmetic and pharmaceutical industries but it is probably only a fraction of this natural resource. The use of sericin extracts in

cosmetics has been a long tradition. Today, we know that sericins possess moisturizing (Padamwar et al., 2005), UV-absorbent, antioxidant, and to some extent antimicrobial properties (Sarovart et al., 2003; Zhaorigetu et al., 2003).These properties have mystified their addition to the skin creams and hair care products. It is claimed that sericins prevent wrinkles and other manifestations of skin aging (Padamwar and Pawar, 2004). Sericin properties have also their industrial applications, for example as a coating of air filters to enhance their cleaning efficacy (Sarovart et al., 2003). The UV-absorption provides natural protection of the silk textiles. The fabrics made from sericin depleted silk are prone to light-induced damage (Becker et al., 1995). However, without a special treatment, standard silk fabrics do not provide sufficient skin protection against strong UV radiation (Reinert et al., 1997).

1.4.2 Niosomes

1.4.2.1 Definition/introduction

At present, there is no available drug delivery system which achieves the site specific delivery with controlled release kinetics of drug in predictable manner. Paul Ehrlich, in 1909, initiated the era of development for targeted delivery when he envisaged a drug delivery mechanism that would target directly to diseased cells. Since then, numbers of carriers have been utilized to carry drug at the target organ/tissue, which include immunoglobulins, serum proteins, synthetic polymers, liposomes, microspheres, erythrocytes and niosomes (Theresa M.A, 1998). Among different carriers, liposomes and niosomes are well documented drug delivery.

Systems Drug targeting can be defined as the ability to direct a therapeutic agent specifically to desired site of action with little or no interaction with non target tissue. (Breimer, D.D. and Speiser, R, 1985).

Niosome or non-ionic surfactant vesicles are microspheres with the size of 0.1–2 μm in diameter. Niosomes are microscopic lamellar structures, which are formed from the mixture of non-ionic surfactant of the alkyl or dialkyl polyglycerol ether class and cholesterol with subsequent hydration in an aqueous media. Structurally, niosomes are similar to liposomes, in that they are also made up of a bilayer. However, the bilayer in the case of niosomes is made up of non-ionic surface active agents rather than phospholipids as seen in the case of liposomes. Most surface active agents when immersed in water yield micellar structures. However, some surfactants can yield bilayer vesicles which are niosomes. Niosomes may be unilamellar or multilamellar structures depending on the preparation method. The niosome is composed of surfactant bilayers with the hydrophilic ends of the surfactants exposed on the outside and inside of the vesicle, while the hydrophobic chains face each other within the bilayer. Hence, the vesicle holds hydrophilic drugs within the space enclosed in the vesicle, while hydrophobic drugs are embedded within the bilayer itself. **Figure 13** shows the structure of niosome and where the drug is located within the vesicle.

1.4.2.2 Formation of niosomes

Niosomes are formed from the self-assembly of nonionic amphiphile and aqueous solvent (Uchegbu and Vyas, 1998). The association of amphiphile monomers into vesicles on hydration is the result of the fact that there is an existing of a high

interfacial tension between water and hydrocarbon portion of the amphiphile causing them to associate (Uchegbu et al., 1995).

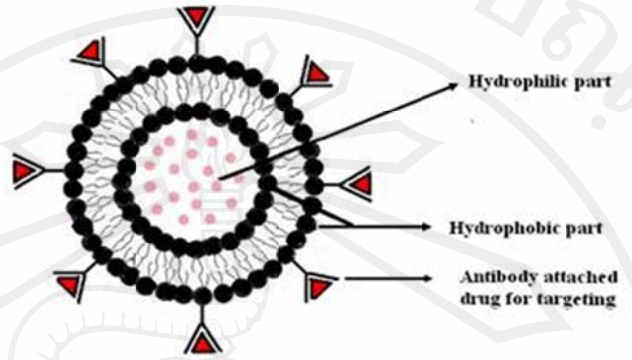


Figure 13 Schematic drawings of a niosome
Source: http://www.pharmainfo.net/../Niosome%20structure.jpg

- Water soluble molecules ρρ Non-ionic surfactant monomers
- || Lipid soluble molecules

1.4.2.3 Niosome preparation methods

Various types of niosomes can be prepared by different methods which imply that there are several mechanisms to get the formation of niosomes.

A. Hand-shaken method

Hand-shaken method or the conventional method is firstly introduced by Bangham et al., (1965). The steps involved in this method are the preparation of lipid for hydration, hydration of the lipid film by agitation and sizing of the lipid lamellar dispersion to a homogeneous distribution of the vesicles. First, the lipids must be dissolved and mixed in an organic solvent (usually chloroform or chloroform mixed with methanol) to assure a homogeneous mixture of the lipids. Then, the solvent is removed by evaporation to form a thin lipid film on the sides of a round bottom flask, and left to dry overnight to remove the residual solvent. Hydration of

the dried lipid film is simply accomplished by adding an aqueous solution, which may include drugs or active ingredients before agitating by swelling on the water bath at the temperature above the gel-liquid crystal transition temperature (Tc or Tm) of the lipids **(Figure 14).** The resulting niosome which is a large, multilamellar vesicle (LMVs) is obtained. The size of the niosome can be reduced by sonication or extrusion to obtain the required sizes.

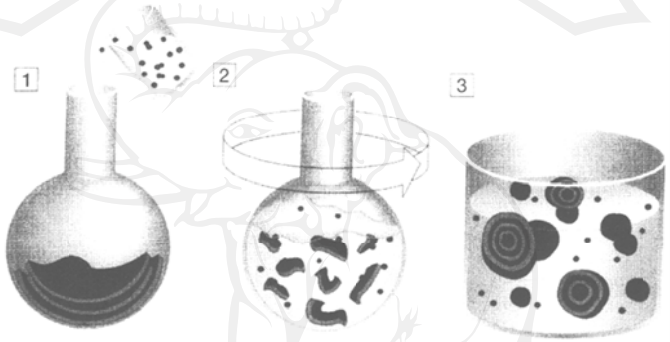


Figure 14 Schematic representation of the three steps of niosome preparation by hand-shaken method. 1: Addition of an aqueous phase to the dry thin lipid film. 2: Swelling and peeling of the lipid film under vigorous agitation. 3: Milky suspension of the equilibrated niosome (Lasch et al., 1995)

B. Sonication method

This method is probably the most widely used method for the preparation of small unilamellar vesicles (SUVs), which has two techniques.A typical production method of the vesicles is by sonication of the solution as described by Cable, (1989). In this method, an aliquot of drug solution in buffer is added to the surfactant/cholesterol mixture in a 10-ml glass vial. The mixture is probe sonicated at 60°C for 3 minutes using a sonicator with a titanium probe to yield niosomes.

Probe sonication**:** The tip of a sonicator is directly immersed into the niosomal dispersion. In this method, the energy input into the dispersion is very high. The dissipation of energy at the tip results in local overheating, and therefore the vessel of the dispersion must be immersed into an ice/water bath. During the sonication of up to one hour, more than 5% of the lipids can be de-esterified. Also, with the probe sonicator, titanium will slough off and contaminate the dispersion. But, this contamination can be removed by centrifugation.

Bath sonication**:** The tube of the niosomal dispersion is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier than the probe sonication method. Materials being sonicated can be kept in a sterile container or under an inert atmosphere unlike the probe units.

C. Freeze-dried rehydration method

Freeze-dried rehydration vesicles (FRVs) are formed from the preformed vesicles. Very high entrapment efficiency, even for macromolecules, can be achieved. Drying brings the lipid layers and materials to be encapsulated into close contact. Upon reswelling, the chances for entrapment of the adhered molecules are larger. Dehydration is best performed by freeze-drying. Rehydration must be done extremely carefully. The significant advantages of this method are that the drugs do not expose high temperature by solvent evaporation and lyophilization can reduce the risk of chemical degradation of many sensitive substances such as DNA and protein.

D. Reverse-phase evaporation method

Niosomes with large internal aqueous space and high capture by reverse-phase evaporation (REV) were introduced by Szoka and Papahadjopoulos in (1978). Historically, this method provided a breakthrough in niosome technology,

since it allowed for the first time of the preparation of niosomes with a high aqueous space-to-lipid ratio and able to entrap a large percentage of the aqueous materials.

Reverse-phase evaporation is based on the formation of the inverted micelles. These inverted micelles are formed upon sonication of a mixture of a buffered aqueous phase, which contains the water soluble molecules to be encapsulated into the niosomes and an organic phase in which the amphiphilic molecules are solubilized. The slow removal of the organic solvent leads to the transformation of these inverted micelles into a gel-like and viscous state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The excess phospholipids in the environment contribute to the formation of a complete bilayer around the remaining micelles, which results in the formation of niosomes. Niosomes by reverse phase evaporation method can be prepared from cholesterol and surfactants (1:1) are dissolved in a mixture of ether and chloroform. An aqueous phase containing drug is added to this and the resulting two phases are sonicated at 4-5°C. The clear gel formed is further sonicated after the addition of a small amount of phosphate buffered saline (PBS). The organic phase is removed at 40°C under low pressure. The resulting viscous niosome suspension is diluted with PBS and heated on a water bath at 60°C for 10 min to yield niosomes.

E. Detergent depletion method

The detergent depletion method is used for the preparation of a variety of niosomal formulations. Detergents can be depleted from the mixed detergent-lipid micelles by various techniques, which lead to the formation of homogeneous niosomes. In practice, all lipids below their phase transition

temperature can be used with this method. Not all detergents are suited for detergent depletion method and only a few detergents can be used for this method such as sodium cholate, alkyl (thio) glucoside and alkyloxy- polyethylenes. Mixed micelles are prepared by adding the concentrated detergent solution to multilamellar niosomes. The final concentration of the detergent should be well above the critical micelle concentration (CMC) of the detergent. Equilibrium of the mixed micelles in the aqueous phase takes quite sometimes, and does not happen during a short period. The use of different detergents results in different size distributions of the vesicles and the ratios of large unilamellar vesicles to oligolamellar vesicles and to multilamellar vesicles. Faster depletion rates can produce the smaller size liposomes.

F. Supercritical carbon dioxide method

Typically, most niosome preparation methods have the multi-step and require large amounts of organic solvent, which are toxic to human and environments. Techniques for the preparation of niosomes have been developed in order to decrease toxicity as well as the improvement of stability and solubility. Supercritical fluid is a substance above its critical temperature (Tc) and critical pressure (Pc). At the critical point, supercritical fluids have the density as liquid and low viscosity with better flow property as gas. Carbon dioxide is a gas, which is widely used to produce supercritical fluid because of its low critical temperature (Tc = 31.1 ˚C) and pressure (Pc = 73.8 bar). It has high solvating power at the conditions near the critical point and similar to non-polar solvent. The procedure is consisted of two main parts; 1) the high-pressure part, the lipid components or cholesterol are dissolved under pressure in supercritical carbon dioxide and 2) the low-pressure part, the homogeneous supercritical solution is expanded and simultaneously mixed with

the aqueous phase to yield vesicles. Carbon dioxide is released when pressure is reduced. It is lesser toxic than an organic solvent.

G. Microfluidization (Khandare, et al.,1994)

Micro fluidization is a recent technique used to prepare unilamellar vesicles of defined size distribution. This method is based on submerged jet principle in which two fluidized streams interact at ultra high velocities, in precisely defined micro channels within the interaction chamber. The impingement of thin liquid sheet along a common front is arranged such that the energy supplied to the system remains within the area of niosomes formation. The result is a greater uniformity, smaller size and better reproducibility of niosomes formed.

H. Multiple membrane extrusion method (Khandare, et al., 1994)

Mixture of surfactant, cholesterol and dicetyl phosphate in chloroform is made into thin film by evaporation. The film is hydrated with aqueous drug solution and the resultant suspension extruded through polycarbonate membranes, which are placed in series for up to 8 passages. It is a good method for controlling niosome size.

1.4.2.4 Characterization of niosomes

A. Morphology

Morphology of vesicle has been proposed as a tool for understanding the formation, stability and entrapment efficiency of the vesicles (El Maghraby et al., 2000b; Zasadzinski et al., 2001; Waninge et al., 2003; Abreu et al., 2007). Several techniques can be used for morphology determination such as electron microscope and small angle x-ray scattering (SAXs). Manosroi et al. (2005) have characterized the morphology of vesicles entrapped with kojic acid by the Bangham method with

sonication using transmission electron microscope (TEM). The stable oligolamellar vesicles could be formed. Fernandez et al. (2008) have used SAXS to determine the interbilayer distances of DMPG vesicles in the presence of sodium salts. The results have indicated that loose multilamellar structures start to appear with 50 mM of NaCl.

B. Vesicle diameter

Niosomes, similar to liposomes, assume spherical shape and so their diameter can be determined using light microscopy, photon correlation microscopy and freeze fracture electron microscopy. Freeze thawing (Khandare, J.N. et al.,1994) keeping vesicles suspension at –20°C for 24 hrs and then heating to ambient temperature) of niosomes increases the vesicle diameter, which might be attributed to fusion of vesicles during the cycle.

C. Charge

The charge density on vesicles can be estimated from mobility measurements in an electrical field (microelectrophoresis). The mobility data can be converted into zeta potential and then calculated to charge density using dynamic light scattering (Grit and Crommelin, 1993). Charges inducing agent for examples phosphatidyl glycerol (PG), phosphatidic acid (PA) or phosphatidyl serine (PS) were regularly added to bilayer to improve the physical stability against aggregation or fusion, and also increase the biological activity (Leonards, 1988; Arouri et al., 2009). Manosroi et al. (2008) have reported that the stability of luciferase plasmid can be enhanced by entrapping in cationic liposomes.

D. Microviscosity

Microviscosity indicates bilayer fluidity of molecules which increases

when organization of the liquid-crystal structure increases. This parameter depends on the bilayer composition, the temperature and the aqueous environment. Microviscosity can be observed by fluorescence polarization with various types of fluorescent probes such as diphenylhexatriene (DPH), trimethylamino-DPH (TMA) or transparinaric acid (TPA). Crommelin and Schreier (1994) have indicated that TMA-DPH is located at the lipid-water interface and thus provides information on the motion of molecule in that particular region. Lentz (1993) has studied the order and dynamics within the acyl chain region of liposomal bilayers using the fluorescent probes [6-diphenyl-1, 3, 5-hexatriene (DPH) and parinaric acid (PA)]. The results can provide unique qualitative information about the state of the lipid bilayer at various positions along the acyl chains. Roy et al. (2005) have investigated microviscosity of some N-acylamino acid surfactants using DPH as probe molecule. The result has indicated that the self-assemblies of these surfactants have high microviscosities in consistence with their bilayer membrane structures.

E. Entrapment efficiency

After preparing niosomal dispersion, unentrapped drug is separated by dialysis (Chauhan and Luorence,1989), centrifugation (Yoshioka, et al., 1994; Gayatri, et al., 2000), or gel filtration (Szoka and Papahadyopoulos, 1980). as described above and the drug remained entrapped in niosomes is determined by complete vesicle disruption using 50% n-propanol or 0.1% Triton X-100 and analysing the resultant solution by appropriate assay method for the drug. Where,

Entrapment efficiency (EF) = (Amount entrapped total amount) x 100

F. Phase transition temperature (Tc) and enthalpy change (ΔH)

The phase transition temperature is the temperature that induces a change of physical state of molecules from gel phase (closely packed molecule) to liquid crystalline phase (loosely packed molecule and fluid). At phase transition temperature, the molecules obtain enough freedom of motion to spontaneously arrange themselves into a crystalline form. Several techniques have been used to investigat the phase transition temperature such as dynamic light scattering (DLS), differential scanning calorimetry (DSC) and NMR (Bammel et al., 1990; Leung and Ho, 1991; Feitosa et al., 2000; Michel et al., 2000). Enthalpy change (ΔH) has been employed to study the energy driven - formation of vesicles. It is usually analyzed by DSC (Fang et al., 2003). Barriocanal et al., (2004) have reported the enthalpy change for the formation of egg phosphatidylcholine vesicles by the thin film hydration technique is exothermic, whilst that for dimyristoylphosphatidylcholine is endothermic. This may be from the influence of hydrocarbon chains on the hydration process and also on the process of vesicle formation.

G. *In-vitro* release

A method of *in-vitro* release rate study includes the use of dialysis tubing. A dialysis sac is washed and soaked in distilled water. The vesicle suspension is pipetted into a bag made up of the tubing and sealed. The bag containing the vesicles is placed in 200 ml of buffer solution in a 250 ml beaker with constant shaking at 25°C or 37°C. At various time intervals, the buffer is analyzed for the drug content by an appropriate assay method (Yoshioka, et al., 1994).

1.4.2.5. Advantages of niosomes (http://www.pharmainfo.net/ Reviews/niosome-unique-drug-delivery-system; Surender, et al., 2010)

The vesicle suspension being water based offers greater patient compliance

over oil based systems, Since the structure of the niosomes offers place to accommodate hydrophilic, lipophilic as well as ampiphilic drug moieties, they can be used for a variety of drugs. The characteristics such as size and lamellarity. of the vesicle can be varied depending on the requirement. The vesicles can act as a depot to release the drug slowly and of controlled release. They are osmotically active and stable. They increase the stability of the entrapped drug. Handling and storage of surfactants do not require any special conditions. They can increase the oral bioavailability of the drugs.

1.4.2.6 Comparison of niosomes and liposomes

Niosomes and liposomes can be compared as the following:

1. Niosomes are now widely studied as an alternative to liposomes, which exhibit certain disadvantages such as they are expensive. Their ingredients like phospholipids are chemically unstable because of their predisposition to oxidative degradation. Liposomes require special storage and handling. Purity of natural phospholipids which are the compositions of liposome is variable.

2. Differences in characteristics exist between liposomes and niosomes, since niosomes are prepared from uncharged single-chain surfactant and cholesterol whereas liposomes are prepared from double-chain phospholipids (neutral or charged) (Don et al., 1997; Handjani et al., 1979).

3. Niosomes behave *in-vivo* like liposomes, prolonging the circulation of the entrapped drug and altering its organ distribution and metabolic stability

Encapsulation of various antineoplastic agents in these carrier has been shown to decrease drug induced toxic side effects, while maintaining, or in some instances, increasing the anti-tumor efficacy (Sheena et al.,1998). Such vesicular drug carrier systems alter the plasma clearance kinetics, tissue distribution, metabolism and cellular interaction of the drug (Azmin et al., 1985; McCormack and Gregordias, 1998). They can be expected to target the drug to its desired site of action and/or to control its release (Baillie et al., 1985).

4. For liposomes, the properties of niosomes depends both on the compositions of the bilayer and the method of production (Szoka and Papahadyopoulos, 1980). It was observed by Baillie et al., (1985) that the intercalation of cholesterol in the bilayers decreases the entrapment volume during formulation and thus the entrapment efficiency. As the concentration of cholesterol increases, entrapment efficiency decreases.

5. The entrapment efficiency increases with an increase in the concentration and lipophilicity of surfactants. Chandraprakash et al., (1990) have loaded methotrexate in non-ionic surfactant vesicles using lipophilic surfactants like Span 40, Span 60 and Span 80 and found that Span 60 (HLB = 4.7) gave the highest percent age of entrapment while Span 85 (HLB = 9.8) gave the least entrapment. They also observed that as the HLB value of the surfactant decreased, the mean size was reduced.

However niosomes are similar to liposomes in functionality. Niosomes also increase the bioavailability of the drug and reduce the clearance like liposomes.

Niosomes can also be used for the targeted drug delivery, similar to liposomes. As with liposomes, the properties of the niosomes depend both- on the compositions of the bilayer, and the production method.

1.4.2.7 Stability of niosomes (Uchegbu et al., 1998)

The stable noisommal dispersions exhibit a constant particle size and constant level of the entrapped drug. The precipitation of the membrane components must not be observed. Ideally, these systems should be stored in dry forms (proniosomes) and when they are reconstituted with the appropriate medium, they should exhibit similar dispersion characteristics to the original dispersion. Proniosomes can minimize physical stability problems of niosomes such as aggregation, fusion, leaking and show additional convenience in transportation, distribution, storage and dosing (Hu et al., 1999).

The stability of niosomal systems are influenced by the nature of surfactant /lipid, nature of the encapsulated drug, temperature of storage, detergents and thermodynamic considerations. They are several methods to enhance the stability of these niosomes. Decreasing the air-water interface may prevent the crystallization of surfactant monomers. The addition of the polymerized surfactants to the formulation, the use of membrane spanning lipids, the interfacial polymerization of surfactant monomers in situ, the inclusion of a charged molecule in bilayer, and the entrapment of the hydrophobic drugs or macromolecular prodrugs can increase the stability of the niosomal dispersions.

1.4.2.8. Applications of niosomes

The application of niosomal technology is widely varied and can be

used to treat a number of diseases. The following are a few uses of niosomes which are either proven or under researched.

Drug Targetting: Niosomes can be used to target drugs to the reticulo-endothelial system. The reticulo-endothelial system (RES) preferentially takes up niosome vesicles. The uptake of niosomes is controlled by circulating serum factors called opsonins. These opsonins mark the niosome for clearance. Such localization of drugs is utilized to treat tumors in animals known to metastasize to the liver and spleen. This localization of drugs can also be used for treating parasitic infections of the liver. Niosomes can also be utilized for targeting drugs to organs other than the RES. A carrier system (such as antibodies) can be attached to niosomes (as immunoglobulins bind readily to the lipid surface of the niosomes) to target them to specific organs. Many cells also possess the intrinsic ability to recognize and bind specific carbohydrate determinants. This can be exploited by niosomes to direct carrier system to particular cells.

Anti-neoplastic Treatment: Most antineoplastic drugs cause severe side effects. Niosomes can alter the metabolism, prolong circulation and half life of the drug, thus decreasing the side effects of the drugs. Niosomal entrapment of doxorubicin and methotrexate (in two separate studies) showed beneficial effects over the unentrapped drugs, such as the decreased rate of proliferation of the tumor and higher plasma levels accompanied by slower elimination.

Leishmaniasis: Leishmaniasis is a disease in which a parasite of the genus *Leishmania* invades the cells of the liver and spleen. Commonly prescribed drugs for

the treatment are derivatives of antimony (antimonials), which in higher concentrations can cause cardiac, liver and kidney damage. Use of niosomes in the tests conducted showed that it was possible to administer higher levels of the drug without the triggering of the side effects, and thus allowing the greater efficacy in treatment.

Delivery of Peptide Drugs: Oral peptide drug delivery has long been faced with a challenge of bypassing the enzymes which would breakdown the peptide. Use of niosomes to successfully protect the peptides from gastrointestinal peptide breakdown is being investigated. In an *in-vitro* study conducted by Yoshida et al, oral delivery of a vasopressin derivative entrapped in niosomes showed that the entrapment of the drug significantly increased the stability of the peptide.

Use for the study of the immune response: Due to their immunological selectivity, low toxicity and greater stability, niosomes are being used to study the nature of the immune response provoked by antigens.

Niosomes as Carriers for Haemoglobin: Niosomes can be used as carriers for haemoglobin within the blood. The niosomal vesicle is permeable to oxygen and hence can act as a carrier for haemoglobin in anemic patients.

Utilizing in Transdermal Drug Delivery Systems : One of the most useful aspects of niosomes is that they greatly enhance the uptake of drugs through the skin. Transdermal drug delivery utilizing niosomal technology is widely used in cosmetics, in fact, it was one of the first use of the niosomes. Topical use of niosomes entrapped with antibiotics to treat acne is performed. The penetration of the drugs through the

skin is greatly increased as compared to the un-entrapped drug. Recently, transdermal vaccines utilizing niosomal technology is also being researched. A study conducted by P. N. Gupta et al has shown that niosomes (along with liposomes and transfersomes) can be utilized for topical immunization using tetanus toxoid. However, the current technology in niosomes allows only a weak immune response, and thus more research needed to be done in this field.

Other Applications: Niosomes can also be the utilized for sustained drug release and localized drug action to greatly increase the safety and efficacy of many drugs. Toxic drugs which need higher doses can possibly be delivered safely using niosomal encapsulation.

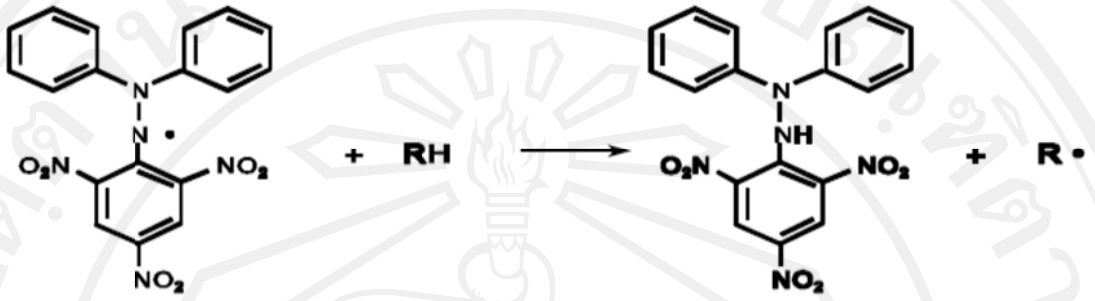
1.4.3 *In vitro* biological assays

1.4.3.1 DPPH free radical scavenging activity assay

The free radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydracyl), a stable free radical, has been widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) or hydrogen donating activities of various compounds since it is a simple, rapid and sensitive method (Suja et al., 2005; Letelier et al., 2008).

DPPH, a radical generating substance, has a deep violet color due to its unpaired electron. Free radical scavenging ability can be followed by the loss of absorbance at 515 nm as the pale yellow non-radical form is produced. After DPPH solution react with the samples, the absorbance of the resulting solutions are measured and compared with the absorbance of DPPH in the absence of sample solution. The lower absorbance represents the higher activity. The reaction of the DPPH radical in

the presence of the antioxidant compound during the DPPH assay is shown in **Figure 15**.



Deep violet color at 515 nm Pale yellow (non-radical form)

Figure 15 Reaction of the DPPH radical in the presence of the antioxidant compound during the DPPH assay (Prakash, 2001)

1.4.3.2 Tyrosinase inhibition assay

The human epidermis is composed of three important cell types including melanocytes, keratinocytes and Langerhans. Melanocytes are located in the basal layer in the epidermis. The melanocytes produce melanin to protect the skin from UV radiation. The process by which melanin is formed is called melanogenesis. Two basic types of melanin are eumelanin, which is a black pigment and pheomelanin which is a yellow to red pigment. The varieties of human skin color depend on the amount of eumelanin and pheomelanin.

The melanogenesis pathway has been elucidated by Prota, 1980 (**Figure 16**). The process starts from the hydroxylation of tyrosine (amino acid) to dopa and the oxidation of dopa to dopaquinone. These steps are under enzymatic control while the remaining steps occur spontaneously. The tyrosinase, copper-containing protein, is a key enzyme for melanogenesis. Tyrosinase existing in the skin can catalyze the oxidation of tyrosine to DOPA and subsequently to DOPA quinine

which will mediately change to melanin, a brown to black color pigment. Therefore, any compound which can inhibit this enzyme, can inhibit the formation of melanin.

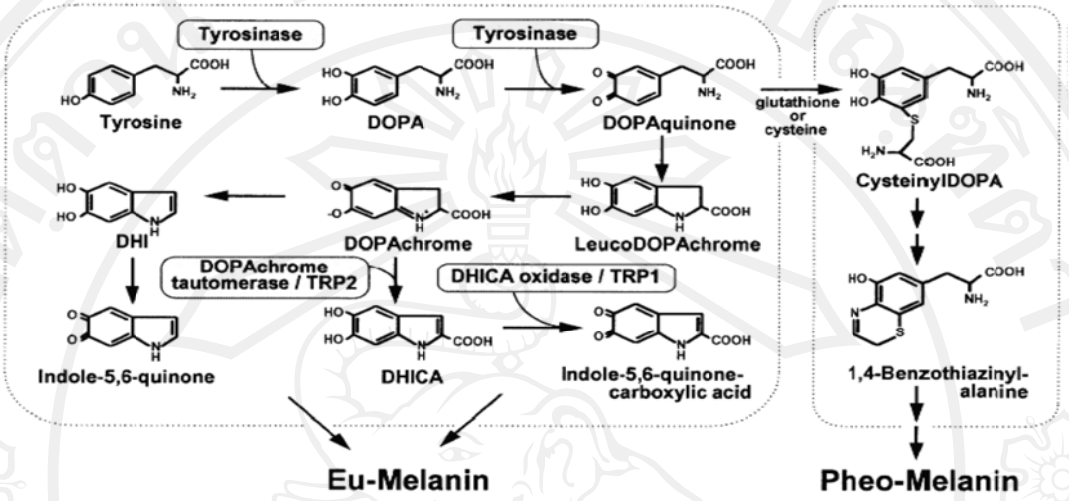


Figure 16 Melanogenesis pathway (Prota, 1980)

1.4.3.3 Microbial limit test

The microbial limit tests are designed to perform the qualitative and quantitative estimations of specific viable microorganisms present in the samples. It includes the tests for total viable count (bacteria and fungi) and *Escherichia coli*. There are four methods for this test including membrane filtration method, pour plate method, spread plate method, and serial dilution method (Prakash, 2001).

The US Food and Drug Administration (FDA) has presented the serial dilution as a preferred method for microbe testing in the bacteriological analytical manual (BAM) of the FDA and the AOAC due to its accuracy with 95% confident levels (Garthright and Blodgett, 2003).

In serial dilution method, the total of 12 test tubes are used where 9 tubes containing 9 mL of soybean-casein digest medium for dilution and 3 tubes containing 10 mL of the same medium for the control. The serial dilution is done in the 9 tubes with the following steps. First, add 1 mL of the test fluid to three test tubes

(triplicate) and mix to make 10-times dilutions. Second, add 1 mL of each of 10-times dilutions to each of another three test tubes (triplicate) and mix to make 100-times dilutions. Third, add 1 mL of each of 100-times dilutions to each of the remaining three test tubes and mix to make 1,000-times dilutions. Then, all 12 test tubes are incubated at 30-35 °C for at least 5 days. No microbial growth should be observed for the control test tubes. If the determination of the result is difficult or the result is not reliable, place a 0.1 mL fluid from each of the 9 test tubes to an agar medium or fluid medium, incubate all media for 24-72 hr at 30-35 °C, and check them for the absence or presence of microbial growth. The number of viable microorganisms is usually presented in the term of colony forming unit (number of colony x dilution factor) per volume or gram of sample (CFU/mL).

1.4.4 *In vivo* biological assays

1.4.4.1 Animals

A. Rabbit skin irritation testing

Determination of the irritant and/or corrosive effects on skin of mammals is useful and reliable in the assessment and evaluation of the toxic characteristics of a substance being exposed on skin. The primary skin irritation is usually evaluated by the Draize patch test on rabbits (Shah et al., 2007). After the tested products are applied on the rabbit skin, the rabbits are examined for the presence of erythema and oedema according to Draize dermal irritation scoring system (0: no erythema or no oedema; 1: barely perceptible erythema or oedema; 2: well defined erythema or slight oedema; 3: moderate to severe erythema or moderate oedema; 4: severe erythema or oedema) at the grading intervals of 1, 24, 48 and 72 hr (Draize, 1959). However, the European Union (EU) has recently imposed that safety

for human health must be guaranteed without animal tests of cosmetic ingredients or the mixtures of ingredients since 11 March 2009, with the exception of the repeated dose toxicity, toxicokinetics and reproduction toxicity tests, which will be prohibited since 11 March 2013 (2003/15/EC, 2003). Thus, the safety assessment of cosmetic ingredients after 2009 has to be done without animal tests (Pauwels and Rogiers, 2004).

1.4.4.2 Human volunteers

Since EU has required the European cosmetic manufacturer or initial importer to hold available "proof of the effect claimed for the cosmetic product, where justified by the nature of the effect or product" (Salter, 1996). Several efficacy study methodologies are provided by cosmetic manufacturers. In case of moisturizer and anti-wrinkle products, confocal Raman microspectroscopy (CRM), corneometer, TEWAmeter, vapometer, mexameter, cutometer and skin visiometer have been proposed to test for the efficacy claim of the products. The principles of each techniques are described as the followings:

A. TEWL meter/ vapometer (Miteva et al., 2006)

TEWL meter or vapometer has been used to measure the rate of transepidermal water loss (TEWL) which is a parameter indicating water barrier function of the skin, based on diffusion principles according to Fick's law (Miteva et al., 2006) as the following equation:

$$dm/dt = -DAdp/dx$$

where A represents the surface in m^2, m is the water transported (g) and t is the time (hr), D is the diffusion constant (0.877 g/m·hr), p is the vapour pressure of the atmosphere (mmHg) and x is the distance from the skin surface to the point of

measurement (m). The TEWL value is given in unit of g/m^2hr**.** Elmahjoubi et al. (2009) have dedicated the TEWL method as a good alternative to water flux measurements for assessing the full-thickness skin barrier function.

B. Cutometer (Bazin and Fanchon, 2006)

Cutometer measures the skin elasticity based on a suction and elongation technique. The cutometer exhibits deformation-time curves from which the following parameters are calculated: Ue, Uv, Us, Uf, Ur/Ue and Ur/Uf. Ue is the immediate deformation skin extensibility. Uv is the deviation which reflects the viscoelastic contribution of the skin. Us is the total deviation of the skin. Uf is the immediate recovery of the skin after the removal of vacuum. Finally, due to typical viscoelastic properties of the skin, the deformation returns very slowly to the original state. The relative parameter Ur/Ue reflects the net elasticity of the skin without viscous deformation and the Ur/Uf represents the biological elasticity, i.e. the ratio of immediate retraction to the total extension. Absolute parameters are influenced by skin thickness, whereas relative parameters are not and can be compared without preliminary standardization to skin thickness (Barbosa-Barros et al., 2008). **Figure 17** showed deformation-time curves of the viscoelasticity of the skin from cutometer.

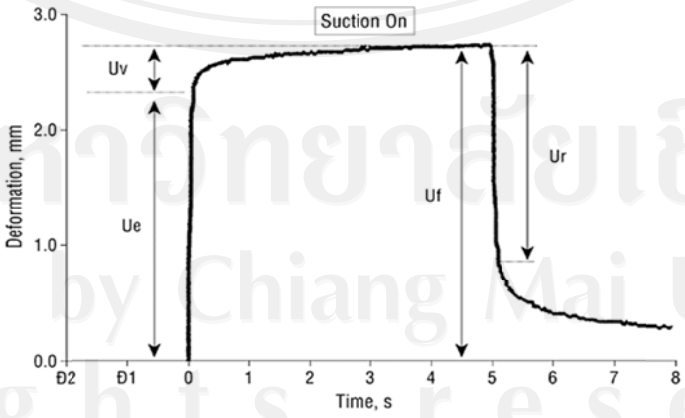


Figure 17 Deformation-time curve of the viscoelasticity of the skin (Fong et. al., 1997)

1.4.5 Applications of sericin and oil in cosmetics

No data were published on the amounts of sericins used by the cosmetic and pharmaceutical industries, but it is probably only a fraction of this natural resource. The use of sericin extracts in cosmetics has a long tradition. Low-molecular-weight sericin is used in various blends for cosmetics, in skin, hair and nail products medical and pharmaceutical applications (Zhang 2002; Padamwar and Pawar, 2004). Today,we know that sericins possess moisturizing (Padamwar et al, 2005), UV-absorbent, antioxidant, and has some extent antimicrobial properties (Sarovart et al., 2003; Zhaorigetu et al., 2003a). These properties their addition to the skin creams and hair care products. It is claimed that sericins prevent wrinkles and other manifestations of skin aging (Padamwar and Pawar, 2004). Sericin properties also drive their industrial applications, for example as a coating of hair filters to enhance their cleansing efficacy (Sarovart et al., 2003). The UV-absorption provides natural protection of the silk textiles. The fabrics made from sericin depleted silk are prone to light-induced damage (Becker et al., 1995). However, without a special treatment, standard silk fabrics do not provide sufficient skin protection against strong UV radiation (Reinert et al., 1997).

Sericin enhances the elasticity of skin and has anti-wrinkle and anti-aging effects when used in lotion, cream, or ointment (Voegeli et al., 1993; Yamada et al., 1998; Ogawa. and Yamada, 1999; Henne and Hoppe., 1985). Sericin gel shows moisturizing properties with an increase of hydroxyproline content in the stratum corneum (outermost layer of the epidermis) and decreased skin impedance (Padamwar et al., 2002). Sericin gels with pluronic and Carbopol restore natural moisturizing factors as well as to prevent transepidermal water loss from the skin. A mixture of silk

fibroin (70–95%) and sericin (5–30%) powder shows antistatic features and the ability to absorb moisture (Kirikawa et al., 2000). Skin problems like dermatitis can be controlled with these treatments (Yasuda et al., 1998). Sericin enhances the light-screening effect of UV filters like triazines and cinnamic acid esters (Yoshioka et al., 2001).Nail cosmetics with sericin (0.02–20%) prevent nail brittleness and impart nail gloss (Yamada et al., 2001). Sericin (0.02–2%) in cosmetics for hair and in bath preparations with 0.01–1% olive oil and fatty acids reduces damage to the hair surface by binding to hair (Hoppe; Koerbaecher and Roeckl, 1984). Sericin hydrolysates with low molecular weights are used as conditioners for skin and hair (Hata, 1987). Shampoos containing sericin and pelargonic acid with pH less than 6 are used for cleansing hair (Engel, W. and Hoppe, U., 1987) include. Other cosmetic applications of the sericin powder (Yamada, H. and Yuri O., 1998), lotion with moisturizing and conditioning effects (Yamada et al., 2001) creams with the improved cleansing properties (Sakamoto and Yamakishi., 2000), as well as and sweat and sebum (fat secreted from the sebaceous glands of the skin) absorbing cosmetics (Miyashita, 1999)

1.4.6 Structure and biochemical changes in aging skin

The skin is not a uniform surface (Barton et al., 1998; Fiedler et al., 1999). The surface area of the exposed skin was increased by approximately 30% by the fine wrinkles as compared with a flat surface. A consequence of the wrinkles was that topical applications do not uniformly cover the skin surface (Brown et al., 1986). There were significant regional differences in the topography, since these fine wrinkles were not found on either the soles the feet or palms of the hands.

1.4.6.1 Physiology of human skin

The development of skin as an organ commences during the fetal stage; however, its final development occurs only postnatally. In a human adult, the skin structure can be categorized into three main layers, from outside to inside, are the epidermis, stratum corneum (SC), dermis and subcutaneous tissue.

Epidermis is contains no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries extending to the upper layers of the dermis. The main type of cells which make up the epidermis are Markel cells, keratinocytes, with melanocytes and Langerhans cells also present. The epidermis can be further subdivided into the following strata: corneum, lucidum, granulosum, spinosum, basale. Cells are formed through mitosis at the basale layer. The daughter cells move up the strata changing shape and composition as they die due to isolation from their blood source. The cytoplasm is released and the protein keratin is inserted. This process is called keratinization and takes place within about 27 days. This keratinized layer of skin is responsible for keeping water in the body and keeping other harmful chemicals and pathogens out, making skin a natural barrier to infection.

Stratum corneum (SC) is highly hydrophobic and contains 10-15 layers of interdigitated corneocytes (thickness of 10-20 μm). Its organization can be described by the 'brick and mortar' model (Elias, 1983). Due to its highly organized structure, SC is the major permeability barrier to external materials. It is regarded as the rate-limiting factor in the penetration of therapeutic agents through the skin.

Dermis is the layer of skin beneath the epidermis that consists of connective tissue and cushions the body from stress and strain. The dermis is tightly connected to the epidermis by a basement membrane. It also harbors many Mechanoreceptors

(nerve endings) that provide the sense of touch and heat. It contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. The blood vessels in the dermis provide nourishment and waste removal from its own cells as well as from the Stratum basale of the epidermis. The dermis is structurally divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region. The elasticity of the dermis is attributed to a network of protein fibres, including collagen (type I and III) and elastin, which are embedded in an amorphous glycosaminoglycan ground substance. The dermis also contains scattered fibroblasts, macrophages, mast cells, leukocytes, hair follicles, sebaceous gland and sweat gland (Scheuplein, 1965).

Papillary region is composed of loose areolar connective tissue. This is named for its fingerlike projections called papillae that extend toward the epidermis. The papillae provide the dermis with a "bumpy" surface that interdigitates with the epidermis, strengthening the connection between the two layers of skin.

Reticular region is lies deep in the papillary region and is usually much thicker. It is composed of dense irregular connective tissue, and receives its name from the dense concentration of collagenous, elastic, and reticular fibres that weave throughout it. These protein fibres give the dermis its properties of strength, extensibility, and elasticity. Also located within the reticular region are the roots of the hair, sebaceous glands, sweat glands, receptors, nails, and blood vessels.

Hypodermis is not part of the skin, and lies below the dermis. Its purpose is to attach the skin to underlying bone and muscle as well as supplying it with blood vessels and nerves. It consists of loose connective tissue and elastic. The main cell types are fibroblasts, macrophages and adipocytes (the hypodermis contains 50% of

body fat). Fat serves as padding and insulation for the body. Another name for the hypodermis is the subcutaneous tissue. Microorganisms like Staphylococcus epidermidis colonize the skin surface. The density of skin flora depends on region of the skin. The disinfected skin surface gets recolonized from bacteria residing in the deeper areas of the hair follicle, gut and urogenital openings (Proksch et al., 2008).

1.4.6.2 Collagen

Collagen is a group of naturally occurring proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals (Muller et al., 2003). It is the main component of connective tissue, and is the most abundant protein in mammals (Di Lullo et al., 2002). Making up about 25% to 35% of the whole-body protein content. Collagen, in the form of elongated fibrils, is mostly found in fibrous tissues such as tendon, ligament and skin, and is also abundant in cornea, cartilage, bone, blood vessels, the gut, and intervertebral disc. In muscle tissue it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscles (Sikorski, Z.E. 2001). Gelatin, which is used in food and industry, is collagen that has been irreversibly hydrolyzed. Collagen fibrils/aggregates are arranged in different combinations and concentrations in various tissues to provide varying tissue properties. In bone, entire collagen triple helices lie in a parallel, staggered array. Forty nm gaps between the ends of the tropocollagen subunits probably serve as nucleation sites for the deposition of long, hard, fine crystals of the mineral component, which is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ with some phosphate. It is in this way that certain kinds of cartilage turn into bone. Type I collagen gives bone its tensile strength.

Types and associated disorders, only 29 types of collagen have been identified and described. Over 90% of the collagen in the body, however, is of collagen One: skin, tendon, vascular, ligature, organs, bone (main component of bone), collagen Two: cartilage (main component of cartilage), collagen Three: reticulate (main component of reticular fibers), commonly found alongside type I, collagen Four: forms bases of cell basement membrane, collagen Five: cells surfaces, hair and placenta, collagen-related diseases most commonly arise from genetic defects or nutritional deficiencies that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes involved in normal collagen production.

Type	Notes
I	This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth.
II	Hyaline cartilage, makes up 50% of all cartilage protein. Vitreous humour of the eye.
III	This is the collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus
IV	basal lamina; eye lens. Also serves as part of the filtration system in capillaries and the glomeruli of nephron in the kidney.
V	most interstitial tissue, assoc. with type I, associated with placenta
VII	forms anchoring fibrils in dermal epidermal junctions
VIII	some endothelial cells
IX	FACIT collagen, cartilage, assoc. with type II and XI fibrils
X	hypertrophic and mineralizing cartilage
XI	cartilage

Type	Notes
XII	FACIT collagen, interacts with type I containing fibrils, decorin and glycosaminoglycans
XIII	transmembrane collagen, interacts with integrin a1b1, fibronectin and components of basement membranes like nidogen and perlecan.
XIV	FACIT collagen
XV	-
XVI	-
XVII	transmembrane collagen, also known as BP180, a 180 kDa protein
XVIII	source of endostatin
XIX	FACIT collagen
XX	-
XXI	FACIT collagen
XXII	-
XXIII	MACIT collagen -
XXIV	-
XXV	-
XXVI	-
XXVII	-
XXVIII	-
XXIX	epidermal collagen

The skin was the largest, heaviest and functionally most versatile organ of the human body. The skin has important functions including regulation of body temperature, protection against environmental insult and moisture loss as well as various immune functions. The appearance of the skin changes with increasing age. There was a loss in elasticity, tautness, smoothness and softness, and a general

decrease in the overall functionality of the skin as well as an increase in dryness and roughness of the skin surface. The formation of wrinkles, a typical phenomenon of skin aging, was a complex procedure and is a central topic in cosmetic research and product development. As they form a scaffold of connective tissue within the dermis, collagen fiber bundles are responsible for many skin-typical phenomena including the formation of wrinkles. Fibroblasts, the cells of the dermis that actively form connective tissue, supply the raw material e.g. collagen, reticular and elastic fibers for the extracellular as well as for the intercellular matrix. Inactive fibroblasts are termed fibrocytes, although they may become active under certain conditions such as regeneration. Fiber formation primarily takes place extracellularly. Building blocks which resemble bamboo shafts, the microfibrils, are secreted and attached to the cell surface of the fibroblasts. This was also the area where polymerization of the raw material into fiber-like components takes place. Microfibrils grow and mature until they reach their typical length. By incorporating further raw materials present in the surrounding matrix they then aggregate and form bundles of defined collagen fibrils and fibers. Contrary to the elastic fibers, which are arranged in a meshwork within the connective tissue, the elasticity of the collagen fiber was insignificant (< 5 percent = high module of elasticity). A certain degree of elasticity may be attributed to the fact that the fibrils cross-link covalently to each other to form a latticework. The amount of cross-linking correlates with the required elasticity. The largest possible shift exists at right-angled fibered crossings. In areas in which a stronger mechanical stress occurs, the collagen fiber systems are predominantly oriented along the lines of stress.

Our illustration gives a schematic representation of collagen fibered bundles formation, the age-dependent decrease of collagen formation, fibroblasts and the

latticework of collagen fibered bundles. Fibroblasts, which were shown as spindle-shaped connective tissue cells, with irregularly formed cell extensions, produce raw material, while the fibered formation takes place extracellularly (collagen, elastic, reticular). In regeneration processes, more collagen than elastic fibers was produced. The meshwork structure of the connective tissue provides for a uniform elasticity of the skin. The age-correlated decreases of the production of collagen as well as the increase in degradation of the collagen fiber bundles disturb the optimal function of the cross-linking principle. This leads to irregularities in skin elasticity and a reduction in the collagen supply of the skin (Otto Bucher.1962).

1.4.7 Sericin and oil role of NMFs

Silk consists of amino acid with 18 kinds of hydroxyl group, particularly, sericin and fibroin having the property in moisturizing the skin. Sericin protein is the water soluble which could be dissolved in polar solvent and was hydrolyzed in the acidic or alkaline solvent which, in this connection, sericin has divided into 2 groups according to molecular weight which was the molecular weight group with the lower weight $\leq$ 20 Kda, the group which was popular for used in making cosmetics and skin care products including hair care, health products and the group with the higher weight of $\leq$ 20 Kda, the group which was popular for used in various medical biomaterials: such as, film bands, for used in medical biomaterials on working biomembranes, hydrogel and duty filaments and cloths, coating materials, etc, with the factor for used in the extractions having the effect on molecular weight and quantity of sericin: such as, temperature, pH and time used in the extractions (Yu-Qing, 2002). Besides these, the silkworm having sericin quantity as high as approximately 33% and specific characteristic carbohydrate of approximately 3 %

will result in the sericin to be capable in flocculation with keratin which was an important protein of stratum corneum and important mechanism in increasing good skin hydration and skin moisturizing with the capability in moisture absorption and release, creation of film in well protecting and entrapping the moisture, thus, making the skin moisturized tenderly mild, which, in this connection, film will flocculate in blended substance on the skin and can flocculate exceptionally well with water, thus, it was a valuable cosmetic substance in giving moisture in order to prevent the loss of water from the skin and function duty in skin protection. When applying sericin on the skin, it was create glutinous film and the skin can be protected after the film gets dried and that moisture was obviously and clearly be seen to be added to the skin (Pandamwa et al., 2004, Hua et al., 2002 and Chen et al., 2001), reduced the loss of water from the skin through epidermis and reduced wrinkle lines. On the capability in originating the films to sericin molecules, the more high quantity of molecules the better the creation of the films. A trade report finds that silk amino acid acquired from fibroin degradation used as the mixture of free amino acid, depeptides and tripeptides by using concentrate acid or alkaline which, in this connection, silk amino acid will dissolved in water and that a clear solvent will be acquired that could be well blended with the products of the mixture of water and alcohol and substance with negative and positive charges and amphoteric. So, it could be widely mixed in the cosmetics. Silk amino acid has the property of the exceptionally good maintaining on the balance of skin moisture when comparing with the other humectants; such as, glycerin, making it suitable to hairs and skins in increasing the flexibility and mildly tenderness when touching. This was because of the reason that the free amino acid can absorb well into cortex layer. A study report in 1983 finds that the application of silk amino acid just

only 0.1% in shampoo and perm lotion products can restore damaged hair condition well. Sericin from dark yellow silk which consists of carotenoid has the property in partly helping anti-free radicals and protection on the degeneration of cell conditions in the body as well as giving the types of more amino acid than the white silkworm, as well.

The important substance that enables the skin to maintain moisture was the natural moisturizing factor (NMF). NMF will function in absorbing water from the atmosphere including water existing in the cells, as well, in order to enable the skin to maintain the moisture. But, because of the reason that NMF can dissolve in water, it, therefore, will usually be washed away when contacting with the water and this is the reason why our skin was dried when we have had a contact with water for a long period of time or frequently. But, the lipid bilayer surrounding the cells will protect corneocyte from losing NMF when we used sericin that has amino acid, the necessary acid, which up to 30% is found in protein from silkworms and we found that the condition of such acid will be deteriorated in accordance with body condition, particularly, from various free radicals. Such amino acid will flocculate with water in stratum corneum layer making the skin able to maintain the moisture and more smoothness. In addition, glycosoaminoglycans (GAG's or Mucopolysaccharides) and Proteo-Glycan will catch water and maintain it in the skin, thus, making the skin moist, as well.

In lipid bilayer which was cholesterol that helps moisturize the skin, it was, therefore, necessary to use skin moisturizer to help in moisturizing, particularly, amino acid group extracted from silkworms in which the quantity and type were most likely similar to the skin of human's beings, enabling it to stay on and coat the skin

for protection it from free radicals as well as protecting the loss of skin water. The cause of making our skin dried mainly derives from the followings; staying in the place with low moisture; such as, in the air-conditioned room which had result in the skin to be much dryer because dry atmosphere will make excessive water evaporate from the skin. Excessive cleaning of fat from the skin will reduce the skin fat down to only 64.4%; the secretion of fat from fat gland will be excessively less; natural moisturizing factor (NMF) degenerated, particularly, those who are getting older and older, NMF will reduce. (Padamwar, et al., 2005; Soler C., 2005)