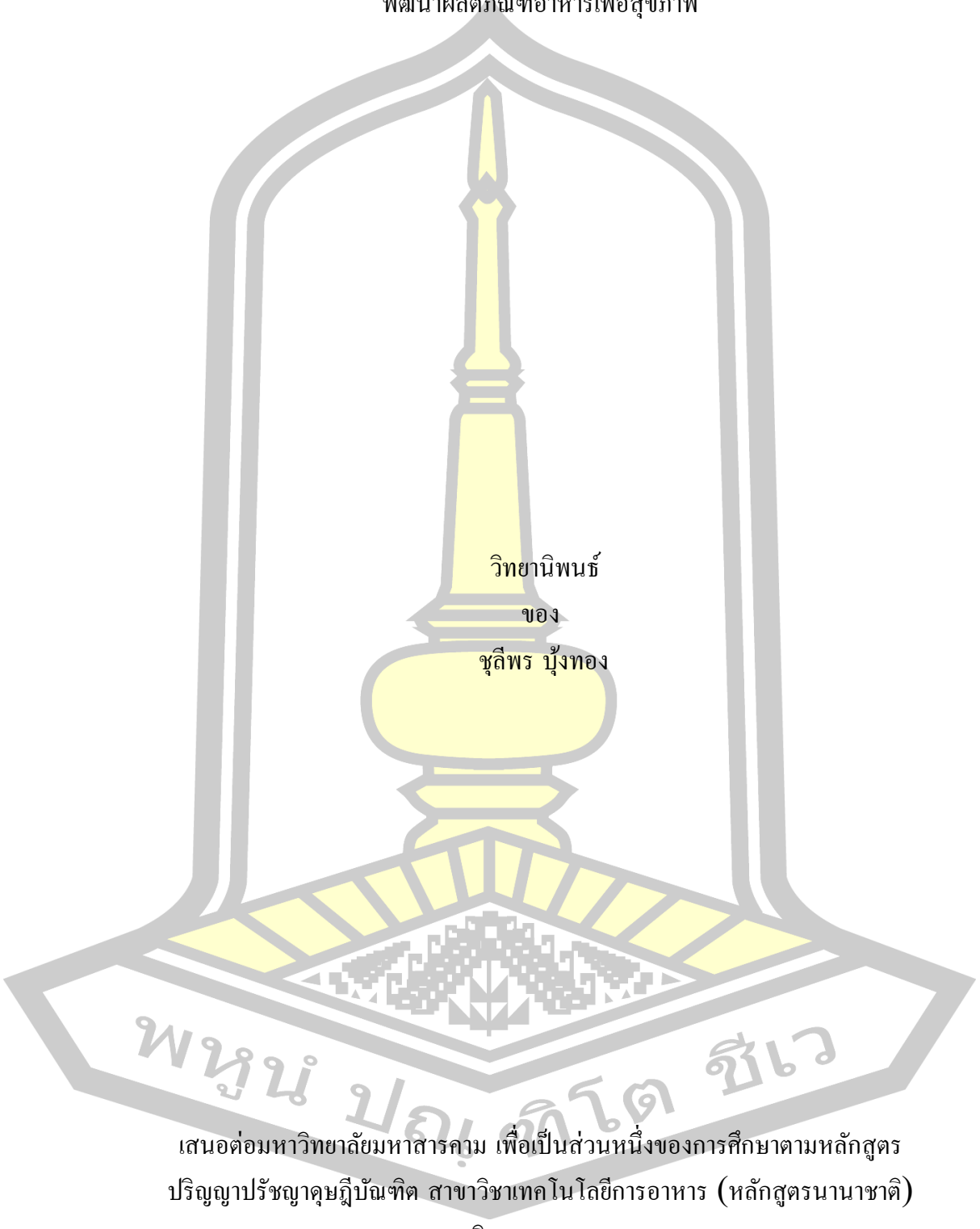
# Chemical and Biological Properties of Silk Protein and Application of Silk Protein for Functional Food Product Development

Chuleeporn Bungthong

A Thesis Submitted in Partial Fulfillment of Requirements for
degree of Doctor of Philosophy in Food Technology (International Program)
August 2020

# การศึกษาคุณสมบัติทางเคมีและฤทธิ์ทางชีวภาพของโปรตีนไหมและการนำไปใช้ประโยชน์ในการพัฒนาผลิตภัณฑ์อาหารเพื่อสุขภาพ



วิทยานิพนธ์

ของ

ชุลีพร ปุ้งทอง

เสนอต่อมหาวิทยาลัยมหาสารคาม เพื่อเป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีการอาหาร (หลักสูตรนานาชาติ)
สิงหาคม 2563

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**TITLE** Chemical and Biological Properties of Silk Protein and Application of Silk Protein for Functional Food Product Development

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**DEGREE** Doctor of Philosophy **MAJOR** Food Technology (International Program)

**UNIVERSITY** Mahasarakham University **YEAR** 2020

## ABSTRACT

Silk proteins have a number of advantageous substances including proteins and pigments. The proteins, sericin and fibroin, are widely studied for medical applications due to their good physiochemical properties and biological activities such as anti-oxidation, anti-inflammatory, biocompatibility, acceleration of cell proliferation, and activation of collagen synthesis. Recently, many studies have focused on the use of silk proteins for the production of drugs or cosmetics; however there has been little information on the development of food products from silk-cocoon proteins. Therefore, the objective of this study was to study an appropriate silk protein extraction method to obtain the chemical and biological properties of silk protein for development into functional food products. Four strains of silk cocoons were selected on the basis of the availability of strains in Northeastern Thailand, namely Leaungsaraburi, Nangsew, Nangtui and Eri. The extraction method of silk protein involved distilled water at 100°C for 2, 4, 6 and 8 hours and also with enzyme (Alcalase). The resulting extracts were evaluated for protein content, amino acids, total phenolic content (TPC), phenolic compounds, total flavonoid content (TFC), flavonoid compounds, DPPH radical-scavenging activity, ABTS radical scavenging capacity assay, FRAP assay, anti-glycation, α-amylase and α-glucosidase inhibitory activities. Furthermore, the molecular mass distribution was assessed by SDS-PAGE. The results showed that longer extraction times provided higher contents of all parameters using extraction times of 2-6 hours; however, these values started to decline after 6 hours of extraction. Interestingly, using enzyme extraction gave the highest values of all parameters for all cultivars studied. The SDS-PAGE results showed that silk protein molecules broke down after 4 hours of extraction. The molecular weights of protein were in range of 10-15, 45-65 and over 75 kDa whereas using the enzyme, the molecular weights of the proteins were between 25-35 and 35-45 kDa. In a toxicity assessment, the samples tested did not show toxicity to the cells studied in Cancer cells of the breast (MCF-7), lung (HCT116) and in normal cells that are not cancerous cells (Vero). However, the major concern of the product is its bitterness taste. Subjectively, we had tested the extract with someone from the company (Siam Natural Product, Co. Ltd). It was quite obvious that the water extract provided a better taste with less bitterness than the enzyme extract. More importantly, the operating cost to produce the enzyme extract is rather more expensive for

industry. Therefore, water extraction for 6 hrs was selected for further development of the possible silk food product. A silk drink product was developed using a mixture-design program with factors consisting of 90-100% silk protein extract (X1) and 0-5% honey (X2). The most acceptable formula consisted of 92.4% of silk protein extract and 7.6% of honey.

In conclusion, this research has demonstrated that the silk cocoon has proved to possess proteins with high bioactivities, extractable using water and enzyme. This potential of silk protein extracts can be developed into valuable functional foods and nutraceuticals.

Keyword : Silk, Silk protein extract, Biological Properties, Silk protein drink

# ACKNOWLEDGEMENTS

This dissertation was granted by the Thailand Research Fund (TRF) and Siam Natural Product, Co. Ltd.for support via a scholarship. First of all, I would like to thank Assoc. Prof. Dr. Sirithon Siriamornpun, Assist. Prof. Dr. Sudathip Inchuen, Assist. Prof. Dr. Srinual Jantathai, Assist. Dr. Wasan Duangkhamchan and Assoc. Prof. Dr. Natthida Weerapreeyakul, for recommendations led to improve this dissertation.

I was very fortunate to have many friends both within and outside the Faculty of Technology during my doctoral life. I thank them all for their being very supportive.

I am deeply indebted to the Department of Food Technology and Nutrition and laboratory equipment center, Mahasarakham University for providing access to the equipments and instruments.

Chuleeporn Bungthong

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# CHAPTER 1
# INTRODUCTION

## 1.1 Background

Thailand is well known for the traditional production of silk, which has been carried on for generations, making Thai silk a prestigious symbol of the country internationally. Silk is a natural protein fiber produced from silkworm (*Bombyx mori*) cocoons. It consists of 70-80% of a two bundled fibrous protein called fibroin and 20–30% of an amorphous matrix of a water-soluble globular protein called sericin which functions as a gum to bond the two fibroin filaments together. In the silk production industry, sericin must be removed and discarded as waste from the cocoon cooking and silk degumming processes. During the last decade, sericin has emerged as a valuable commercial resource in many industries, such as those making cosmetics and pharmaceuticals, and food, as well as in the production of many functional biomaterials (Zhaorigetu et al. 2001). Moreover, sericin has also been found to be useful as a degradable biomaterial, a biomedical material and for use as polymers for forming articles and functional membranes (Zhang 2002). Hence, recovery of sericin not only can reduce the environmental impact but also can generate revenue as a natural value-added material in silk production.

Sericin can be extracted from silk by detaching it from the fibroin part. Only fibroin part is demanded in the silk industry so removal of sericin is needed and is done by degumming process which later on is discarded in the effluent. Restoration of sericin from the degumming liquor can lighten the load in effluent, thus lowering the environmental impression and we get a biopolymer having untold profitable properties (Jajpura and Rangi 2015). Thai traditional extraction method use boiled silk cocoons with hot water at 80-100°C for sericin dissolved out of the cocoon, However it is time consuming. Therefore, and extraction method needs to be to obtain higher yield and better quality of sericin as well as its health promoting properties.

Therefore, the aim of the present study is to investigate the extraction methods on chemical and biological properties of silk protein. This research expects to obtain

high quality and quantity information of important compounds in processed silk protein, which would be used for development of a natural additive in functional food from silk protein extract with increasing value of by-product from processing and high potential for consumers.

## 1.2 Research Objectives

The objectives of study are:

1.2.1 To investigate the physical and chemical properties of silk cocoon from different strains.

1.2.2 To investigate the effects of extraction method on the physical, chemical and biological properties in silk protein and to optimize the extraction methods of silk protein extract.

1.2.3 To develop the process and product of functional food from silk protein extract.

## 1.3 Outcomes

1.3.1 Obtain the data of physical and chemical properties of silk cocoon.

1.3.2 Obtain an appropriate extraction method of silk protein extract.

1.3.3 Obtain the knowledge of effect of extraction method on chemical and biological properties in silk protein extract.

1.3.4 Application of silk protein extract to a function food product.

## 1.4 Hypothesis

1.4.1 Different silk strains could have different physical and chemical properties.

1.4.2 Different extraction methods could affect the chemical and biological properties of obtained silk protein.

1.4.3 Addition of silk protein extract in the product could increase health promoting properties.

**1.5 Scope of research**

1.5.1 Study the physical and chemical properties in silk from four Thai silk strains namely, 1) Leaungsaraburi 2) Nangsew 3) Nangtui (from Baan Hua Saban, Phutthaisong District, Buriram Province) and 4) Eri (from Nong Ya Plong Community, Mancha Khiri, Khon Kaen Province). The physical properties include color, weight, width and length of silk cocoon, the chemical properties include protein content.

1.5.2 Study the extraction method of silk protein by extracted with distilled water at 100°C for different time including are 2 4 6 and 8 hrs and extracted with enzyme.

1.5.3 Study the physical, chemical and biological properties of silk protein. The physical properties include color. The chemical properties include pH, moisture content, protein content, and amino acids. The biological properties which will be shown are TPC, TFC, DPPH, FRAP assay, ABTS radical scavenging capacity assay, anti-glycation, α-amylase and α-glucosidase inhibitory activities and molecular mass by SDS-PAGE.

1.5.4 Product development of silk protein extract and assessment of its stability for chemical and biological properties of the developed products.

**1.6 Definition of key words**

1.6.1 Silk : is a natural protein fiber, some forms of which can be woven into textiles. The protein fiber of silk is composed mainly of fibroin and is produced by certain insect larvae to form cocoons. The best-known silk is obtained from the

cocoons of the larvae of the mulberry silkworm *Bombyx mori* reared in captivity (sericulture).

1.6.2 Silk sericin : is a water-soluble globular protein that is easily soluble in hot or boiling water, highly hydrophilic and obtained as a by-product in the silk industry. Its variable amino acid composition and diverse functional groups confer upon it attractive bioactive properties, which are particularly interesting for biomedical applications. Because of its antioxidant character, moisturizing ability and mitogenic effect on mammalian cells, sericin is useful in cell culture and tissue engineering

1.6.3 Protein hydrolysates : are products derived from protein degradation by cutting polypeptide chains into free amino acids or peptides by using chemicals or enzymes to improve nutrition and some properties of proteins such as solubility emulsifiers and foam properties.

1.6.4 Proteases : also known as proteinases or peptidases hydrolyze the peptide bond between amino acid residues in a polypeptide chain. Proteases may be specific and limited to one or more sites within a protein, or they may be nonspecific, digesting proteins into individual amino acids. The ability to digest a protein at specific points is critical to mass spectrometry. Proteases are found in all organisms and are involved in all areas of metabolism.

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Silk cocoon

The silk cocoon is a natural protein fiber, some forms of which can be woven into textiles. The protein fiber of silk is composed mainly of fibroin which is produced by certain insect larvae to form cocoons. The best-know silk is obtained from the cocoons of the larvae of the mulberry silkworm *Bombyx mori* reared in captivity (sericulture). The shimmering appearance of silk is due to the triangular prism-like structure of the silk fiber, which allows silk cloth to refract incoming light at different angles, thus producing different colors (Sutherland et al. 2010).

There are four types of natural silk which are commercially known and produced in the world. Among them mulberry silk is the most important and contributes as much as 90% of world production. Therefore, the term “silk” in general refers to the silk of the mulberry silkworm. Three other commercially important types fall into the category of non-mulberry silks namely: Eri silk, Tasar silk and Muga silk. There are also other types of non-mulberry silk, which are mostly wild and exploited in Africa and Asia; they are Anaphe silk, Fagara silk, Coan silk, Mussel silk and Spider silk (Banday et al. 2018).

There are several types of silk commercially known and produced around the world. Some of the common types of silk include;

1) Mulberry silk

The bulk of the commercial silk produced around the world comes from this variety and often generally refers to mulberry silk. Mulberry silk comes from the silkworm, *Bombyx mori L.*, which solely feeds on the leaves of mulberry plants. These silkworms are completely domesticated and reared indoors. Mulberry silk contributes to around 90% of world silk production.

2) Non-Mulberry Silk

2.1) Eri silk

These belong to either of two species namely *Samia ricini* and *Philosamia ricini* (also called the castor silkworm) is a domesticated one reared on castor oil plant leaves to produce a white or brick-red silk popularly known as Eri silk. Since the filament of the cocoons spun by these worms is neither continuous nor uniform in thickness, the cocoons cannot be reeled and, therefore, the moths are allowed to emerge and the pierced cocoons are used for spinning to produce the Eri silk yarn.

2.2) Tasar Silk

Tasar silkworms belong to the genus Antheraea and they are all wild silkworms. There are many varieties such as the Chinese tasar silkworm *Antherae pernyi* Guerin, which produces the largest quantity of non-mulberry silk in the world. The Indian tasar silkworm *Antheraea mylitte* Dury, next in importance, and the Japanese tasar silkworm *Antheraea yamamai* Querin, which is peculiar to Japan and produces green silk thread. The Chinese and Japanese tasar worms feed on oak leaves and other allied species. The Indian tasar worm feeds on leaves of Terminalia and several other minor host plants. The worms are either uni- or bi-voltine and their cocoons, like the mulberry silkworm cocoons, can be reeled in as raw silk.

2.3) Muga silk

The muga silkworms (*Antheraea assamensis*) also belong to the same genus as tasar worms, but produce an unusual golden-yellow silk thread which is very attractive and strong. These are found only in the state of Assam, India and feed on *Persea bombycina* and *Litsaea monopetala* leaves and those of other species. The quantity of muga silk produced is quite small and is mostly used for the making of traditional dresses in the State of Assam (India) itself.

**2.2 Composition of silk cocoon**

The cocoon layer of silkworm *Bombyx mori* is mainly composed of silk fibroin and sericin (Fig. 2.1), which is a globular glue protein. When silk is spun, sericin covers two fibroin filaments, binding them together into silk fibers that ultimately

construct a cocoon where the silkworm progresses from larva to adult. Indeed, the cocoon built using sericin as an adhesive is the insect's home and protects against wind, rain, frost and sunlight. It also helps silkworm pupa development and metamorphosis. Generally, because of the different silkworm varieties, fibrous silk fibroin accounts for 65 to 85% of the cocoon layer, while the sericin layer accounts for 15 to 35%. Most of the commercial varieties of silkworm cocoons are composed of approximately 75% silk fibroin fiber and 25% sericin. In its natural formation, silk comprises natural impurities like fat, wax, inorganic salt and coloring matter. The composition of silk is given in Table 2.1.

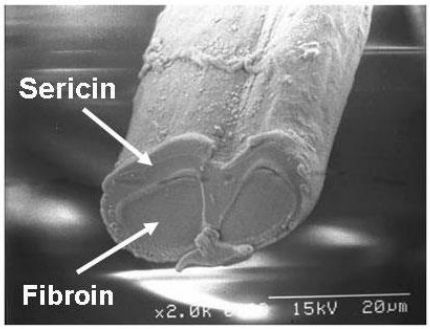



**Figure 2.1** Structure of the silk cocoon.
From: https://textiletoday.com.bd/wp-content/uploads/2015/02/31.jpg

**Table 2.1** Composition of the silk cocoon

| Component | (%) |
|---|---|
| Fibroin | 70-80 |
| Sericin | 20-30 |
| Carbohydrates | 1.2-1.6 |
| Inorganic matter | 0.7 |
| Wax matter | 0.4-0.8 |
| Pigment | 0.2 |
| Total | 100 |

From : ( Jajpura and Rangi, 2015)

1) Fibroin

Silk fibroin is the protein that forms the filament of silkworm and provides the unique physical and chemical properties. Silk adopts various secondary structures,

including α-helix, β-sheet, and crossed β-sheet (Rajput and Kumar Singh, 2015). Fibroin is a glycoprotein composed of two equimolar protein subunits of 370 and 25 kDa covalently linked by disulfide bonds. Fibroin filament is made of both crystalline and amorphous domains. The amorphous domains are characterized by the presence of amino acids with bulkier side chains, whereas the crystalline domains are characterized by a high percentage of alanine, glycine, and serine (12, 30, 44 per cent, respectively), which contain short side chains to permit close packing densities for overlying sheets. The β-sheet form (silk II or β-silk) and crystalline form (silk I) have been reported for silk fibroin, having relative molecular masses of 350–415K. Anti-parallel β-sheet structure forming microfibrils are responsible for the crystalline nature of the silk fiber. The microfibrils are orzganized into fibril bundles, with several bundles leading finally to a single silk thread.

Fibroin is a fibrous protein with a semi-crystalline structure that provides stiffness and strength. Silk fibroin consists of a heavy (H) chain (~390 kDa) and a light (L) chain (~26 kDa) linked together via a single disulfide bond at the C-terminus of the H-chain, forming an H–L complex. A glycoprotein P25 (~25 kDa) is also non-covalently linked to the H–L complex. The H-chain, L-chain, and P25 are assembled in a ratio of 6:6:1 to form silkworm silk (Hakimi et al. 2007).The amino acid composition of fibroin from *Bombyx mori* consists mainly of Gly, Ala, and Ser (Fig. 2.2).

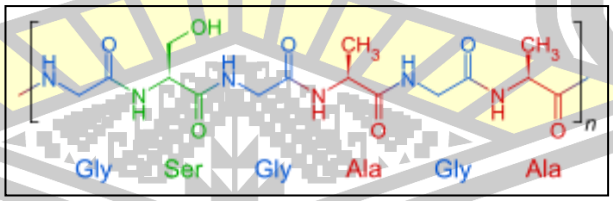



**Figure 2.2** Primary structure of fibroin.

From: https://commons.wikimedia.org/wiki/File:Silk_fibroin_primary_structure.svg

The hydrophobic domains of the H-chain contain a repetitive hexapeptide sequence of Gly-Ala-Gly-Ala-Gly-Ser and repeats of Gly-Ala/Ser/Tyr dipeptides, which can form stable anti-parallel β-sheet crystallites. The amino acid sequence of the L-chain is non-

repetitive, so the L-chain is more hydrophilic and relatively elastic (Vepari and Kaplan, 2007). The main crystal structures of silkworm fibroin are silk I and silk II. The little and unstable silk III structure also exists in regenerated SF solution at the air/water interface. Silk I is a metastable structure with a crank or S zigzag structure spatial conformation, belonging to the orthorhombic system. Silk II is an anti-parallel β-sheet structure, belonging to the monoclinic system. Strong hydrogen bonds between adjacent segments contribute greatly to the rigidity and tensile strength of fibroin. The silk I structure can be easily converted to silk II via methanol or potassium phosphate treatment (Fig. 2.3).

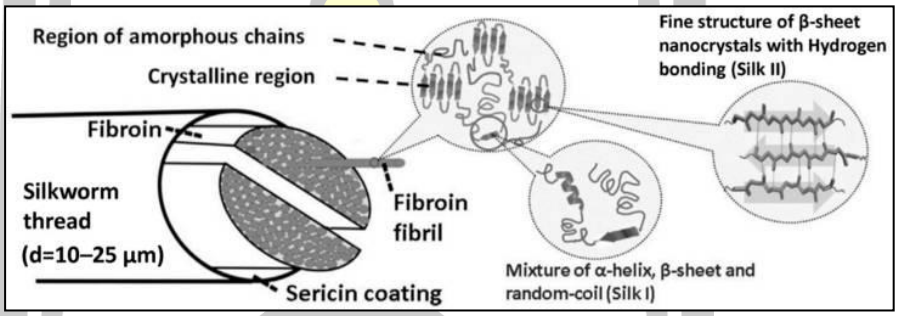



**Figure 2.3** Schematic presentation of the fibroin structure.
From : (Volkov, Ferreira, and Cavaco-Paulo 2015)

2) Sericin

Sericin is a water-soluble globular protein that is easily soluble in hot or boiling water, highly hydrophilic, with adhesive characteristics such as gelatin. This protein allows the adhesion of silk filaments to maintain the structural integrity of the cocoon during its formation (Volkov, Ferreira, and Cavaco-Paulo 2015). Furthermore, sericin contains 18 amino acids where serine (32%), aspartic acid (18%) and glycine (16%) are the more significant compounds. Additionally, this protein is composed of 45.8% of hydroxy amino acids (serine and threonine), 42.3% of polar amino acid and 12.2% of non-polar amino acids ( Fig. 2.4) . In order to produce biodegradable materials, sericin can be copolymerized and blended along with other polymers. This is possible as the sericin possesses a strong polar side chains such as hydroxyl, carboxyl and amino that allow an easy crosslinking (Zhang 2002).

The molecular weight of sericin depends on its extraction method. For instance, there is a range of 40-400 kDa when this is recovered from cocoons and a range of 80-310 kDa when it is extracted directly from the gland worm (Barajas-Gamboa et al. 2016b). These ranges will depend on their extraction conditions as the reactive type (acidic, alkaline, enzymes) and other factors such as temperature, pressure, pH and the processing time.

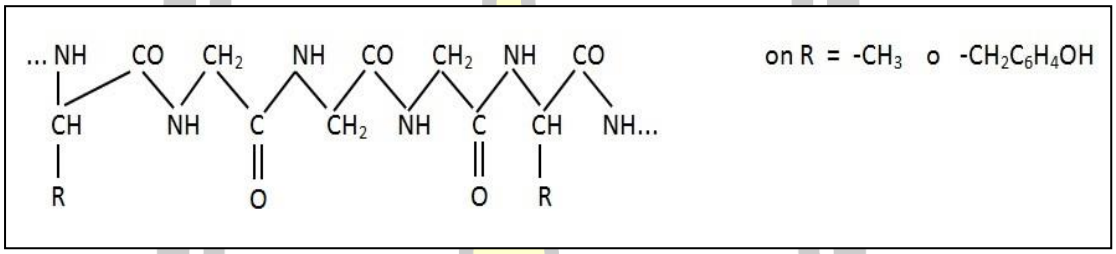



**Figure 2.4** Structure of sericin
From : https://en.wikipedia.org/wiki/Sericin

Chemically, fibroin and sericin together have amino acids in disparate balance. Table 2.2 tabulates the different amino acid percentages in fibroin and sericin (Aramwit et al. 2010). Fibroin is rich in glycine and alanine acids, whereas sericin is rich in serine and aspartic acids. By reason of dissimilarity in the percentage of amino acids, both proteins put on view different features.

**Table 2.2** Amino acid composition of fibroin and sericin.

| Symbol | Amino acid | Fibroin | Sericin |
|---|---|---|---|
| Gly | Glycine | 4 | 14 |
| Ala | Alanine | 29 | 5 |
| Ser | Serine | 12 | 33 |
| Tyr | Tyrosine | 5 | 3 |
| Val | Valine | 2 | 3 |
| Asp | Aspartic acid | 1 | 15 |
| Arg | Arginine | 1 | 3 |
| Glu | Glutamic acid | 1 | 8 |
| Ile | Isoleucine | 1 | 1 |
| Leu | Leucine | 1 | 1 |
| Phe | Phenylalanine | 1 | 1 |
| Thr | Threonine | 1 | 8 |
| Cys | Cystine | 0 | 0 |
| His | Histidine | 0 | 1 |
| Lys | Lysine | 0 | 4 |
| Met | Methionine | 0 | 0 |
| Pro | Proline | 0 | 1 |
| Trp | Tryptophan | 0 | 0 |

Values are presented in molar percent

## 2.3 Extraction method for silk sericin

Silkworm is the only source generating silk sericin; hence silk sericin is attained from cocoons, silk fabric, and silk waste or from the degumming liquor of silk industry. Across the world overall yearly cocoon production is around 400,000 metric tons and so from the degumming process only, 50,000 metric tons of silk sericin is junked in effluent every year. Nowadays most of the silk sericin is retrieved from silk degumming process. Large amount of silk sericin can be attained from cocoon waste or silk waste as compared to silk degumming liquor. However very less consideration was given to it, but now silk sericin is becoming the centre of attraction to researchers. (Vaithanomsat and Punyasawon 2008).

Silk sericin can be extracted from silk by detaching it from the fibroin part. Only fibroin part is demanded in the silk industry so removal of silk sericin is needed and is done by degumming process which later on is discarded in the effluent. Restoration of silk sericin from the degumming liquor can lighten the load in effluent, thus lowering the environmental impression and we get a biopolymer having untold profitable properties. Several methods based on heat treatment are currently available for the extraction of silk sericin from silk cocoons :

2.2.1 The conventional soap-alkaline degumming process involves chemicals whereby the cocoons are boiled at atmospheric pressure in a solution of sodium bicarbonate ($Na_2CO_3$) and Marseille soap, resulting in the complete removal of silk sericin (Yun et al. 2013). While this method is suitable for obtaining relatively clean and isolated fibroin for further application, it is very difficult to recover and separate silk sericin from the soap (Oh et al. 2011). Because Marseille soap (made from olive oil) is very expensive (Mahmoodi et al. 2010), other alternatives, such as degumming with heat and alkali only, are also applied to separate SS from fibroin on an industrial scale. Indeed, $Na_2CO_3$, urea and ureamercaptoethanol as well as sodium chloride, may all be used (Dash et al., 2007 ; Yun et al., 2013). However, even these processes make it difficult to recover high quality SS for further studies or applications, owing to the purification steps needed to remove the chemical impurities (Aramwit, Siritientong, and Srichana 2012). Enzymatic procedures and degumming in acidic solutions have

also been developed, but these methods are likewise limited in application (Mahmoodi et al. 2010).

2.2.2 Degumming by boiling in water under ambient or increased pressure has the advantage of introducing no impurities (Aramwit, Siritientong, and Srichana 2012). Therefore, simple hot water treatment is the method of choice for further applications of silk sericin and has been applied to extract silk sericin from *B. mori* cocoons (Chlapanidas et al. 2013). In a recent study, silk sericin was also successfully and completely removed from raw silk by heating the silk to 110°C in a water bath inside an infrared dyeing machine. This approach yielded a higher content of protein with better quality as compared with silk sericin obtained by extraction in an autoclave at 120°C (Gupta et al. 2013). The higher yield obtained with infrared heating was attributed mainly to the fact that the process consisted of radiation heating, where the energy was transferred directly to the material in the form of electromagnetic waves. The radiation caused the water to behave like an abrading agent, easing the detachment of silk sericin from the silk in addition to increasing its solubility in water via heat. This could also explain the lower degree of protein degradation observed in this method. Fluorescence spectroscopy measurements showed that using infrared extraction, denaturation and degradation of the silk sericin molecules was less compared with those resulting from conventional high pressure and temperature autoclave extraction. While the process of recovering silk sericin from *B. mori* cocoons seems to be relatively well-established, the extraction of silk sericin from wild silkworm (*A. mylitta*) cocoons has not yet been fully optimized (Yun et al. 2013). Till date, the most popular degumming method for *A. mylitta* cocoons is extraction by boiling in $Na_2CO_3$ solutions (Kundu & Kundu, 2012 ; Mandal et al., 2011 ; Nayak et al., 2012). In a recent study, Yun et al., ( 2013) concluded that this $Na_2CO_3$ extraction method was the most suitable for the extraction of SS from *A. mylitta* silk sericin cocoons.

2.2.3 Degumming by chemical properties and antityrosinase activities of silk sericin extracted from various methods. For extraction with high temperature and high pressure, cocoons of *B. mori* were autoclaved at 120°C and 15 $lbf/in^2$ for 60 min. For the preparation by acid- and alkali-degraded was added to the cocoons 1.25% citric acid solution or 0.5% sodium carbonate solution, respectively, and boiled for 30 min.

Cocoons were soaked into aqueous 8 M urea solution for 30 min and then refluxed at 85°C for 30 min for degumming with urea solution. The silk sericin extracted with high temperature and pressure and by urea solution had higher yields compared with the other methods. Moreover, the silk sericin extracted by urea solution appeared to give the most clearly distinguishable protein bands in SDS-PAGE. When obtained with high temperature and high pressure, the silk sericin shows an endothermic degradation at 220°C, higher temperature than those obtained from other methods (210°C), implying that the use of chemicals during the extraction process influences the thermal stability of silk sericin. Other tests results revealed that the extraction process of silk sericin could affect the chemical structure of protein and change the amino acid composition. Among the four methods used in this study, extraction with urea had the most significant impact on silk sericin conformation. Still, regarding the inhibition of tyrosinase, urea-extracted silk sericin showed the highest antityrosinase activity, while the alkali-degumming has a minor. (Aramwit et al. 2010).

2.2.4 The use of enzyme to extract silk sericin from cocoon can be attributed to the discovery of cocoonase, a group of proteinases, which can attack the silk sericin bindings. The enzymes trypsin, papain, and bacterial enzymes were the main types used for the degumming process (Devi 2012). Trypsin, a proteolytic enzyme, hydrolyses the peptide bond between the carboxyl group of lysine or of arginine and amino groups of adjacent amino acids. Since silk sericin has a relatively high lysine and arginine content, it is easily hydrolysed by trypsin. Papain exhibits a wide specificity in its action towards polypeptides and is an effective cocoon degumming enzyme. Alkalase, a bacterial enzyme, and many other fungal protease enzymes have been standardized and found to be economically viable without chemical hazards.

After extraction silk sericin is reformed to powder form. In different studies researchers have tried various processes like membrane filtration, ethanol precipitation freeze drying/tray drying and spray drying to isolate silk sericin from the degumming solution. The membrane process is a technique that concentrates the amount of silk sericin in liquor without the use of heat. The advantage of the spray drying process over others is that it includes minimal possibility of degradation of proteinous molecules which are sensitive to heat. Dried powder of silk sericin from solution is obtained very rapidly in one-step.

## 2.4 Characteristics of silk sericin

The structure and molecular weight affects functional properties of silk sericin. The chemical structure and molecular weight of silk sericin mainly depends on two factors: method of separation of silk sericin and fibroin and method of recovering silk sericin from degumming liquor and as mentioned earlier the addition of chemicals or exposure to heat (high temperature) causes the degradation of silk sericin polypeptides during degumming and its recovery process, so methods are chosen very carefully to get maximum benefits from silk sericin. Characteristics of silk sericin are next given to have a better understanding about this biopolymer.

2.4.1 Various contents

Different characteristics of silk sericin like moisture content (Aramwit and Sangcakul, 2007) nitrogen content, ash content, color (Kim et al. 2009) and solubility are reported in literature and are given in Table 2.3

**Table 2.3** Various characteristics of silk sericin.

| Parameters | (%) |
|---|---|
| Moisture content | 10-17 |
| Nitrogen Content | 9-16 |
| Ash content | 0.8-6 |
| Color | Cream to light yellow |
| Solubility | soluble in hot water |

2.4.2 Morphology

The surface morphology of the spray dried silk sericin powder to be like wrinkled particles which can be due to the collapse of hollow spherical structures when rapidly evaporated. Silk sericin by virtue of its hydrophilic nature gets agglomerated by immediate picking of moisture when exposed to atmosphere (Kurioka, Kurioka, and Yamazaki 2004).

2.4.3 Molecular weight

Silk sericin extracted by different methods has different molecular weights. It has been reported that molecular weight of silk sericin analysed using non reducing SDS-PAGE analysis lies in the range of 90-125 kDa. Silk sericin extracted without any heat treatments show three distinct bands. Heat treatments attack on the peptides linkage and the samples showed broader bands due to mixture of different molecular weight peptides (Teramoto and Miyazawa, 2005).

2.4.4 Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical which absorbs at 517 nm and is normally used to study the radical scavenging effect. As antioxidants donate protons to this radical, the absorption decreases. Antioxidant activity of silk sericin is assayed by calculating the decrease in absorption. In another research it has been found that silk sericin completely inhibits lipid peroxidation showing silk sericin have antioxidant activity.

**2.5 Applications of silk sericin**

2.5.1 Cosmetic applications

Silk sericin is properties such as biocompatibility, biodegradability and wettability allow the development of cosmetic products for skin, nails and hair. Moisturizers have had a special development; they are mainly used to prevent and delay the dehydration of the top layer of the skin. This condition occurs when the water of the stratum corneum (outermost layer of the epidermis) is lost faster of what it receives from the inner layer, and also can be possible due to a reduction of lipids of the stratum corneum. The dehydration can be evidenced when skin is brittle and rough, although water is being constantly supply from inside the body (Barel, A.D., Paye, M., and Maibach 2001). Normal and healthy skin has a wet, clean, soft, flexible, malleable, and practically wrinkle-free look. The smoothness of the skin is determined by its content of water, which should be at least 10% to keep this condition. When water content lowers this level, keratin, epidermis major component, becomes less flexible. Besides keratin, the corneum stratum has a special humectant mixture known as Natural Moisturizing Factor (Padamwar and Pawar 2004). This

factor is defined as a group of hydrosoluble and/or hydrodispersible molecules, which are present in the intercellular spaces of the stratum. They also can be found at the skin free surface, as a result of the physiological processes that occur at skin level. Wide varieties of moisturizers are available in the market. They contain wetting agents such as vegetable glycerine, water, jojoba oil, vitamin E oil and sorbitol among other products. The properties of silk sericin have been evaluated in different cosmetic formulations, such as creams and lotions. It has been found that a powder mixture of silk fibroin (70-95%) and silk sericin (5-30%) has antistatic characteristics and capacity to absorb moisture and both detection and UV filtration are enhanced. Other cosmetic applications include silk sericin powder and other products that absorb sweat and grease secreted from the sebaceous glands of the skin.

2.5.2 Medical applications

Silk sericin has both antioxidant and anticoagulant properties (Kundu et al.2008). These characteristics have led to the development of multiple investigations in order to apply these in the medical field. Some examples include applications in anticarcinogenic and healing products, and in tissue engineering.

- Anticarcinogenic

The antioxidant effect of silk sericin can represent a significant health benefit. Studies have shown that this protein allows a reduction in the oxidative stress in the human organs such as the colon; as well as a reduction in the number of cancer cells. Studies have shown that, the silk sericin that is taken orally (by mouth) by rats and mice, helps to effectively suppress the 1,2-dimethylhydrazine agent. As this agent is a cancer growth promoter, a reduction of the incidence of colorectal cancer was observed when this agent was. Additionally, it has been reported that silk sericin inhibits the growth of cloned tumor cells and activates the apoptosis factor, leading to an apoptosis of cancer cells in rats. Furthermore, silk sericin that is not digested by the colon, it has a strong antioxidant effect, which reduces the oxidative stress and colon tumorigenesis (Zhaorigetu et al., 2001 Kaewkorn et al. 2012).

- Healing

Silk sericin has good hydrophilic properties, it is also biocompatible and biodegradable, it actives the collagen production in wounds, and induces

epithelialization. It is also reported that silk sericin promotes both attachment and proliferation of fibroblasts and keratinocytes in the human skin.

- Bandage

Development of new wound dressing materials have been possible due to healing properties of silk sericin. Clinical evaluation of a silk sericin/ PVA scaffold, which was used in patients with skin grafts, was evaluated. Results showed an accelerated healing and patient pain reduction, compared to wounds that were treated with the commercial bandage Bactigras® (Siritientong et al. 2014).

- Tissue engineering

Materials that can be used in drug delivery, grafts and immobilizing matrices such as matrices in 2D (films) and 3D (scaffold) are one of the mail goals of biomedical research. Films and scaffold have been successfully made using a mixture of gelatine and silk sericin extracted from silkworm *Antheraea mylitta*. Fabricated supports have evenly distributed pores, good compressive strength and high swellability. In addition, they show high porosity, low immunogenicity and improvement in both cell attachment and viability. These properties are critical for tissue engineering and biomedical applications, which reveals the potential use of silk sericin in future development of bio-polymeric grafts (Mandal, Priya, and Kundu 2009).

2.5.3 Food applications

Today, FDA has included silk sericin and its derivatives in the “Generally Recognized as Safe - GRAS” list (Food and Drug Administration 2001b). The main characteristic of this protein is its antioxidant function; therefore, it has been proposed a functional food. However, commercial foods that contain this protein or any related products are still not available. Oxygen is responsible for the production of free radicals in the organism. This as a consequence of physiologic processes that are involved in the correct functioning of the human body, including breathing and reactions at cell level. For this reason, the biological production of antioxidant compounds is needed, in order to balance the concentration of the free radicals. Naturally, the body produces its own antioxidants, called endogenous (Samaranayaka, and Li-Chan 2011). Free radicals are produced inside the body due not only by physiological processes, but also by external factors such as environmental

contamination and smoking. This last situation leads to the overproduction of these radicals, which cannot be stabilised by the endogenous antioxidant. As a solution, it is suggested the intake of exogenous antioxidants in a daily basis, which help to avoid the oxidative reactions increase. These reactions are related to human illness such as cancer, rheumatoid arthritis, and diabetes and also to neurodegenerative diseases such as Alzheimer and arteriosclerosis. Additionally, there are evidences that show some beneficial effects of silk sericin in stabilizing free- radicals such as hydroxyls, super oxides, DPPH and ABTS (Chlapanidas et al., 2013 ; Dash et al., 2007). Furthermore, silk sericin has antioxidant effect during of the linoleic acid peroxidation and in vitro lipid peroxidation. Beneficial effects of silk sericin have been linked to its proteinic characteristic and other substances (flavonoids, katekins, kercitinas, epicatequins and carotenoids) present in this protein after the extraction process. Additionally, silk sericin can suppress peroxidation of lipids and inhibit tyrosinase enzyme activity. This enzyme catalyzes the tyrosinase oxidation, which is the amino acid responsible for the skin melanin biosynthesis, and the enzymatic browning in foods. In addition, there are evidences of favorable effects in the intestinal health of rodents, as silk sericin helps to modular both fermentation and barrier processes. Due to these properties, silk sericin could be used in developing fortified foods and nutritional supplements. Silk sericin has a high content of bioactive peptides that are specific fragments of proteins. Their amino acid sequence is directly related to the beneficial effects on corporal functions, specifically on systems such as cardiovascular, nervous, gastrointestinal and immune (Samaranayaka, and Li-Chan, 2011). However, peptides of silk sericin have been also studied with the aim to improve both, the antioxidant and inhibitor tyrosinase activity, related to an increased intestinal absorption as consequence of the size protein reduction.

**2.6 Protein Hydrolysates**

Protein hydrolysates are products derived from protein degradation by cutting polypeptide chains into free amino acids or peptides by using chemicals or enzymes to improve nutrition and some properties of proteins such as solubility emulsifiers and foam properties (Kristinsson and Rasco 2000).

2.6.1 Protein digestion with acid solution

Protein digestion with acid solution is a relatively low cost method. Protein digestion is fast and provides good flavor, but tryptophan which is the essential amino acid is destroyed. In addition, protein digestion by using high concentration of hydrochloric acid in high temperature causes to 3-monochlorapropane-1, 2-diol (3-MCPD), which is toxic to the liver, kidney, thyroid gland and mucous membrane of the mouth and tongue, it also causes a carcinogen. The level of digestibility is difficult to control, resulting the product quality is not stable and also the smell of acid residue solution in solution. The acid solution is commonly used in protein digestion in foods such as HCl and $H_2SO_4$.

2.6.2 Protein digestion with alkaline solution

Protein digestion with alkaline solution if digested in severe conditions causes the racemization reaction of amino acids, which will change the structure from L-form to D-form and the human body can not utilize resulting bad flavor and loss of important nutrients. In addition causes the β-elimination of serine and cysteine by compounding dehydroalanine,which can react with other amino acids to form various compounds such as lysinoalanine, ornithioalanine and lathionine. This causes loss of important nutrients and some of these compounds cause toxicity in the food. Alkaline solutions used in protein digestion include NaOH, KOH and $Ba(HO)_2$

2.6.3 Protein digestion with enzyme

Protein digestion in this method can be use protease cut the peptide bond of the protein molecule into a short peptide and free amino acids. This digestion has the advantage that the enzyme has a specificity for the high substrate and the conditions is not severe. Therefore, the structure of amino acids will not be destroyed. Enzyme digestion is relatively high compared to chemical methods but will cause bitter compounds due to the hydrophobic group of protein molecules. The enzymes (Trade name) used in protein digestion Flavourzyme®, Alcalase® and Neutrase®.

In the production of protein hydrolysates use protease enzymes which are enzymes in the hydrolysis group, catalyzes the degradation of the peptide bond of a protein into a peptide and free amino acids. can be divided into different types of proteases. The grouping of proteases is divided into several categories

A) Peptide bonding is divided into two groups:

- Endopeptidase is an enzyme that decomposes a peptide bond independently within a protein molecule to a short peptide. These enzymes are made from plants or microorganisms that are highly effective in biodegradation because of their specificity to many protein substrates and molecules of peptides results in rapid degradation.

- Exopeptidase is an enzyme that breaks down the peptide bond on the molecular ends. The bonding end of the free amino group is called the amino-peptidase while the bonding end of the carboxyl group is called carboxylidase.

B) Mechanism is divided into four groups:

- Serine proteases are alkali proteases have optimal of pH in range 7-11, have imidazole and serine at the active site and restrained by disopropyl-phospho-fluoride (PDF). Examples of these enzymes are trypsin, elastase and thrombin.

- Sulfhydryl proteases are neutral proteases have optimal of pH in range 6-7.5, have sulfhydryl at the active site. These enzymes are extracted from high plant such as papain from papaya and bromelain from pineapple.

- Metal-containing proteases are proteolytic enzymes contains ion and metal in the enzyme molecule or in the degradation reaction have good reaction in the neutral of pH (6.6-7.5), which is classified as a neutral protease and inhibited with metal chelating agents such as EDTA and 1, 10-phenanthroline. Examples of this enzyme are carboxypeptidase A, cytosol aminopeptidase, prolidase and carnosinase.

- Acid proteases are proteases with a pH range of degradation reactions in the pH range of 2-4 and do not show the amino acids at the active site. There are more than one carboxyl group from aspartic acid at the active site. Examples of these enzymes are rennin and pepsin (Belitz, Grosch and Schieberle 2009)

In addition, the industry uses a variety of commercial enzymes for protein digestion. Most of these enzymes are derived from microorganisms, each of which has different enzymes and conditions for different types of protein digestion.

Flavourzyme® is an enzyme derived from *Aspergillus oryzae* microorganisms. Both the endopeptidase and the exopeptidase, which are less time to digest. Protein hydrolysis has no bitter taste and the optimum temperature is 50 °C and the optimum pH is 5.0-7.0.

Alcalase® is an enzyme derived from *Bacillus licheniformis*. The endopeptidase function has the optimum temperature in the range of 55-60°C and the optimum pH in the range of 8.0-8.5.

Neutrase® is an enzyme derived from *Bacillus licheniformis* or *Bacillus amyloliquefaciens* were endopeptidase with suitable temperature in the range of 45-55°C and pH at 5.5-7.0.

# CHAPTER 3

# Materials and Methods

This research is experimental research was determined and analyzed physical, chemical and biological properties in silk protein as well as evaluate their antioxidant activities with determination of protein. In addition, determination of protein content, amino acid and biological properties of silk protein from different strains and extraction method are investigated. Finally, Product development of silk protein extract and assessment of its stability for chemical and biological properties of the developed products. Therefore, experimental design is followed by:

3.1 Experimental plans
3.2 Instruments and equipment
3.3 Materials
3.4 Chemicals
3.5 Methods
3.6 Statistical analysis

## 3.1 Experimental plans

This research was divided into four experiments including

3.1.1 Experiment I : Study physical and chemical properties of silk cocoons. Four Thai silk strains namely, 1) Leaungsaraburi 2) Nangsew and 3) Nangtui (from Baan Hua Saban, Phutthaisong District, Buriram Province) and 4) Eri (from Nong Ya Plong Community, Mancha Khiri, Khon Kaen Province) was investigated.

- The physical properties including color, weight, width and length of silk cocoon was studied.

- The chemical properties including moisture content and protein content of silk cocoon was studied.

3.1.2 Experiment II : Study the extraction method of silk protein.

(1) Water extraction : Extraction temperature at 100°C for 2 4 6 and 8 hrs.

( 2) Enzymatic extraction : Protease ( Enzyme Alcalase® from Novozymes) was used for enzymatic extraction.

3.1.3 Experiment III : Study the physical, chemical and biological properties of silk protein extract. The parameters of these properties was determined as follows :

(1) The physical properties : color, turbidity.

(2) The chemical properties : moisture content, pH, protein content and amino acids composition.

(3) The biological properties : TPC, TFC, DPPH, FRAP assay, ABTS radical scavenging capacity assay, anti-glycation and α-amylase and α-glucosidase inhibitory activities and molecular mass (SDS-PAGE).

3.1.4 Experiment IV : Development of functional product from silk protein extract.

(1) Product development of silk protein extract and assessment of its stability for chemical and biological properties of the developed products.

(2) Sensory evaluations of silk protein product prototype was conducted by 30 panelists using a nine-point hedonic scale where nine is like extremely and one dislike extremely.

**3.2 Instruments and equipment**

3.2.1 High performance liquid chromatography system with diode array detector (HPLC 20A, Shimadzu)

3.2.2 Gel electrophoresis

3.2.3 Ultraviolet-Visible spectrophotometer (Lambda 12, Perkin Elmer, USA)

3.2.4 Centrifuge (Rotina 48 R)

3.2.5 pH meter

3.2.6 Rotary evaporator (Buchi)

3.2.7 Freeze dry

3.2.8 Hot air oven (Memmert)

3.2.9 Incubator shaker

3.2.10 Colorimeter

3.2.11 Ultrasonicator

3.2.12 Beaker

3.2.13 Erlenmeyer flask

3.2.14 Volumetric flask

3.2.15 Pipette

3.2.16 Vial

3.2.17 Micro pipette

3.2.18 Test tubes

3.2.19 Water bath

3.2.20 Whatman filter paper No. 1

**3.3 Materials**

Silk from 4 Thai silk strains are 1) Leaungsaraburi 2) Nangsew 3) Nangtui (from Baan Hua Saban, Phutthaisong District, Buriram Province) and 4) Eri (from Nong Ya Plong Community, Mancha Khiri, Khon Kaen Province).

**3.4 Chemicals**

3.4.1 Enzyme Alcalase® from Novozymes

3.4.2 Bradford solution

3.4.3 Bovine Serum Albumin (BSA)

3.4.4 2,2-Diphenyl-1-picrylhydrazyl , DPPH (Fluka)

3.4.5 2,4,6-Tripiridyl-s-triazine, TPTZ (Fluka)

3.4.6 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS (Fluka)

3.4.7 Folin-Ciocalteu's reagent (Fluka)

3.4.8 Sodium nitrite (Merck)

3.4.9 Hydrochloric Acid (Merck)

3.4.10 Sodium carbonate (Merck)

3.4.11 Sodium sulphate (Merck)

3.4.12 Aluminium chloride (Merck)

3.4.13 Sodium hydroxide (Merck)

3.4.14 Acetate buffer (Merck)

3.4.15 Methanol (Merck)

3.4.16 Acetic acid (Fisher Scientific)

3.4.17 Potassium persulfate (Merck)

3.4.18 Maleate buffer (Sigma)

3.4.19 Acarbose (Sigma)

3.4.20 Potato starch

3.4.21 Sodium phosphate buffer (Sigma)

3.4.22 Dimethyl sulfoxide (DMSO) (Sigma)

3.4.25 Enzyme entry: EC 3.2.1.1 (Sigma)

3.4.23 3,5-Dinitrosalicylic acid (Sigma)

3.4.24 Ethanol (Merck)

3.4.25 Sodium chloride (Fluka)

3.4.26 Potassium hydroxide (Fluka)

3.4.27 Hexane (Merck)

3.4.28 Boric acid (Sigma)

3.4.29 Methyl red (Sigma)

3.4.30 Bromocresol green (Sigma)

**3.5 Methods**

The details of methods which was used throughout the study are provided as below:

3.5.1 Determination of the physical and chemical properties four Thai silk strains as below:

3.5.1.1 The physical properties

(1) Color measurement in silk cocoon by Colorimeter

Silk cocoon to color measure and shows the color values in

terms of Lightness (L*), redness (a*), and yellowness (b*) by randomly measuring 3 points. Samples was measured in 3 replicates.

(2) Weight, width and length measurement in silk cocoon

Silk cocoon to weight measure by weight machine and to width and length measure by ruler with scale. Samples was measured in 3 replicates.

3.5.1.2 The chemical properties

(1) Moisture content (AOAC 2000)

Dry the empty dish and lid in the oven at 105°C for 3 hrs and transfer to desiccator to cool. Weight the empty and lid. Weight 3 g of sample to dish. Spread the sample to the uniformity. Place the dish with sample in the oven. Dry for 3 h at 105°C. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweight the dish and its dried sample. The moisture content (%) in the following equation:

$$\text{moisture content (\%)} = \frac{(W_1 - W_2)}{W_1} \times 100$$

$W_1$ = weight (g) of sample before drying, $W_2$ = weight (g) of sample after drying

(2) Protein content (AOAC 2000)

Place sample (0.5-1.0 g) in digestion flask. Add 5 g Kjedahl catalyst and 200 ml of conc. $H_2SO_4$. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until the solution becomes clear. Cool and add 60 ml of distilled water cautiously. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 drops of indicator in receiver. Rotate flask to mix content thoroughly, then heat until all $NH_3$ is distilled. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

$$\text{Protein (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W}$$

A= volume (ml) of 0.2 N HCl used sample titration

B= volume (ml) of 0.2 N HCl used in blank titration

N= Normality of HCl

W= weight (g) of sample

1.4007 = atomic weight of nitrogen

6.25 = the protein-nitrogen conversation factor

3.5.2 Determination of physical ,chemical and biological properties of silk protein as below:

3.5.2.1 Extraction method

(1) Extracted with distilled water (Sangwong, Sumida, and Sutthikhum 2016)

Silk cocoon was cut to small pieces. 2 g of silk cocoons was extracted with 200 mL of distilled water treated in 100ºC distilled water for 2 4 6 and 8 hrs. The aqueous solution obtained was collected, and centrifuged (10 min, 5,000×g). The extracts was filtrated through Whatman No. 1 paper under vacuum to remove insoluble material, which is fibroin. After this filtering process to obtain silk protein extract.

(2) Extracted with protease (Vaithanomsat and Punyasawon, 2008)

Silk cocoon was cut to small pieces and soaked in distilled water for 24 h at 10°C, separated and added the distilled water 1:100 (w/v). Enzyme Alcalase® from Novozymes 0.5% (v/v) was added and adjusted pH to 8.0 incubated at 50°C for 120 min . The enzymatic reaction was stopped by heating at 100°C for 20 min and centrifuged (15 min, 6,000×g). The extracts was filtrated through Whatman No. 1 paper under vacuum. After this filtering process to obtain silk protein extract.

3.5.2.2 The physical properties of silk protein extract.

Color measurement in silk protein by Colorimeter

Silk protein to color measure and shows the color values in

terms of Lightness (L*), redness (a*), and yellowness (b*) by randomly measuring 3 points. Samples was measured in 3 replicates.

3.5.2.3 The chemical properties of silk protein extract.

(1) pH measurement by pH meter

(2) Protein determination by Bradford assay (Bradford, 1976)

Silk protein was diluted with distilled water. Sample (0.5 ml) was added to 1.0 ml of Bradford solution and incubated at room temperature for 5 min. Bovine Serum Albumin (BSA) was used as a standard reference protein. The absorbance of samples was measured at 595 nm with visible spectrophotometer.

(3) Amino acid analysis

The extraction of amino acids in this study was performed according to Liyanaarachchi, Mahanama, Somasiri and Punyasiri (2018). Amino-acid analysis involved an LC–MS-MS (Shimadzu LCMS-8030) triple-quadrupole mass spectrometer, in electrospray ionization (ESI) mode, followed by a Shimadzu HPLC (Shimadzu, Kyoto, Japan) (Chumroenphat, Somboonwatthanakul, Saensouk & Siriamornpun 2019), with some modifications. The conditions were: 0.2 ml/min was the flow rate, the temperatures of autosampler and column oven were set at 4 °C and 38 °C, respectively. Mobile phases used were: (A) demineralized ion (DI) water: formic acid 0.1%, and (B) methanol 50% in DI water: formic acid 0.1% (v/v). The analyses were performed in triplicate.

3.5.2.4 The biological properties of silk protein extract (SPE) .

(1) Determination of total phenol content

(1.1) Total phenolic content (TPC)

Total phenolics in silk protein was analyzed following the Folin-Ciocalteau method (Kubola and Siriamornpun, 2008). Briefly, 0.3 ml of extract samples was mixed with 2.25 ml of 10% Folin-Ciocalteu reagent dissolved in distilled water. After 5 min incubation, 2.25 ml of 6% sodium carbonate ($Na_2CO_3$) solution was added and the mixtures were left to stand for 90 min at room temperature. The absorbance of the solution samples was measured at 725 nm using a

spectrophotometer. The TPC in beans was expressed as mg gallic acid equivalents (GAE) per g dry weight (mg GAE/g DW)

(1.2) The phenolic compounds

HPLC–DAD system for analysis of phenolic compounds HPLC analysis will be performed using Shimadzu LC-20AC pumps, SPD-M20A with diode array detector and chromatographic separations will be performed on a LUNA C-18 column (4.6 × 250 mm i.d., 5 µm). The composition of solvents and used gradient elution conditions will be described previously by (Uzelac et al. 2005) with some modifications. The solvent system will be used a gradient of mobile phase A containing 0.36% phospholic acid in water; solution B will be used acetonitrile. The following gradient will be used: 0–15 min, from 5% A to 9% A, 91% B with a flow rate 0.8 ml/min; 15–22 min, from 9%A, 91% B to 11% A, 89% B with flow rate 0.8 ml/min; 22–38 min, from 11% A, 89% B to 18% A, 82% B with flow rate 0.8 ml/min; 38-43 min, from 18% A, 82% B to 23% A, 77% B with flow rate 0.8 ml/min; 43-44 min, from 23% A, 77% B to 30% A, 70% B with flow rate 0.8 ml/min; 44-55 min, from 30% A, 70% B to 20% A, 80% B with flow rate 0.8 ml/min; 60-65 min 5% A, 95% B with flow rate 0.8 ml/min. Operating conditions will be as follows: column temperature, 38 °C; injection volume, 20 µl; UV-diode array detection at 280 nm and 320 nm.

(2) Determination of total flavonoid content

(2.1) Total flavonoid content (TFC)

Total flavonoid content in silk protein was determined using a modified method, as described previously by (Kubola and Siriamornpun 2008). Briefly, 0.5 ml of each sample solution was mixed with 2.25 ml of distilled water and 0.15 ml of 5% $NaNO_2$ solution (w/v). The solution was allowed to stand for 6 min and then 0.3 ml of 10% $AlCl_3$ (w/v) was added to the solution. After 5 min, 0.1 ml of 1 M NaOH (w/v) solution was added and then the absorbance was measured at 510 nm using a spectrophotometer. Results was expressed as mg rutin equivalents (RE) per g dry weight (mg RE/g DW).

(2.2) The flavonoid compounds

HPLC–DAD system for analysis of flavonoid compounds HPLC analysis will be performed using Shimadzu LC-20AC pumps, SPD-M20A with diode array detector and chromatographic separations will be performed on a LUNA C-18 column (4.6 × 250 mm i.d., 5 µm). The composition of solvents and used gradient elution conditions will be described previously by (Uzelac et al., 2005) with some modifications. The solvent system will be used a gradient of mobile phase A containing 0.36% phospholic acid in water; solution B will be used acetonitrile. The following gradient will be used: 0–15 min, from 5% A to 9% A, 91% B with a flow rate 0.8 ml/min; 15–22 min, from 9%A, 91% B to 11% A, 89% B with flow rate 0.8 ml/min; 22–38 min, from 11% A, 89% B to 18% A, 82% B with flow rate 0.8 ml/min; 38-43 min, from 18% A, 82% B to 23% A, 77% B with flow rate 0.8 ml/min; 43-44 min, from 23% A, 77% B to 30% A, 70% B with flow rate 0.8 ml/min; 44-55 min, from 30% A, 70% B to 20% A, 80% B with flow rate 0.8 ml/min; 60-65 min 5% A, 95% B with flow rate 0.8 ml/min. Operating conditions will be as follows: column temperature, 38 °C; injection volume, 20 µl; UV-diode array detection at 370 nm.

(3) DPPH radical scavenging activity

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, will be modified from that of (Braca et al. 2001). Aqueous extract (0.1 ml) was added to 3 ml of a 0.001 M DPPH in methanol. Absorbance at 517 nm will be determined after 30 min, and the percent inhibition of activity will be calculated as The DPPH radical scavenging activities of the samples was calculated as % inhibition of $DPPH^{\bullet}$ in the following equation:

$$DPPH^{\bullet} \text{ scavenging activity } (\%) = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

$A_{control}$ is the absorbance of the $DPPH^{\bullet}$ solution without test sample and $A_{sample}$ is absorbance of the test sample ($DPPH^{\bullet}$ solution with extract sample).

(4) Ferric reducing antioxidant power (FRAP) assay

FRAP assay will be based on the reduction of $Fe^{3+}$ –TPTZ to a blue coloured Fe $^{2+}$ –TPTZ (Benzie and Strain, 1996).

The FRAP reagent will be freshly prepared by mixing 100 ml of acetate buffer (300 mM, pH 3.6), 10 ml TPTZ solution(10 mM TPTZ in 40 mM/HCl), 10 ml $FeCl_3 \cdot 6H_2O$ (20 nM) in a ratio of 10:1:1 and 12 ml distilled water, at 37 °C. To perform the assay, 1.8 ml of FRAP reagent, 180 µl Milli-Q water and 60 µl sample, standard or blank will be added to the same test tubes, and incubated at 37 °C for 4 min; absorbance will be measured at 593 nm, using FRAP working solution as blank. The reading of relative absorbance should be within the range 0–2.0; otherwise, the sample should be diluted. In the FRAP assay, the antioxidant potential of sample will be determined from a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression equation to calculate the FRAP values of the sample.

(5) Antioxidant activity by ABTS assay

The ABTS radical cation method (Lee et al. 2015) was modified to evaluate the free radical-scavenging effect of one hundred pure chemical compounds. The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1 : 44, v/v). To determine the scavenging activity, 100 µL ABTS reagent was mixed with 100 µL of sample in a 96-well microplate and was incubated at room temperature for 6 min. After incubation, the absorbance was measured 734 nm, and 100% methanol was used as a control. The ABTS scavenging effect was measured using the following formula:

Inhibition (%) = [1-(Abs. sample/Abs. control)] x 100

(6) Evaluation of anti-AGEs formation activity

The inhibitory capacities of AGEs formation of the silk protein and its fractions was measured using a method of Vinson and Howard (1996). The total volume of glycation reaction solution (2.5 ml) was prepared by mixing 500 µl of silk protein, 500 µl of 20 mg/ml BSA in phosphate buffer, 500 µl of 0.5 M glucose

in phosphate buffer and 1 ml of 0.1 M phosphate buffer at pH 7.4 containing 0.02% (w/v) sodium azide. This mixture was incubated at 37˚C for 5 days in the dark and then the amount of fluorescent AGEs formed was determined using a fluorescent spectrometer with an excitation wavelength of 330 nm and emission wavelength of 410 nm. The percentage of anti-AGEs formation was calculated based on the resulted fluorescent intensity (FI) using the following equation:

$$\text{Inhibition}(\%) = [1-(FI_{\text{sample}} - FI_{\text{sample blank}})/(FI_{\text{control}} - FI_{\text{control blank}})] \times 100$$

(7) Inhibitory activity against enzyme α-glucosidase

The α-glucosidase inhibitory activity was measured using edit method of Matsui et al. ( 2019). 10 µl of silk protein was mixed with 130 mL phosphate buffer (pH 7.0) and 1 unit/mL of α-glucosidase solution (10 µL) were mixed and the solution was incubated at 37 °C for 10 min. The reaction was started by adding 50 µL 2.5 mM *p*-Nitrophenyl- α -D-glucopyranoside (PNP-G). The absorbances were measured at 405 nm by UV/Vis absorbance spectrophotometer microplate reader. The rate of α-glucosidase inhibition was calculated as a percentage by the following equation:

$$\%\ \text{inhibition} = [(A_{\text{control}} - A_{\text{sample}})] / (A_{\text{control}}) \times 100$$

(8) Inhibitory activity against enzyme α-amylase

The α-amylase (A8220, Sigma–Aldrich) enzyme inhibitory activity was measured using the method reported by Kusano et al. (2011) with slight modifications. Substrate was prepared by boiling 100 mg potato starch in 5 mL phosphate buffer (pH 7.0) for 5 min, then cooling to room temperature. The sample (50 µL), substrate (300 µL) and 5 µg/mL α-amylase solution (20 µL) were mixed and the solution was incubated at 37 °C for 15 min. The reaction was stopped by adding 50 µL 1 M HCl, and then 50 µL iodine solution was added. The absorbances were measured at 650 nm by a microplate reader. Acarbose was used as positive control. The inhibition percentage of α-amylase was assessed by the following formula:

$$\%\ \text{inhibition} = 100 \text{ x } (\Delta A_{\text{control}} - \Delta A_{\text{sample}}) / \Delta A_{\text{control}}$$

(9) Estimation of molecular mass of silk proteins by SDS-PAGE

The molecular mass of silk proteins was estimated by Gel electrophoresis. The separating gel and staking gel were 12.5% and 5%, respectively. The sample solution was mixed with 2x buffer and heated in hot water, then sample was loaded into the well. The electric current of 150 V, 50 mA was applied to the gel. At the end of electrophoresis, the gel was stained with silver staining technique. Standard molecular mass marker was applied for estimating the molecular mass. (Sangwong, Sumida, and Sutthikhum 2016)

3.5.3 Product development of silk protein extract (SPE) and assessment of its stability for chemical and biological properties of the developed products.

After choose the potent SPE as natural food additive to develop functional food product by following step as below:

Step for develop product from SPE

Idea for generation and screening

↓

Product information and feasibility study for industry process

↓

Development product of prototype

↓

Testing of product (sensory evaluation and chemicals)

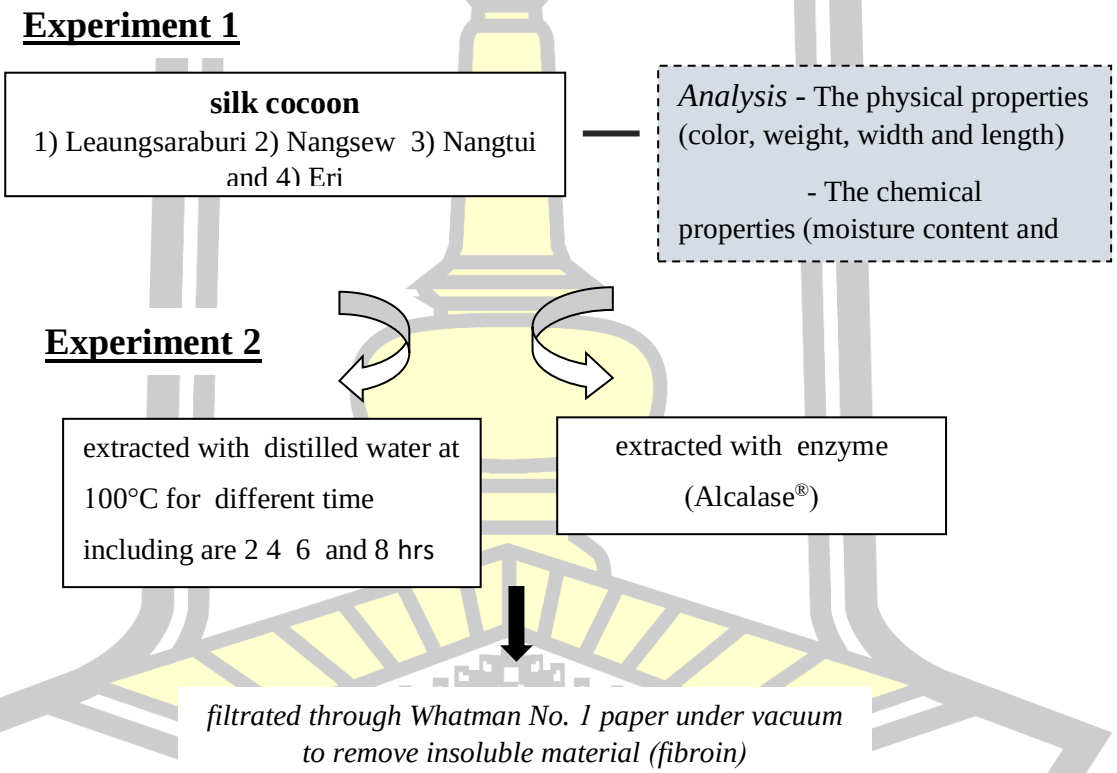
3.5.4 Sensory evaluation

Sensory evaluations of silk protein product prototype was conducted by 30 panelists using a 9-point hedonic scale where nine is like extremely and one dislike extremely. Three coded samples was served and water is provided for rinsing between samples. Control was used to compare with the product prototype for sensory test.

### 3.6 Statistical analysis

For physical, chemical and biological properties in silk cocoon and silk protein was used in this research was completely randomized design (CRD). Analysis of variance was used to test any difference in resulting from these methods. Duncan method was used to determine significant differences at $p < 0.05$.

For Sensory evaluations of silk protein product was used in this research was randomized completely block design (RCBD). Analysis of variance was used to test any difference in resulting from these methods. Duncan method was used to determine significant differences at $p < 0.05$.



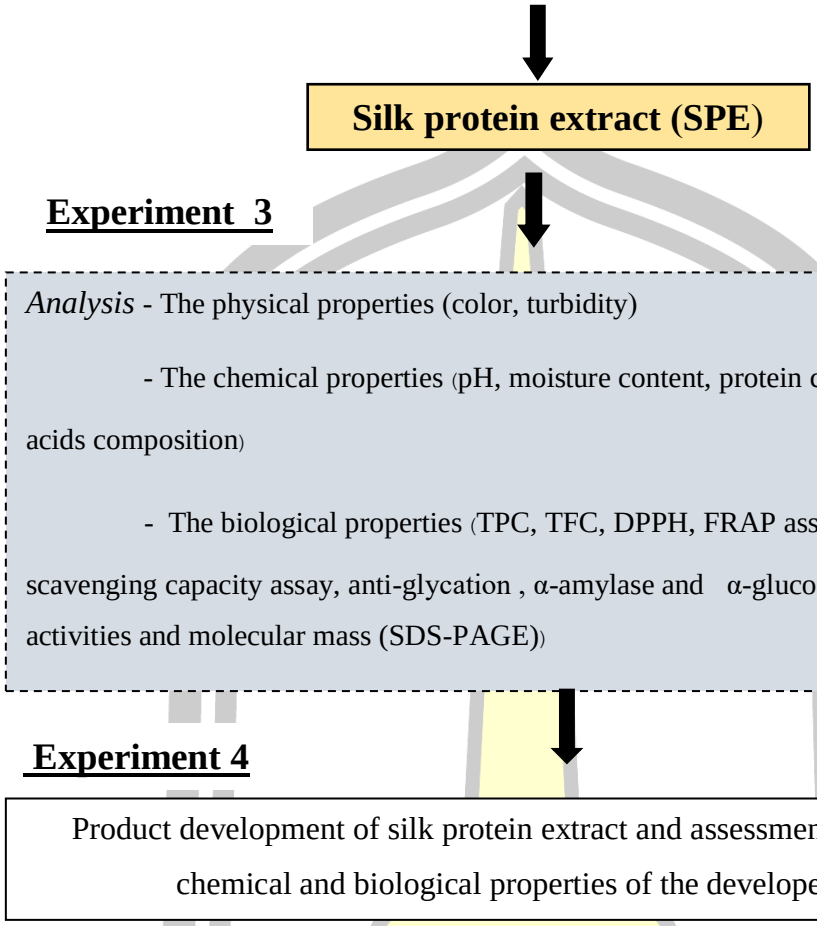



**Figure 3.5** Flow chart of experiment procedure.

# CHAPTER 4

# Results and Discussion

We studied the physical and chemical properties of silk cocoon from different strains on the extraction methods of silk protein and studied the effect of extraction method on physical, chemical and biological properties in silk protein to develop the process and product of functional food from silk protein extract.

## 4.1 The physical and chemical properties of Thai silk strains

4.1.1 The physical properties

Silk cocoon colors of silkworm vary, with naturally occurring shades of yellow and white cocoons (Figure 4.6). Leaungsaraburi, Nangsew and Nangtui were yellow cocoons and Eri was a white cocoon. The yellow of the silk cocoons is derived from a flavonoid (Kurioka and Yamazaki 2002). These pigments are absorbed from mulberry leaves, transferred from the midgut to the silk gland via hemolymph, and accumulated in the silk fiber. Silk cocoons were different in appearance, color, size and weight due to the differences of strains (Table 4.6)


Figure 4.6 Physical appearance of silk cocoons from four Thai silk strains.
(A: Leaungsaraburi, B: Nangsew, C: Nangtui, D: Eri)

**Table 4.4** The physical properties of silk cocoon.

| Thai silk strains | Color | | | Size | | Weight (g/ 1cocoon) |
|---|---|---|---|---|---|---|
| | Lightness (L*) | redness (a*) | yellowness (b*) | Width (cm) | Length (cm) | |
| Leaungsaraburi | 79.74±0.21$^{b}$ | 8.84±0.06$^{b}$ | 62.66±0.08$^{a}$ | 1.53±0.04$^{a}$ | 4.40±0.13$^{b}$ | 0.19±0.03$^{a}$ |
| Nangsew | 71.89±0.07$^{d}$ | 10.98 ±0.03$^{a}$ | 56.74±0.12$^{b}$ | 1.25±0.11$^{b}$ | 3.70±0.98$^{d}$ | 0.07±0.02$^{c}$ |
| Nangtui | 77.49±0.64$^{c}$ | 8.22±0.03$^{c}$ | 43.48 ±0.06$^{c}$ | 1.12±0.12$^{c}$ | 4.20±0.11$^{c}$ | 0.11±0.02$^{b}$ |
| Eri | 87.71±0.42 $^{a}$ | 1.12±0.10$^{d}$ | 11.04 ±0.11$^{d}$ | 1.22±0.23$^{b}$ | 4.80±0.21$^{a}$ | 0.08±0.02$^{c}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the same column were significantly different at the level $p < 0.05$.

4.1.2 The chemical properties

The levels of moisture content for the samples examined varied no significantly, from 7.27 -7.33 % and protein content of Thai silk strains were in the range of 51.24- 57.61% (Table 4.5).

**Table 4.5** The chemical properties of silk cocoon.

| Thai silk strains | Moisture content (%) $^{ns}$ | Protein content (%) |
|---|---|---|
| Leaungsaraburi | 7.33±1.53 | 55.63 ± 0.46$^{c}$ |
| Nangsew | 7.31±1.52 | 56.11 ± 0.21$^{b}$ |
| Nangtui | 7.27±1.00 | 57.61 ± 0.28$^{a}$ |
| Eri | 7.29±1.04 | 51.24 ± 0.31$^{d}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the same column were significantly different at the level $p < 0.05$, ns is no significant differences.

**4.2 The physical, chemical and biological properties of silk protein as below:**

Study of the extraction methods for silk protein were compared between water extraction and enzymatic extraction.

4.2.1 The physical properties of silk protein extract (SPE).

(1) Color measurement in silk protein by Colorimeter

The appearance of SPE as the water extraction time increased the lightness and redness of each strains were decreased but yellowness and turbidity were increased. (Fig. 4.7). For the enzymatic extraction was clear. (Table 4.6).

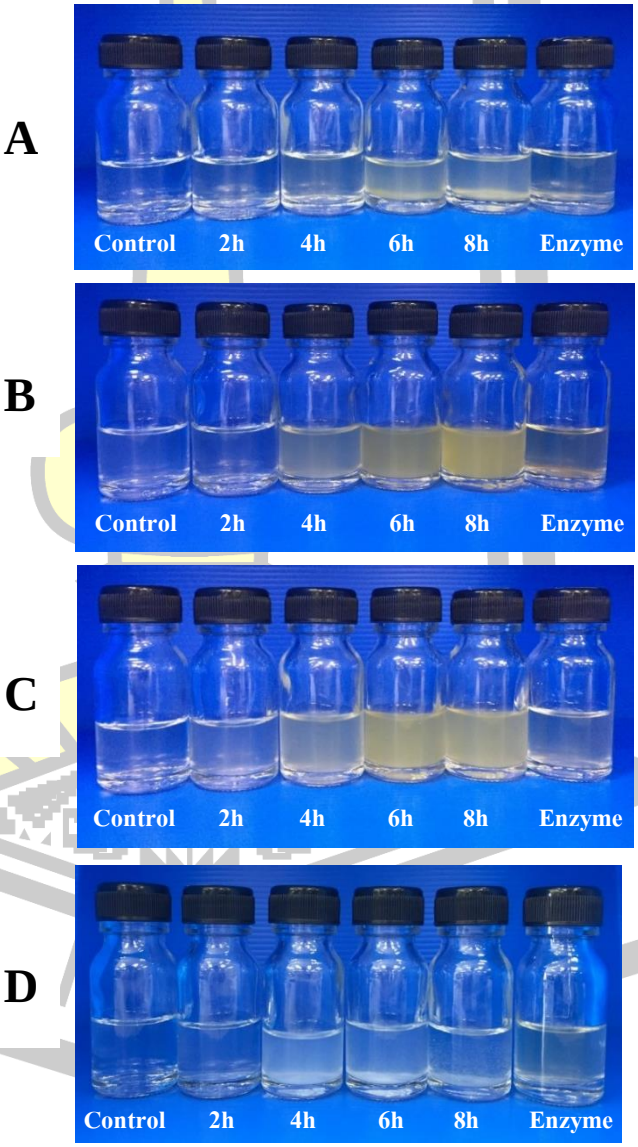



**Figure 4.7** The effect of extraction method on characteristic of silk protein extract in Thai silk strains. (A: Leaungsaraburi, B: Nangsew, C: Nangtui, D: Eri)

**Table 4.6** The effect of extraction method on the physical properties of silk protein extracts in Thai silk strains.

| Thai silk strains | Extraction method | Color measurement | | | Turbidity (NTU) |
|---|---|---|---|---|---|
| | | Lightness (L*) | redness (a*) | yellowness (b*) | |
| Leaungsaraburi | 1) Water extraction | | | | |
| | control | 29.98 ± 0.08$^{b}$ | 0.13± 0.01$^{a}$ | 1.22 ±0.13$^{e}$ | 12.56 ± 0.16$^{f}$ |
| | 2 hrs | 29.61 ± 0.02$^{b}$ | 0.11 ± 0.01$^{a}$ | 1.63 ± 0.02$^{d}$ | 15.32 ± 0.12$^{e}$ |
| | 4 hrs | 29.32 ± 0.09$^{bc}$ | 0.09 ± 0.01$^{b}$ | 2.17 ± 0.08$^{c}$ | 46.98 ± 0.22$^{c}$ |
| | 6 hrs | 29.00 ± 0.01$^{c}$ | 0.01 ± 0.01$^{c}$ | 3.13 ± 0.11$^{b}$ | 86.02 ± 0.13$^{b}$ |
| | 8 hrs | 28.89 ± 0.02$^{e}$ | 0.01 ± 0.01$^{c}$ | 3.28 ± 0.12$^{a}$ | 96.11 ± 0.11$^{a}$ |
| | 2) Enzymatic extraction | 37.66 ± 0.05$^{a}$ | 0.01 ± 0.01$^{c}$ | 3.10 ± 0.14$^{b}$ | 22.82 ± 0.15$^{d}$ |
| Nangsew | 1) Water extraction | | | | |
| | control | 29.68 ± 0.13$^{b}$ | 0.11± 0.01$^{a}$ | 1.17 ±0.07$^{e}$ | 11.80 ± 0.13$^{f}$ |
| | 2 hrs | 28.78 ± 0.02$^{c}$ | 0.10 ± 0.01$^{a}$ | 1.43 ± 0.09$^{d}$ | 15.53 ± 0.22$^{e}$ |
| | 4 hrs | 28.87 ± 0.11$^{c}$ | 0.04 ± 0.01$^{b}$ | 2.57 ± 0.13$^{c}$ | 62.11 ± 0.12$^{c}$ |
| | 6 hrs | 27.46 ± 0.06$^{d}$ | 0.02 ± 0.01$^{c}$ | 3.73 ± 0.17$^{a}$ | 89.27 ± 0.14$^{b}$ |
| | 8 hrs | 27.33 ± 0.09$^{d}$ | 0.02 ± 0.01$^{c}$ | 3.85 ± 0.12$^{a}$ | 98.04 ± 0.09$^{a}$ |
| | 2) Enzymatic extraction | 37.79 ± 0.12$^{a}$ | 0.01 ± 0.01$^{c}$ | 3.07 ± 0.14$^{b}$ | 23.06 ± 0.11$^{d}$ |
| Nangtui | 1) Water extraction | | | | |
| | control | 29.08 ± 0.06$^{b}$ | 0.13± 0.01$^{a}$ | 1.19 ±0.09$^{d}$ | 12.31 ± 0.21$^{f}$ |
| | 2 hrs | 28.61 ± 0.02$^{c}$ | 0.12 ± 0.01$^{a}$ | 1.23 ± 0.11$^{d}$ | 17.25 ± 0.13$^{e}$ |
| | 4 hrs | 27.47 ± 0.02$^{d}$ | 0.07 ± 0.01$^{b}$ | 1.55 ± 0.12$^{c}$ | 57.54 ± 0.11$^{c}$ |
| | 6 hrs | 27.14 ± 0.05$^{d}$ | 0.05 ± 0.01$^{b}$ | 3.63 ± 0.09$^{a}$ | 90.26 ± 0.12$^{b}$ |
| | 8 hrs | 27.03 ± 0.03$^{d}$ | 0.03 ± 0.01$^{c}$ | 3.86 ± 0.12$^{a}$ | 99.35 ± 0.23$^{a}$ |
| | 2) Enzymatic extraction | 37.62 ± 0.07$^{a}$ | 0.02 ± 0.01$^{c}$ | 3.04 ± 0.02$^{b}$ | 21.32 ± 0.14$^{d}$ |
| Eri | 1) Water extraction | | | | |
| | control | 29.15 ± 0.19$^{d}$ | 0.12± 0.01$^{a}$ | 1.06 ± 0.16$^{d}$ | 11.12 ± 0.09$^{e}$ |
| | 2 hrs | 29.86 ± 0.22$^{d}$ | 0.08 ± 0.01$^{b}$ | 1.18 ± 0.19$^{d}$ | 21.25 ± 0.07$^{d}$ |
| | 4 hrs | 30.12 ± 0.14$^{c}$ | 0.03 ± 0.01$^{c}$ | 1.65± 0.22$^{c}$ | 51.54 ± 0.11$^{c}$ |
| | 6 hrs | 30.47 ± 0.21$^{c}$ | 0.02 ± 0.01$^{c}$ | 2.45 ± 0.09$^{b}$ | 73.26 ± 0.12$^{b}$ |
| | 8 hrs | 32.22 ± 0.23$^{b}$ | 0.02 ± 0.01$^{c}$ | 2.96 ± 0.18$^{a}$ | 83.35 ± 0.08$^{a}$ |
| | 2) Enzymatic extraction | 37.89 ± 0.18$^{a}$ | 0.01 ± 0.01$^{c}$ | 2.67 ± 0.15$^{b}$ | 20.32 ± 0.13$^{d}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column within the same strains were significantly different at the level $p < 0.05$.

4.2.2 The chemical properties of SPE.

The SPE of each strain were pH value ranged from 6.60 – 6.93 and protein content was increased with increased extraction time for water extraction (Table 4 .7). The amino acid composition and content of each of SPE in different strains is shown in Tables 4.8-4.11. The results indicated that amino acid composition and content was improved when longer extraction time was applied. The higher amino acid content was observed except for after 6 hours and total essential amino acid were found to be the highest using enzyme extraction due to the action of the enzyme that hydrolyzes protein peptide bonds and breakdown of proteins into smaller polypeptides or single amino acids (Devi 2012). The amino acid composition differs between strains of silk. The three major amino acids were serine, glycine and threonine, as found in mulberry strains ( yellow cocoon) and non-mulberry strains (white cocoon). Among the other major amino acids present, there were tyrosine and valine. In mulberry silks, there were glycine, alanine and serine. The overall composition of acidic amino groups (i.e. aspartic and glutamic acids) in the mulberry strain were greater than that of the basic amino acids (Sen and Murugesh Babu 2004).

**Table 4.7** The effect of extraction method on the chemical properties of SPE in Thai silk strains.

| Thai silk strains | Extraction method | pH$^{ns}$ | Protein content (mg/g) |
|---|---|---|---|
| Leaungsaraburi | 1) Water extraction | | |
| | control | 6.69 ± 0.07 | 0.50 ± 0.68$^{e}$ |
| | 2 hrs | 6.68 ± 0.01 | 0.89 ± 0.23$^{d}$ |
| | 4 hrs | 6.67 ± 0.03 | 1.16 ± 0.74$^{c}$ |
| | 6 hrs | 6.67 ± 0.01 | 1.30 ±0.72$^{b}$ |
| | 8 hrs | 6.66 ± 0.03 | 1.44 ± 0.23$^{a}$ |
| | 2) Enzymatic extraction | 6.71 ± 0.01 | 1.07 ± 0.36$^{c}$ |
| Nangsew | 1) Water extraction | | |
| | control | 6.69 ± 0.04 | 0.80 ±0.56$^{e}$ |
| | 2 hrs | 6.68 ± 0.01 | 1.15 ±0.42$^{d}$ |
| | 4 hrs | 6.63± 0.03 | 1.24 ± 0.55$^{c}$ |
| | 6 hrs | 6.62 ± 0.01 | 1.46 ±0.72$^{b}$ |
| | 8 hrs | 6.63 ± 0.03 | 2.18± 0.23$^{a}$ |
| | 2) Enzymatic extraction | 6.71 ± 0.01 | 1.11± 0.42$^{d}$ |
| Nangtui | 1) Water extraction | | |
| | control | 6.68 ± 0.05 | 0.81±0.39$^{e}$ |
| | 2 hrs | 6.66± 0.01 | 1.13±0.20$^{d}$ |
| | 4 hrs | 6.63 ± 0.01 | 1.92±0.25$^{c}$ |
| | 6 hrs | 6.62 ± 0.01 | 2.13 ±0.45$^{b}$ |
| | 8 hrs | 6.60 ± 0.01 | 2.24±0.66$^{a}$ |
| | 2) Enzymatic extraction | 6.70 ± 0.01 | 1.14±0.12$^{d}$ |
| Eri | 1) Water extraction | | |
| | control | 6.71 ± 0.05 | 0.81±0.36$^{e}$ |
| | 2 hrs | 6.70± 0.01 | 1.02±0.22$^{d}$ |
| | 4 hrs | 6.77 ± 0.01 | 1.06±0.31$^{c}$ |
| | 6 hrs | 6.76 ± 0.01 | 2.01 ±0.30$^{b}$ |
| | 8 hrs | 6.76 ± 0.01 | 2.07±0.43$^{a}$ |
| | 2) Enzyme extraction | 6.82 ± 0.01 | 1.08±0.50$^{c}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column within the same strains were significantly different at the level $p < 0.05$. $^{ns}$ = no significantly different.

**Table 4.8** The effect of extraction method on amino acid contents of SPE in Leaungsaraburi strains.

| Group | Amino acid content (µg/g DW) | Extraction method | | | | | |
|---|---|---|---|---|---|---|---|
| | | Water extraction (hrs) | | | | | Enzyme |
| | | control | 2 | 4 | 6 | 8 | |
| Essential amino acids | Phenylalanine | 0.21±0.28$^{f}$ | 0.55±0.32$^{e}$ | 1.20±0.42$^{d}$ | 1.84±0.21$^{b}$ | 1.77±0.24$^{c}$ | 35.03 ±0.24$^{a}$ |
| | Valine | 0.78±0.31$^{e}$ | 1.87±0.29$^{d}$ | 2.58±0.23$^{c}$ | 3.35±0.23$^{b}$ | 2.21± 0.37$^{c}$ | 34.16 ±0.15$^{a}$ |
| | Tryptophan | 0.34±0.10$^{f}$ | 0.81±0.22$^{e}$ | 1.61±0.19$^{c}$ | 2.39±0.19$^{b}$ | 1.29±0.11$^{d}$ | 30.93 ±0.27$^{a}$ |
| | Threonine | 1.04±0.24$^{e}$ | 7.83±0.17$^{d}$ | 17.48±0.49$^{c}$ | 19.75±0.16$^{b}$ | 18.59±0.24$^{c}$ | 21.28 ±0.29$^{a}$ |
| | Isoleucine | 0.98±0.39$^{e}$ | 1.01±0.48$^{d}$ | 2.62±0.49$^{c}$ | 3.69±0.34$^{b}$ | 2.21±0.72$^{c}$ | 10.21 ±0.25$^{a}$ |
| | Methionine | 0.76 ±0.41$^{d}$ | 0.87±0.40$^{d}$ | 1.39±0.24$^{c}$ | 2.49±0.14$^{b}$ | 1.09 ±0.20$^{c}$ | 4.86 ±0.42$^{a}$ |
| | Histidine | 2.38 ±0.30$^{e}$ | 7.39±0.13$^{d}$ | 8.21±0.16$^{c}$ | 9.54±0.43$^{b}$ | 8.46±0.19$^{c}$ | 12.09 ±0.5$^{a}$ |
| | Arginine | 2.09±0.11$^{e}$ | 3.21±0.19$^{d}$ | 3.94±0.48$^{c}$ | 4.46±0.38$^{b}$ | 4.31±0.32$^{c}$ | 7.46±0.11$^{a}$ |
| | Lysine | 2.13±0.27$^{e}$ | 5.68±0.12$^{d}$ | 11.36±0.36$^{c}$ | 12.74±0.11$^{b}$ | 11.02±0.25$^{c}$ | 15.82±0.35$^{a}$ |
| | Leucine | 0.32±0.24$^{e}$ | 1.09±0.71$^{d}$ | 1.24±0.53$^{c}$ | 1.46±0.11$^{b}$ | 1.28±0.18$^{c}$ | 8.14±0.20$^{a}$ |
| | *Total essential amino acids* | 11.03±0.36$^{f}$ | 30.31±0.66$^{e}$ | 51.63±0.50$^{d}$ | 61.98±0.27$^{b}$ | 52.23±0.57$^{c}$ | 179.98±0.23$^{a}$ |
| Non-essential amino acids | Glycine | 2.11±0.56$^{f}$ | 16.77±0.48$^{e}$ | 27.09±0.62$^{d}$ | 33.89±0.43$^{b}$ | 30.98±0.39$^{c}$ | 51.02±0.48$^{a}$ |
| | Glutamic acid | 1.04±0.17$^{e}$ | 5.05±0.62$^{d}$ | 8.75±0.68$^{c}$ | 17.41±0.37$^{b}$ | 15.07±0.10$^{c}$ | 23.32±0.39$^{a}$ |
| | Aspartic acid | 0.98±0.21$^{f}$ | 10.98±0.11$^{e}$ | 11.34±0.31$^{d}$ | 13.63±0.42$^{b}$ | 12.26±0.33$^{c}$ | 27.39±0.27$^{a}$ |
| | Glutamine | 1.02±0.16$^{d}$ | 1.25±0.21$^{d}$ | 3.04±0.11$^{c}$ | 4.06±0.59$^{b}$ | 3.03±0.15$^{c}$ | 6.72±0.54$^{a}$ |
| | Serine | 6.41±0.23$^{e}$ | 12.64±0.52$^{d}$ | 18.37±0.89$^{c}$ | 28.22±0.47$^{a}$ | 25.41±0.65$^{b}$ | 37.30±0.45$^{a}$ |
| | Tyrosine | 2.35±0.45$^{f}$ | 5.70±0.47$^{e}$ | 8.65±0.40$^{d}$ | 12.07±0.14$^{b}$ | 11.04±0.48$^{c}$ | 28.42±0.27$^{a}$ |
| | Alanine | 0.85±0.13$^{e}$ | 1.89±0.33$^{d}$ | 2.44±0.39$^{c}$ | 3.13±0.36$^{b}$ | 2.11±0.37$^{c}$ | 13.05±0.62$^{a}$ |
| | Arginine | 2.03±0.22$^{e}$ | 4.03±0.31$^{d}$ | 4.73±0.10$^{c}$ | 5.24±0.55$^{b}$ | 5.18±0.74$^{b}$ | 22.07±0.21$^{a}$ |
| | Asparagine | 1.71±0.50$^{f}$ | 2.04±0.57$^{e}$ | 3.25 ±0.63$^{d}$ | 5.71±0.24$^{b}$ | 4.09±0.90$^{c}$ | 12.09±0.60$^{a}$ |
| | **Total amino acids** | **29.53±0.41$^{f}$** | **90.66±0.52$^{e}$** | **139.31±0.71$^{d}$** | **185.32±0.48$^{b}$** | **161.40±0.64$^{c}$** | **401.36±0.58$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.

**Table 4.9** The effect of extraction method on amino acid contents of SPE in Nangsew strains.

| Group | Amino acid content (µg/g DW) | Extraction method | | | | | |
|---|---|---|---|---|---|---|---|
| | | Water extraction (hrs) | | | | | Enzyme |
| | | control | 2 | 4 | 6 | 8 | |
| Essential amino acids | Phenylalanine | 0.63±0.36$^{f}$ | 1.01±0.57$^{e}$ | 1.63±0.64$^{d}$ | 2.44±0.45$^{b}$ | 2.16±0.23$^{c}$ | 32.07±0.44$^{a}$ |
| | Valine | 0.86±0.48$^{e}$ | 2.19±0.11$^{d}$ | 2.69±0.51$^{c}$ | 3.22±0.92$^{b}$ | 2.95± 0.55$^{bc}$ | 20.76±0.37$^{a}$ |
| | Tryptophan | 1.07±0.57$^{f}$ | 1.81±0.29$^{e}$ | 2.81±0.09$^{d}$ | 2.79±0.19$^{b}$ | 2.97±0.13$^{c}$ | 25.43±0.52$^{a}$ |
| | Threonine | 1.86±0.62$^{f}$ | 10.38±0.19$^{e}$ | 26.92±0.19$^{d}$ | 29.30±0.46$^{b}$ | 28.61±0.23$^{c}$ | 30.63±0.71$^{a}$ |
| | Isoleucine | 0.96±0.46$^{f}$ | 1.95±0.84$^{e}$ | 2.89±0.94$^{d}$ | 3.91±1.21$^{b}$ | 3.24±0.56$^{c}$ | 16.43±0.49$^{a}$ |
| | Methionine | 0.78±0.54$^{f}$ | 1.48±0.26$^{e}$ | 1.98±0.26$^{d}$ | 2.79±0.65$^{b}$ | 2.21±0.54$^{c}$ | 5.07±0.38$^{a}$ |
| | Histidine | 0.04±0.61$^{e}$ | 7.35±0.17$^{d}$ | 8.92±0.19$^{c}$ | 10.29±0.14$^{b}$ | 8.93±0.26$^{b}$ | 13.08±0.59$^{a}$ |
| | Arginine | 0.97±0.52$^{e}$ | 1.57±0.30$^{d}$ | 1.71±0.33$^{c}$ | 2.64±0.24$^{b}$ | 1.69±0.13$^{c}$ | 37.15±0.33$^{a}$ |
| | Lysine | 1.53±0.41$^{e}$ | 2.44±0.41$^{d}$ | 4.64±0.16$^{c}$ | 5.32±0.31$^{b}$ | 4.88±0.52$^{c}$ | 19.63±0.25$^{a}$ |
| | Leucine | 1.02±0.26$^{e}$ | 1.98±0.65$^{d}$ | 2.31±0.65$^{c}$ | 2.59±0.95$^{b}$ | 2.20± 0.15$^{c}$ | 10.07±0.42$^{a}$ |
| | *Total essential amino acids* | 9.72±0.49$^{f}$ | 32.16±0.66$^{e}$ | 56.50±0.53$^{d}$ | 65.29±0.86$^{b}$ | 59.84±0.48$^{c}$ | 210.32±0.57$^{a}$ |
| Non-essential amino acids | Glycine | 2.76±0.38$^{b}$ | 11.73±0.38$^{b}$ | 21.32±0.72$^{b}$ | 34.11±0.48$^{b}$ | 30.23±0.52$^{b}$ | 53.50±0.68$^{a}$ |
| | Glutamic acid | 1.93±0.52$^{f}$ | 8.67±0.85$^{e}$ | 10.37±0.53$^{d}$ | 20.67±0.51$^{b}$ | 18.18±0.87$^{c}$ | 28.45±0.73$^{a}$ |
| | Aspartic acid | 1.57±0.48$^{f}$ | 16.44±0.51$^{e}$ | 22.17±0.93$^{d}$ | 25.64±0.23$^{b}$ | 24.25±0.10$^{c}$ | 28.37±0.69$^{a}$ |
| | Glutamine | 0.74±0.63$^{e}$ | 1.87±0.19$^{d}$ | 3.07±0.19$^{b}$ | 3.79±0.46$^{b}$ | 3.01±0.23$^{c}$ | 11.96±0.58$^{a}$ |
| | Serine | 2.01±0.74$^{f}$ | 10.04±0.71$^{e}$ | 15.19±0.69$^{d}$ | 21.60±0.65$^{b}$ | 18.03±1.75$^{c}$ | 38.21±0.87$^{a}$ |
| | Tyrosine | 0.67±0.40$^{f}$ | 1.07±0.37$^{e}$ | 3.27±0.57$^{d}$ | 6.48±0.28$^{b}$ | 5.35±0.06$^{c}$ | 30.28±0.75$^{a}$ |
| | Alanine | 1.06±0.39$^{e}$ | 1.94±0.57$^{d}$ | 2.48±0.27$^{c}$ | 3.25±0.49$^{b}$ | 2.30±0.18$^{c}$ | 15.63±0.58$^{a}$ |
| | Arginine | 1.37±0.51$^{e}$ | 4.57±0.32$^{d}$ | 5.79±0.33$^{c}$ | 7.64±0.04$^{b}$ | 7.49±0.13$^{b}$ | 27.34±0.37$^{a}$ |
| | Asparagine | 1.96±0.44$^{f}$ | 2.81±1.68$^{e}$ | 3.22±0.98$^{d}$ | 5.06±1.48$^{b}$ | 4.29±0.37$^{c}$ | 15.07±0.49$^{a}$ |
| | **Total amino acids** | **23.79±0.61$^{f}$** | **91.30±0.64$^{e}$** | **143.38±0.68$^{d}$** | **193.53±0.53$^{b}$** | **172.97±0.60$^{c}$** | **469.13±0.59$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.

**Table 4.10** The effect of extraction method on amino acid contents of SPE in Nangtui strains.

| Group | Amino acid content (µg/g DW) | Extraction method | | | | | |
|---|---|---|---|---|---|---|---|
| | | Water extraction (hrs) | | | | | Enzyme |
| | | control | 2 | 4 | 6 | 8 | |
| Essential amino acids | Phenylalanine | 1.32±0.37$^{e}$ | 8.21±0.11$^{d}$ | 9.15±0.31$^{c}$ | 10.56±0.25$^{b}$ | 9.98±0.31$^{bc}$ | 35.21±0.41$^{a}$ |
| | Valine | 2.01±0.39$^{e}$ | 4.03±0.42$^{d}$ | 4.43±0.40$^{c}$ | 5.24±0.75$^{b}$ | 5.18±0.44$^{b}$ | 30.42±0.61$^{a}$ |
| | Tryptophan | 1.67 ±0.47$^{e}$ | 2.06±0.31$^{d}$ | 2.68±0.62$^{c}$ | 3.25±0.51$^{b}$ | 3.13±0.64$^{b}$ | 28.02±0.30$^{a}$ |
| | Threonine | 1.36±0.52$^{f}$ | 14.05±0.33$^{e}$ | 22.04±0.65$^{d}$ | 24.29±0.45$^{b}$ | 23.23±0.39$^{c}$ | 32.03±0.64$^{a}$ |
| | Isoleucine | 0.89±0.47$^{f}$ | 1.31±0.80$^{e}$ | 1.62±0.80$^{d}$ | 2.84±0.82$^{b}$ | 2.45±0.22$^{c}$ | 18.75±0.54$^{a}$ |
| | Methionine | 0.45±0.60$^{f}$ | 1.10±0.62$^{e}$ | 1.58±0.82$^{d}$ | 2.63±0.22$^{b}$ | 2.04±0.60$^{c}$ | 6.89±0.52$^{a}$ |
| | Histidine | 0.16±0.49$^{f}$ | 8.21±0.11$^{e}$ | 9.15±0.11$^{d}$ | 10.56±0.75$^{b}$ | 9.98±0.41$^{c}$ | 15.63±0.36$^{a}$ |
| | Arginine | 0.55±0.64$^{e}$ | 4.03±0.42$^{d}$ | 4.43±0.10$^{c}$ | 5.24±0.35$^{b}$ | 4.63±0.34$^{c}$ | 43.21±0.72$^{a}$ |
| | Lysine | 1.21±0.51$^{e}$ | 2.06±0.31$^{d}$ | 2.96±0.62$^{c}$ | 3.25±0.26$^{b}$ | 3.07±0.24$^{c}$ | 21.09±0.58$^{a}$ |
| | Leucine | 0.36±0.53$^{e}$ | 1.92±0.48$^{d}$ | 2.03±0.21$^{c}$ | 2.42±0.11$^{b}$ | 2.05±0.35$^{c}$ | 12.65±0.39$^{a}$ |
| | *Total essential amino acids* | 9.98±0.49$^{f}$ | 46.98±0.56$^{e}$ | 60.07±0.63$^{d}$ | 70.28±0.27$^{b}$ | 65.74±0.59$^{c}$ | 243.90±0.55$^{a}$ |
| Non-essential amino acids | Glycine | 2.89±0.27$^{f}$ | 14.54±0.49$^{e}$ | 23.37±0.42$^{d}$ | 36.48±0.39$^{b}$ | 31.31±0.55$^{c}$ | 54.44±0.49$^{a}$ |
| | Glutamic acid | 10.87±0.58$^{f}$ | 10.17±0.65$^{e}$ | 14.39±1.40$^{d}$ | 22.42±0.39$^{b}$ | 18.67±0.42$^{c}$ | 30.57±0.73$^{a}$ |
| | Aspartic acid | 1.78±0.49$^{f}$ | 14.05±0.33$^{e}$ | 22.04±0.65$^{d}$ | 26.29±0.15$^{b}$ | 24.23±0.69$^{c}$ | 31.69±0.69$^{a}$ |
| | Glutamine | 0.97±0.57$^{e}$ | 1.18±0.51$^{d}$ | 2.06±0.34$^{c}$ | 3.20±0.35$^{b}$ | 2.98±0.32$^{b}$ | 7.83±0.72$^{a}$ |
| | Serine | 1.86±0.73$^{f}$ | 12.64±0.52$^{e}$ | 18.17±1.89$^{d}$ | 22.22±0.47$^{b}$ | 19.41±0.45$^{c}$ | 39.65±0.69$^{a}$ |
| | Tyrosine | 1.96±0.48$^{f}$ | 5.70±0.47$^{e}$ | 8.65±0.40$^{d}$ | 10.07±0.64$^{b}$ | 9.04±0.38$^{c}$ | 31.09±0.48$^{a}$ |
| | Alanine | 0.69±0.59$^{e}$ | 1.89±0.53$^{d}$ | 2.44±0.39$^{c}$ | 3.13±0.86$^{b}$ | 2.11±0.47$^{c}$ | 38.14±0.65$^{a}$ |
| | Arginine | 0.78±0.61$^{e}$ | 3.03±0.52$^{d}$ | 4.43±0.10$^{c}$ | 5.24±0.45$^{b}$ | 5.18±0.44$^{b}$ | 28.74±0.38$^{a}$ |
| | Asparagine | 1.09±0.48$^{a}$ | 3.04±0.57$^{d}$ | 4.25 1.53$^{c}$ | 5.71±0.24$^{a}$ | 3.09±0.90$^{b}$ | 16.94±0.53$^{a}$ |
| | **Total amino acids** | **32.87±0.60$^{f}$** | **113.22±0.56$^{e}$** | **141.70±0.84$^{d}$** | **205.04±0.75$^{b}$** | **181.76±0.72$^{c}$** | **522.99±0.67$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.

**Table 4.11** The effect of extraction method on amino acid contents of SPE in Eri strains.

| Group | Amino acid content (µg/g DW) | Extraction method | | | | | |
|---|---|---|---|---|---|---|---|
| | | Water extraction (hrs) | | | | | Enzyme |
| | | control | 2 | 4 | 6 | 8 | |
| Essential amino acids | Phenylalanine | 0.12±0.25$^{f}$ | 0.36±0.67$^{e}$ | 0.52±0.44$^{d}$ | 1.49±0.50$^{b}$ | 0.75±0.32$^{c}$ | 24.07±0.53$^{a}$ |
| | Valine | 0.73±0.36$^{e}$ | 2.02±0.11$^{d}$ | 2.43±0.51$^{c}$ | 2.87±0.52$^{b}$ | 2.61± 0.55$^{bc}$ | 28.32±0.37$^{a}$ |
| | Tryptophan | 0.07±0.31$^{f}$ | 0.41±0.37$^{e}$ | 0.50±0.35$^{d}$ | 0.95±0.20$^{b}$ | 0.63±0.47$^{c}$ | 20.73±0.63$^{a}$ |
| | Threonine | 1.31±0.69$^{e}$ | 2.08±0.39$^{d}$ | 3.52±0.19$^{c}$ | 4.53±0.51$^{b}$ | 3.37±0.49$^{c}$ | 21.21±0.54$^{a}$ |
| | Isoleucine | 0.11±0.57$^{f}$ | 0.46±0.77$^{e}$ | 0.53±0.38$^{d}$ | 0.87±0.76$^{b}$ | 0.62±0.42$^{c}$ | 32.62±0.32$^{a}$ |
| | Methionine | 0.23±0.60$^{f}$ | 0.57±0.30$^{e}$ | 0.68±0.37$^{d}$ | 0.86±0.42$^{b}$ | 0.72±0.49$^{c}$ | 2.21±0.41$^{a}$ |
| | Histidine | 0.11±0.32$^{e}$ | 0.21±0.36$^{d}$ | 0.54±0.79$^{c}$ | 0.76±0.36$^{b}$ | 0.57±0.67$^{c}$ | 14.02±0.42$^{a}$ |
| | Arginine | 0.18±0.45$^{f}$ | 0.28±0.62$^{e}$ | 0.39±0.37$^{d}$ | 0.54±0.46$^{b}$ | 0.43±0.25$^{c}$ | 24.72±0.27$^{a}$ |
| | Lysine | 1.64±0.62$^{e}$ | 2.31±0.35$^{d}$ | 3.08±0.32$^{c}$ | 4.71±0.64$^{b}$ | 2.58±0.48$^{d}$ | 10.15±0.36$^{a}$ |
| | Leucine | 1.02±0.61$^{f}$ | 1.56±0.44 $^{e}$ | 2.07±0.92$^{c}$ | 3.65±0.38$^{b}$ | 2.54± 0.47$^{d}$ | 5.71±0.39$^{a}$ |
| | *Total essential amino acids* | 5.52±0.51$^{e}$ | 10.26±0.63$^{d}$ | 14.26±0.86$^{c}$ | 21.23±0.72$^{b}$ | 14.82±0.53$^{c}$ | 183.86±0.54$^{a}$ |
| Non-essential amino acid | Glycine | 1.44±0.65$^{f}$ | 11.03±0.38$^{f}$ | 19.36±0.43$^{f}$ | 28.66±0.51$^{f}$ | 27.32±0.38$^{f}$ | 50.32±0.61$^{f}$ |
| | Glutamic acid | 2.51±0.41$^{f}$ | 8.53±0.69$^{e}$ | 12.09±0.53$^{d}$ | 14.03±0.42$^{b}$ | 13.98±0.37$^{c}$ | 20.45±0.66$^{a}$ |
| | Aspartic acid | 0.54±0.32$^{e}$ | 10.21±0.86$^{d}$ | 10.42±0.73$^{c}$ | 12.87±0.47$^{b}$ | 12.23±0.32$^{d}$ | 25.08±0.37$^{a}$ |
| | Glutamine | 0.79±0.37$^{e}$ | 1.08±0.56$^{d}$ | 1.32±0.29$^{c}$ | 1.57±0.39$^{b}$ | 1.09±0.75$^{d}$ | 5.72±0.68$^{a}$ |
| | Serine | 1.24±0.55$^{e}$ | 11.76±0.32$^{d}$ | 13.87±0.43$^{c}$ | 17.62±0.73$^{b}$ | 15.42±1.49$^{c}$ | 30.02±0.75$^{a}$ |
| | Tyrosine | 1.97±0.53$^{e}$ | 2.37±0.43$^{d}$ | 4.08±0.72$^{c}$ | 5.64±0.43$^{b}$ | 2.79±0.38$^{d}$ | 29.26±0.43$^{a}$ |
| | Alanine | 0.94±0.47$^{e}$ | 1.09±0.39$^{d}$ | 1.27±0.44$^{c}$ | 1.46±0.37$^{b}$ | 0.87±0.45$^{d}$ | 11.90±0.35$^{a}$ |
| | Arginine | 0.08±0.60$^{f}$ | 0.15±0.46$^{e}$ | 0.22±0.33$^{d}$ | 0.78±0.42$^{b}$ | 0.37±0.56$^{c}$ | 18.82±0.37$^{a}$ |
| | Asparagine | 1.07±0.56$^{f}$ | 1.50±0.79$^{e}$ | 1.84±0.37$^{d}$ | 2.10±0.96$^{b}$ | 1.97±0.54$^{c}$ | 12.85±0.56$^{a}$ |
| | **Total amino acids** | **16.10±0.58$^{f}$** | **57.98±0.69$^{e}$** | **78.73±0.62$^{d}$** | **105.96±0.68$^{b}$** | **90.86±0.62$^{c}$** | **388.18±0.59$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.

4.2.4 The biological properties of SPE.

(1) Total phenolic content (TPC) and the phenolic compounds.

The extraction method with water at 6 hours was the highest TPC and phenolic compounds when compared to other times, increasing until 6 hours after which there was a decrease. However, enzymatic extraction provided better values than water extraction (Table 4.12). The most common phenolic compounds in SPE were protocatechuic acid, gallic acid, *p*-hydroxybenzoic acid, ferulic acid and sinapic acid (Table 4.13-4.16).

(2) Total flavonoid content (TFC) and the flavonoid compounds.

The extraction method with water at 6 hours was the highest TFC when compared to other times and it increased until 6 hours, after which there was a decrease. However, enzyme extraction provided better values than water extraction (Table 4.12). SPE of Nangtui strains for water extraction at 6 hours and enzyme extraction provided values of 49.10 and 51.87 mg RE/g DW, respectively. The most common flavonoid compounds were rutin and myricetin (Table 4.13-4.16).

There are many varieties of the silkworm *Bombyx mori* (*B. mori*), and these produce brilliantly colored yellow, pink, golden-yellow, flesh, sasa (yellowish green) and green cocoons. The pigments in yellow, pink, golden-yellow and flesh cocoons are derived from flavonoids and carotenoids (Tabunoki et al. 2004).

**Table 4.12** The effect of extraction method on total phenolic and total flavonoid contents of SPE in Thai silk strains.

| Thai silk strains | Extraction method | Total phenolic content (mg GAE/g DW) | Total flavonoid content (mg RE/g DW) |
|---|---|---|---|
| Leaungsaraburi | 1) Water extraction control | 11.18 ± 1.28$^{f}$ | 3.66 ± 1.31$^{f}$ |
| | 2 hrs | 28.11 ± 1.13$^{e}$ | 11.58 ± 1.09$^{e}$ |
| | 4 hrs | 49.47 ± 1.19$^{d}$ | 32.93 ± 1.12$^{d}$ |
| | 6 hrs | 63.24 ± 1.12$^{b}$ | 43.27 ± 1.19$^{b}$ |
| | 8 hrs | 58.27 ± 1.21$^{c}$ | 40.05 ± 1.08$^{c}$ |
| | 2) Enzymatic extraction | 69.42 ± 1.01$^{a}$ | 48.64 ± 1.16$^{a}$ |
| Nangsew | 1) Water extraction control | 10.51 ±1.48$^{f}$ | 5.94 ± 1.63$^{f}$ |
| | 2 hrs | 43.03 ± 1.05$^{e}$ | 35.74 ± 1.25$^{e}$ |
| | 4 hrs | 58.23 ± 1.05$^{d}$ | 43.03 ± 1.05$^{d}$ |
| | 6 hrs | 78.89 ± 1.82$^{b}$ | 47.29 ± 1.11$^{b}$ |
| | 8 hrs | 76.28 ± 1.05$^{c}$ | 45.06 ± 1.10$^{c}$ |
| | 2) Enzymatic extraction | 81.25 ± 1.16$^{a}$ | 50.38 ± 1.19$^{a}$ |
| Nangtui | 1) Water extraction control | 12.79 ±1.75$^{e}$ | 8.23 ±1.48$^{e}$ |
| | 2 hrs | 51.17 ± 1.20$^{d}$ | 38.69 ± 1.22$^{d}$ |
| | 4 hrs | 71.97 ± 1.20$^{c}$ | 47.02 ± 1.20$^{c}$ |
| | 6 hrs | 87.92 ± 1.08$^{a}$ | 49.10 ± 1.07$^{b}$ |
| | 8 hrs | 80.23 ± 1.17$^{b}$ | 47.79 ± 1.13$^{c}$ |
| | 2) Enzymatic extraction | 87.21 ± 1.03$^{a}$ | 51.87 ± 1.27$^{a}$ |
| Eri | 1) Water extraction control | 10.22 ± 1.11$^{f}$ | 1.57 ± 1.01$^{f}$ |
| | 2 hrs | 18.12 ± 1.20$^{e}$ | 8.98 ± 1.17$^{e}$ |
| | 4 hrs | 22.54 ± 1.03$^{d}$ | 10.43 ± 1.32$^{d}$ |
| | 6 hrs | 32.06 ± 1.14$^{b}$ | 12.67 ± 1.13$^{b}$ |
| | 8 hrs | 30.08 ± 1.07$^{c}$ | 11.85 ± 1.21$^{c}$ |
| | 2) Enzymatic extraction | 40.09 ± 1.21$^{a}$ | 14.09 ± 1.40$^{a}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column within the same strains were significantly different at the level $p < 0.05$.

**Table 4.13** The effect of extraction method on content of phenolic and flavonoid compounds of SPE in Leaungsaraburi strains

| Parameters | Extraction method | | | | | |
|---|---|---|---|---|---|---|
| | Water extraction (hrs) | | | | | Enzyme |
| | control | 2 | 4 | 6 | 8 | |
| Phenolic acid content (µg/g DW) | | | | | | |
| gallic acid | 0.98 ± 0.78$^{f}$ | 3.08 ± 1.08$^{e}$ | 5.81± 0.91$^{d}$ | 9.24 ± 0.56$^{b}$ | 7.16 ± 0.63$^{c}$ | 11.82 ± 0.57$^{a}$ |
| protocatechuic acid | 0.86 ± 0.38$^{f}$ | 4.32 ± 0.44$^{e}$ | 9.83 ± 0.71$^{d}$ | 27.43 ± 0.90$^{b}$ | 24.28 ± 0.28$^{c}$ | 29.57 ± 0.39$^{a}$ |
| *p*-hydroxybenzoic acid | 0.77 ± 0.36$^{e}$ | 4.18 ± 0.70$^{d}$ | 7.98± 0.35$^{c}$ | 8.39 ± 0.48$^{b}$ | 8.18 ± 0.67$^{b}$ | 9.84 ± 0.42$^{a}$ |
| chlorogenic acid | ND | ND | ND | ND | ND | ND |
| vanillic acid | ND | ND | ND | ND | ND | ND |
| caffeic acid | ND | ND | ND | ND | ND | ND |
| syringic acid | ND | ND | ND | ND | ND | ND |
| *p*-coumaric acid | ND | ND | ND | ND | ND | ND |
| ferulic acid | 0.74 ± 0.23$^{d}$ | 0.74 ± 0 63$^{d}$ | 5.08 ± 0.22$^{c}$ | 9.26 ± 0.36$^{b}$ | 5.29 ± 0.21$^{c}$ | 10.14 ± 0.26$^{a}$ |
| sinapic acid | 0.96 ± 0.31$^{e}$ | 5.11± 0.47$^{d}$ | 8.91 ± 0.37$^{c}$ | 9.91 ± 0.30$^{b}$ | 9.84 ± 0.42$^{b}$ | 10.39 ± 0.15$^{a}$ |
| ***Total*** | **4.31 ± 0.26$^{f}$** | **17.43 ± 0.22$^{e}$** | **37.61 ± 0.28$^{d}$** | **64.23 ± 0.18$^{b}$** | **54.75 ± 0.27$^{c}$** | **71.76± 0.24$^{a}$** |
| Flavonoid content (µg/g DW) | | | | | | |
| rutin | 0.23 ± 0.16$^{e}$ | 1.28 ± 0.19$^{d}$ | 8.65 ± 0.32$^{c}$ | 10.87 ± 0.14$^{b}$ | 8.76 ± 0.28$^{c}$ | 12.67 ± 0.17$^{a}$ |
| myricetin | 0.98 ± 0.11$^{e}$ | 2.14 ± 0.25$^{d}$ | 9.07 ± 0.37$^{c}$ | 10.45 ± 0.18$^{b}$ | 8.67 ± 0.38$^{c}$ | 11.32 ± 0.42$^{a}$ |
| quercetin | ND | ND | ND | ND | ND | ND |
| apigenin | ND | ND | ND | ND | ND | ND |
| kaempferol | ND | ND | ND | ND | ND | ND |
| ***Total*** | **1.21 ± 0.30$^{e}$** | **3.42 ± 0.31$^{d}$** | **17.72 ± 0.27$^{c}$** | **21.32 ± 0.47$^{b}$** | **17.43 ± 0.17$^{c}$** | **23.99 ± 0.40$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.
ND: Not detected.

**Table 4.14** The effect of extraction method on content of phenolic and flavonoid compounds of SPE in Nangsew strains.

| Parameters | Extraction method | | | | | |
|---|---|---|---|---|---|---|
| | Water extraction (hrs) | | | | | Enzyme |
| | control | 2 | 4 | 6 | 8 | |
| Phenolic acid content (µg/g DW) | | | | | | |
| gallic acid | 1.32 ± 0.76$^{f}$ | 2.86 ± 1.01$^{e}$ | 6.56 ± 0.71$^{d}$ | 14.87± 0.58$^{b}$ | 10.30 ± 0.55$^{c}$ | 17.90 ± 0.37$^{a}$ |
| protocatechuic acid | 2.97 ± 0.37$^{f}$ | 8.54 ± 0.74$^{e}$ | 10.09 ± 0.56$^{d}$ | 18.85 ± 1.01$^{b}$ | 16.09 ± 0.62$^{c}$ | 20.50 ± 0.45$^{a}$ |
| *p*-hydroxybenzoic acid | 0.12 ± 0.21$^{f}$ | 1.23 ± 0.33$^{e}$ | 2.07 ± 0.48$^{d}$ | 25.66 ± 0.52$^{b}$ | 20.38 ± 1.08$^{c}$ | 26.56 ± 0.55$^{a}$ |
| chlorogenic acid | ND | ND | ND | ND | ND | ND |
| vanillic acid | ND | ND | ND | ND | ND | ND |
| caffeic acid | ND | ND | ND | ND | ND | ND |
| syringic acid | ND | ND | ND | ND | ND | ND |
| *p*-coumaric acid | ND | ND | ND | ND | ND | ND |
| ferulic acid | 1.98 ± 0.44$^{f}$ | 3.98 ± 0.27$^{e}$ | 7.37 ± 0.36$^{d}$ | 14.66 ± 0.74$^{b}$ | 10.35 ± 0.24$^{c}$ | 15.44 ± 0.28$^{a}$ |
| sinapic acid | 2.01 ± 0.70$^{f}$ | 6.02 ± 0.54$^{e}$ | 10.00 ± 0.73$^{d}$ | 18.75 ± 0.38$^{b}$ | 13.81 ± 0.29$^{c}$ | 20.38 ± 0.35$^{a}$ |
| ***Total*** | **8.4 ± 0.33$^{f}$** | **22.63 ± 0.17$^{e}$** | **36.09± 0.32$^{d}$** | **92.79 ± 0.19$^{b}$** | **70.93 ± 0.27$^{c}$** | **100.78 ± 0.50$^{a}$** |
| Flavonoid content (µg/g DW) | | | | | | |
| rutin | 0.76 ± 0.23$^{f}$ | 4.67 ± 0.30$^{e}$ | 10.96 ± 0.28$^{d}$ | 20.88 ± 0.19$^{b}$ | 18.07 ± 0.25$^{c}$ | 21.67 ± 0.20$^{a}$ |
| myricetin | 1.22 ± 0.41$^{f}$ | 8.54 ± 0.36$^{e}$ | 15.09 ± 0.16$^{d}$ | 19.08 ± 0.17$^{b}$ | 17.98 ± 0.43$^{c}$ | 20.32 ± 0.22$^{a}$ |
| quercetin | ND | ND | ND | ND | ND | ND |
| apigenin | ND | ND | ND | ND | ND | ND |
| kaempferol | ND | ND | ND | ND | ND | ND |
| ***Total*** | **1.98 ± 0.37$^{f}$** | **13.21 ± 0.45$^{e}$** | **26.05 ± 0.50$^{d}$** | **39.96 ± 0.56$^{b}$** | **36.05 ± 0.31$^{c}$** | **41.99 ± 0.44$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.
ND: Not detected.

**Table4.15** The effect of extraction method on content of phenolic and flavonoid compounds of SPE in Nangtui strains.

| Parameters | Extraction method | | | | | |
|---|---|---|---|---|---|---|
| | Water extraction (hrs) | | | | | Enzyme |
| | control | 2 | 4 | 6 | 8 | |
| Phenolic acid content (µg/g DW) | | | | | | |
| gallic acid | 1.01 ± 0.54$^{f}$ | 4.39 ± 0.31$^{e}$ | 8.95 ± 0.22$^{d}$ | 16.98 ± 0.17$^{b}$ | 10.56 ± 0.36$^{c}$ | 18.09 ± 0.13$^{a}$ |
| protocatechuic acid | 2.13± 1.01$^{f}$ | 8.44 ± 0.75$^{e}$ | 17.89 ± 0.43$^{d}$ | 28.90 ± 0.40$^{b}$ | 26.09 ± 0.38$^{c}$ | 29.90 ± 0.35$^{a}$ |
| *p*-hydroxybenzoic acid | 0.32 ± 0.24$^{f}$ | 2.26 ± 0.58$^{e}$ | 7.09± 0.37$^{d}$ | 15.11 ± 0.28$^{b}$ | 10.65 ± 0.43$^{c}$ | 22.08 ± 0.51$^{a}$ |
| chlorogenic acid | ND | ND | ND | ND | ND | ND |
| vanillic acid | ND | ND | ND | ND | ND | ND |
| caffeic acid | ND | ND | ND | ND | ND | ND |
| syringic acid | ND | ND | ND | ND | ND | ND |
| *p*-coumaric acid | ND | ND | ND | ND | ND | ND |
| ferulic acid | 1.09 ± 0.61$^{f}$ | 6.67 ± 1.03$^{e}$ | 9.42 ± 0.32$^{d}$ | 16.32 ± 0.51$^{b}$ | 10.81 ± 0.66$^{c}$ | 18.18 ± 0.44$^{a}$ |
| sinapic acid | 1.45 ± 0.32$^{f}$ | 7.36 ± 0.74$^{e}$ | 9.64 ± 0.42$^{d}$ | 19.03 ± 0.39$^{b}$ | 17.23 ± 0.41$^{c}$ | 21.24 ± 0.37$^{a}$ |
| ***Total*** | **6.00 ± 0.56$^{f}$** | **29.12±0.65$^{e}$** | **52.99± 0.38$^{d}$** | **96.34±0.43$^{b}$** | **75.34±0.52$^{c}$** | **109.49 ± 0.43$^{a}$** |
| Flavonoid content (µg/g DW) | | | | | | |
| rutin | 1.87 ± 0.46$^{f}$ | 6.89 ± 0.46$^{e}$ | 16.28 ± 0.27$^{d}$ | 28.76 ± 0.42$^{b}$ | 20.53 ± 0.42$^{c}$ | 29.09 ± 0.37$^{a}$ |
| myricetin | 1.79 ± 0.27$^{f}$ | 5.56 ± 0.66$^{e}$ | 10.17 ± 0.29$^{d}$ | 20.14± 0.30$^{b}$ | 14.47± 0.29$^{c}$ | 22.90 ± 0.51$^{a}$ |
| quercetin | ND | ND | ND | ND | ND | ND |
| apigenin | ND | ND | ND | ND | ND | ND |
| kaempferol | ND | ND | ND | ND | ND | ND |
| ***Total*** | **3.66 ± 0.39$^{f}$** | **12.45 ± 0.52$^{e}$** | **26.45 ± 0.31$^{d}$** | **48.90 ± 0.38$^{b}$** | **35.00 ± 0.41$^{c}$** | **51.99 ± 0.53$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.
ND: Not detected.

**Table 4.16** The effect of extraction method on content of phenolic and flavonoid compounds of SPE in Eri strains.

| Parameters | Extraction method | | | | | |
|---|---|---|---|---|---|---|
| | Water extraction (hrs) | | | | | Enzyme |
| | control | 2 | 4 | 6 | 8 | |
| Phenolic acid content (µg/g DW) | | | | | | |
| gallic acid | 1.98 ± 0.15$^{f}$ | 2.98 ± 0.43$^{e}$ | 4.09 ± 0.22$^{d}$ | 6.50 ± 0.41$^{b}$ | 5.30 ± 0.38$^{c}$ | 9.13 ± 0.44$^{a}$ |
| protocatechuic acid | 0.87 ± 0.27$^{e}$ | 1.22 ± 0.19$^{d}$ | 2.76 ± 0.48$^{c}$ | 4.78 ± 0.47$^{b}$ | 3.06 ± 0.46$^{c}$ | 11.43± 0.40$^{a}$ |
| *p*-hydroxybenzoic acid | 0.11± 0.33$^{e}$ | 0.90 ± 0.28$^{d}$ | 1.07 ± 0.25$^{c}$ | 2.09 ± 0.32$^{b}$ | 1.50 ± 0.57$^{c}$ | 7.14 ± 0.26$^{a}$ |
| chlorogenic acid | ND | ND | ND | ND | ND | ND |
| vanillic acid | ND | ND | ND | ND | ND | ND |
| caffeic acid | ND | ND | ND | ND | ND | ND |
| syringic acid | ND | ND | ND | ND | ND | ND |
| *p*-coumaric acid | ND | ND | ND | ND | ND | ND |
| ferulic acid | 2.00 ± 0.52$^{f}$ | 5.98 ± 0.41$^{e}$ | 6.08 ± 0.31$^{d}$ | 10.07± 0.28$^{b}$ | 8.08 ± 0.16$^{c}$ | 11.65 ± 0.12$^{a}$ |
| sinapic acid | 1.24± 0.28$^{f}$ | 3.11 ± 0.26$^{e}$ | 5.40 ± 0.39$^{d}$ | 7.89 ± 0.29$^{b}$ | 6.12 ± 0.13$^{c}$ | 9.43 ± 0.23$^{a}$ |
| ***Total*** | **6.20± 0.32$^{f}$** | **14.28± 0.39$^{e}$** | **19.40 ± 0.37$^{d}$** | **31.33 ± 0.31$^{b}$** | **24.06± 0.39$^{c}$** | **48.78 ± 0.35$^{a}$** |
| Flavonoid content (µg/g DW) | | | | | | |
| rutin | 0.80 ± 0.44$^{f}$ | 1.87 ± 0.40$^{e}$ | 3.55 ± 0.22$^{d}$ | 5.11 ± 0.17$^{b}$ | 4.12 ± 0.19$^{c}$ | 6.56 ± 0.33$^{a}$ |
| myricetin | 0.56 ± 0.37$^{f}$ | 1.08 ± 0.38$^{e}$ | 3.60 ± 0.31$^{d}$ | 6.98± 026$^{b}$ | 5.08 ± 0.24$^{c}$ | 7.19 ± 0.40$^{a}$ |
| quercetin | ND | ND | ND | ND | ND | ND |
| apigenin | ND | ND | ND | ND | ND | ND |
| kaempferol | ND | ND | ND | ND | ND | ND |
| ***Total*** | **1.36 ± 0.29$^{f}$** | **2.95 ± 0.43$^{e}$** | **7.15 ± 0.35$^{d}$** | **12.09 ± 0.32$^{b}$** | **9.20 ± 0.25$^{c}$** | **13.75 ± 0.31$^{a}$** |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the row were significant differences at $p < 0.05$.
ND: Not detected.

( 3) DPPH radical scavenging activity, Ferric reducing antioxidant power (FRAP) assay and Antioxidant activity by ABTS assay.

The extraction method with water at 6 hours provided the highest DPPH radical-scavenging activity, ABTS+• and FRAP assay when compared to other times, they increased until 6 hours, after which they decreased. However, enzyme extraction was better that all values for water extraction (Table 4.17). SPE of Nangtui strains for water extraction at 6 hours and enzyme extraction provided DPPH radical-scavenging activity, ABTS+• and FRAP assay values of 24.40% inhibition, 30.13% inhibition and 5.11 mg FeSO4/g DW respectively, and 25.87% inhibition, 31.09% inhibition and 6.10 mg FeSO4/ g DW, respectively.

**Table 4.17** The effect of extraction method on DPPH radical-scavenging activity, $ABTS^{+\bullet}$ assay and FRAP assay of SPE in Thai silk strains.

| Thai silk strains | Extraction method | DPPH radical-scavenging activity (% inhibition) | $ABTS^{+\bullet}$ (% inhibition) | FRAP assay (mg $FeSO_4$/ g DW) |
|---|---|---|---|---|
| Leaungsaraburi | 1) Water extraction | | | |
| | control | 1.51 ± 0.19$^{f}$ | 2.09 ± 0.15$^{f}$ | 1.08 ± 0.17$^{e}$ |
| | 2 hrs | 2.79 ± 0.07$^{e}$ | 9.37 ± 0.21$^{e}$ | 1.28 ± 0.26$^{d}$ |
| | 4 hrs | 10.62 ± 0.07$^{d}$ | 16.26 ± 0.19$^{d}$ | 1.32 ± 0.24$^{cd}$ |
| | 6 hrs | 16.33 ± 0.03$^{b}$ | 23.22 ± 0.20$^{b}$ | 1.55 ± 0.14$^{b}$ |
| | 8 hrs | 15.30 ± 0.06$^{c}$ | 22.09 ± 0.22$^{c}$ | 1.40 ± 0.12$^{c}$ |
| | 2) Enzymatic extraction | 17.07 ± 0.11$^{a}$ | 24.62 ± 0.18$^{a}$ | 1.70 ± 0.02$^{a}$ |
| Nangsew | 1) Water extraction | | | |
| | control | 2.17± 0.12$^{f}$ | 2.81 ± 0.08$^{f}$ | 1.24 ±0.13$^{e}$ |
| | 2 hrs | 4.68 ± 0.11$^{e}$ | 7.86 ± 0.15$^{e}$ | 3.16 ± 0.24$^{d}$ |
| | 4 hrs | 13.66 ±0.09 $^{d}$ | 15.99 ± 0.26$^{d}$ | 4.59 ± 0.32$^{c}$ |
| | 6 hrs | 20.90 ± 0.07$^{b}$ | 23.94 ± 0.24$^{b}$ | 5.01 ± 0.22$^{b}$ |
| | 8 hrs | 18.88 ± 0.04$^{c}$ | 22.87 ± 0.31$^{c}$ | 4.53 ± 0.13$^{c}$ |
| | 2) Enzymatic extraction | 21.27 ± 0.14$^{a}$ | 24.51 ± 0.19$^{a}$ | 5.78 ± 0.42$^{a}$ |
| Nangtui | 1) Water extraction | | | |
| | control | 1.81± 0.26$^{f}$ | 2.10 ± 0.07$^{f}$ | 1.26 ±0.11$^{f}$ |
| | 2 hrs | 3.69 ± 0.06$^{e}$ | 11.88 ± 0.16$^{e}$ | 3.73 ± 0.04$^{e}$ |
| | 4 hrs | 11.99 ± 0.04$^{d}$ | 13.50 ± 0.11$^{d}$ | 4.78 ± 0.02$^{d}$ |
| | 6 hrs | 24.40 ± 0.10$^{b}$ | 30.13 ± 0.12$^{b}$ | 5.11 ± 0.06$^{b}$ |
| | 8 hrs | 22.01 ± 0.10$^{c}$ | 25.37 ± 0.28$^{c}$ | 5.07 ± 0.02$^{c}$ |
| | 2) Enzymatic extraction | 25.87 ± 0.23$^{a}$ | 31.09 ± 0.09$^{a}$ | 6.10 ± 0.02$^{a}$ |
| Eri | 1) Water extraction | | | |
| | control | 1.03± 0.14$^{f}$ | 1.58 ± 0.11$^{e}$ | 1.02 ±0.17$^{f}$ |
| | 2 hrs | 2.28 ± 0.18$^{e}$ | 3.25 ± 0.20$^{d}$ | 2.11 ± 0.24$^{e}$ |
| | 4 hrs | 5.21 ±0.2c$^{d}$ | 4.20 ± 0.26$^{c}$ | 2.68 ± 0.16$^{d}$ |
| | 6 hrs | 12.36 ± 0.19$^{b}$ | 4.88 ± 0.17$^{b}$ | 3.45 ± 0.22$^{b}$ |
| | 8 hrs | 11.33 ± 0.13$^{c}$ | 4.19 ± 0.28$^{c}$ | 3.37 ± 0.19$^{c}$ |
| | 2) Enzymatic extraction | 13.87 ± 0.20$^{a}$ | 5.32 ± 0.24$^{a}$ | 3.61 ± 0.10$^{a}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column within the same strains were significantly different at the level $p < 0.05$.

( 4) SPE has the ability of anti-AGEs formation activity for water extraction at 6 hours; this was the highest when compared to other times and it increased until 6 hours and then decreased. However, enzymatic extraction provided better inhibition than water extraction (Table 4.18). AGEs provide a reaction between sugar and protein causing the protein to behave unusually in some people. For people with diabetes who have up to 50 times higher blood sugar than normal people, the chance of a high glycation reaction causes the protein to malfunction. Is a cause of complications in patients with diabetes (Singh et al. 2 0 1 6 ). The anti-glycation capacity of numerous medicinal herbs and dietary plants was comparable or even stronger than that of amino guanidine (Bernonville et al. 2010). Several studies have demonstrated that the anti-glycation activity correlates significantly with the phenolic content of the tested plant extracts (Safari et al. 2018). Therefore, SPE of Nangtui strains for water extraction at 6 hours and enzyme extraction has total phenolic contents of 87.92 and 87.21 mg GAE/g DW, respectively, and cause to anti-AGEs formation activity.

(5) Inhibitory activity against enzyme α-amylase and α-glucosidase.

SPE has inhibitory activity against the enzymes α-amylase and α-glucosidase. This activity was highest for water extraction at 6 hours when compared to other times; it increased until 6 hours after which it decreased. However, enzymatic extraction provided better inhibition than water-extracted protein ( Table 4.18). The enzymes α-amylase and α-glucosidase have important roles in catalyzing the hydrolysis of starch into glucose, resulting in the absorption of glucose from the small intestine into the blood stream, reducing the condition of high blood sugar. Many studies report that plants with flavonoid compounds and phenolic compounds from many medicinal plants have effective inhibitory α-amylase and α-glucosidase (Yin et al. 2014). Some similar substances, such as myricetin, can be found in cashew leaves. It is thus significant that in addition, SPE (water or enzyme extracted) from Nangtui strains have total flavonoid contents of 49.10 and 51.87 mg GAE/g DW respectively, thus capable of causing inhibitory activity against the enzymes α-amylase and α-glucosidase.

**Table 4.18** The effect of extraction method on anti-AGEs formation activity, inhibitory activity against the enzyme α-amylase and inhibitory activity against the enzyme α-glucosidase of SPE from Thai silk strains.

| Thai silk strains | Extraction method | Anti-AGEs formation activity (% inhibition) | Inhibitory activity against enzyme α-amylase (% inhibition) | Inhibitory activity against enzyme α-glucosidase (% inhibition) |
|---|---|---|---|---|
| Leaungsaraburi | 1) Water extraction control | 1.51 ± 0.19$^{f}$ | 2.09 ± 0.15$^{f}$ | 1.08 ± 0.17$^{f}$ |
| | 2 hrs | 2.79 ± 0.07$^{e}$ | 4.37 ± 0.21$^{e}$ | 2.28 ± 0.26$^{e}$ |
| | 4 hrs | 10.62 ± 0.07$^{d}$ | 16.26 ± 0.19$^{d}$ | 3.32 ± 0.24$^{d}$ |
| | 6 hrs | 16.33 ± 0.03$^{b}$ | 23.22 ± 0.20$^{b}$ | 4.55 ± 0.14$^{b}$ |
| | 8 hrs | 15.30 ± 0.06$^{c}$ | 22.09 ± 0.22$^{c}$ | 4.00 ± 0.12$^{c}$ |
| | 2) Enzymatic extraction | 17.07 ± 0.11$^{a}$ | 24.62 ± 0.18$^{a}$ | 5.07 ± 0.02$^{a}$ |
| Nangsew | 1) Water extraction control | 2.17± 0.12$^{f}$ | 2.81 ± 0.08$^{f}$ | 1.24 ±0.13$^{e}$ |
| | 2 hrs | 4.68 ± 0.11$^{e}$ | 7.86 ± 0.15$^{e}$ | 3.16 ± 0.04$^{d}$ |
| | 4 hrs | 14.66 ±0.09 $^{d}$ | 15.99 ± 0.26$^{d}$ | 4.59 ± 0.02$^{c}$ |
| | 6 hrs | 21.90 ± 0.07$^{b}$ | 24.94 ± 0.24$^{b}$ | 5.51 ± 0.02$^{b}$ |
| | 8 hrs | 18.88 ± 0.04$^{c}$ | 22.87 ± 0.31$^{c}$ | 4.53± 0.03$^{c}$ |
| | 2) Enzymatic extraction | 22.27 ± 0.14$^{a}$ | 25.51 ± 0.19$^{a}$ | 6.78 ± 0.02$^{a}$ |
| Nangtui | 1) Water extraction control | 2.26 ± 0.64$^{f}$ | 1.11 ± 0.20$^{e}$ | 1.60 ± 0.36$^{f}$ |
| | 2 hrs | 11.60 ± 0.70$^{e}$ | 3.91 ± 0.18$^{d}$ | 4.50 ± 0.51$^{e}$ |
| | 4 hrs | 15.60 ± 0.39$^{d}$ | 17.66 ± 0.35$^{c}$ | 7.95 ± 0.22$^{d}$ |
| | 6 hrs | 25.07 ± 0.73$^{b}$ | 29.40 ± 0.13$^{a}$ | 10.58 ± 0.39$^{b}$ |
| | 8 hrs | 26.57 ± 0.94$^{c}$ | 22.00 ± 0.29$^{b}$ | 9.65 ± 0.17$^{c}$ |
| | 2) Enzymatic extraction | 26.11 ± 0.69$^{a}$ | 30.67 ± 0.37$^{a}$ | 11.26 ± 0.36$^{a}$ |
| Eri | 1) Water extraction control | 1.58 ± 0.83$^{f}$ | 1.06 ± 0.15$^{f}$ | 0.69 ± 0.51$^{f}$ |
| | 2 hrs | 3.46 ± 0.97$^{e}$ | 3.60 ± 0.20$^{e}$ | 1.15 ± 0.37$^{e}$ |
| | 4 hrs | 5.55 ± 0.48$^{d}$ | 5.65 ± 0.19$^{d}$ | 2.29 ± 0.26$^{d}$ |
| | 6 hrs | 9.30 ± 0.73$^{b}$ | 7.91 ± 0.30$^{b}$ | 3.63 ± 0.35$^{b}$ |
| | 8 hrs | 8.56 ± 0.69$^{c}$ | 6.03 ± 0.27$^{c}$ | 3.11 ± 0.41$^{c}$ |
| | 2) Enzymatic extraction | 10.68 ± 0.77$^{a}$ | 8.60 ± 0.38$^{a}$ | 4.84 ± 0.36$^{a}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column within the same strains were significantly different at the level $p < 0.05$.

(6) Estimation of molecular mass of SPE by SDS-PAGE

The SDS-PAGE results showed that the silk protein molecules break down after 4 hours of water extraction. The molecular weights of protein in Leaungsaraburi strains showed thin protein bands at 4-8 hours; these were in range of 10-20 and 35-45kDa whereas using the enzyme, the molecular weights of extracted proteins were in the range of 25-45kDa. The molecular weights of extracted proteins in Nangsew and Nangtui strains showed thick and clear protein bands at 4-8 hours; they were in the range of 10-45, and over 75kDa whereas using the enzyme, the molecular weights of proteins were in the range of 25-45kDa.

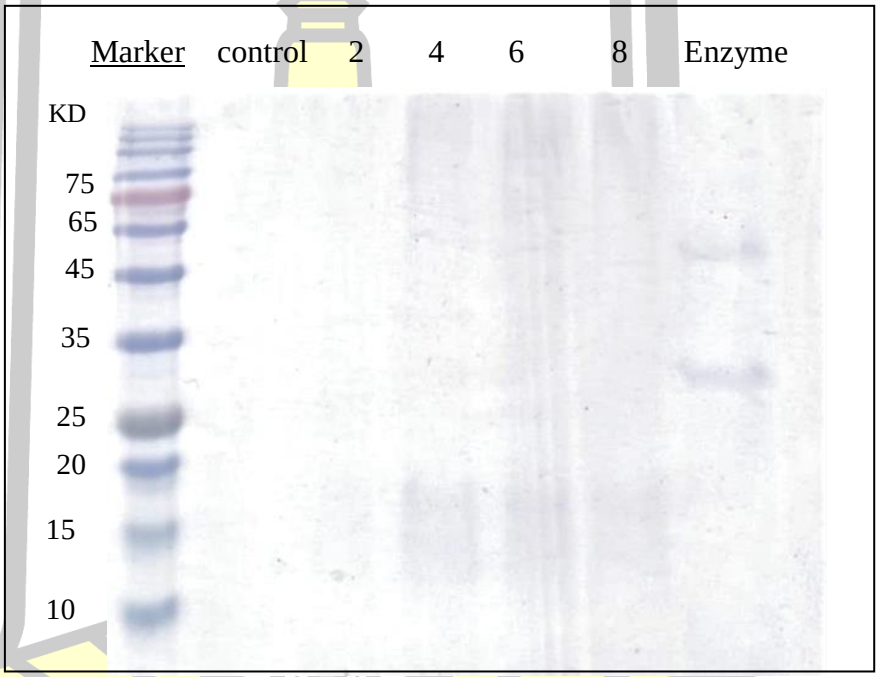



**Figure 4.8** The effect of extraction method (hours of water extraction) on SDS-PAGE profiles of extracts from Leaungsaraburi strains.

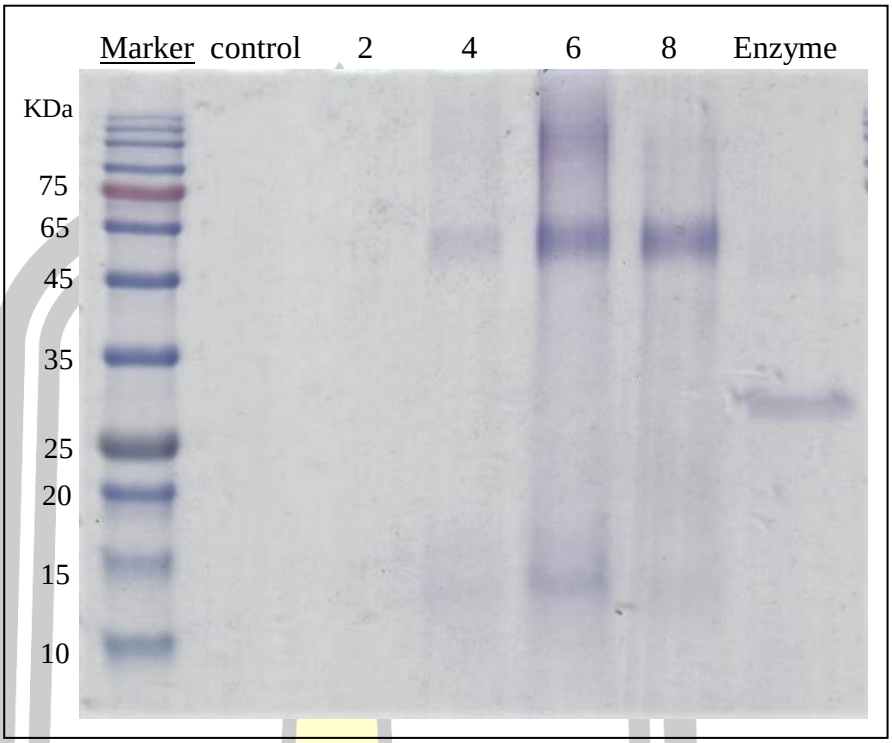



**Figure 4.9** The effect of extraction method (hours of water extraction) on SDS-PAGE profiles of extracts from Nangsew strains.

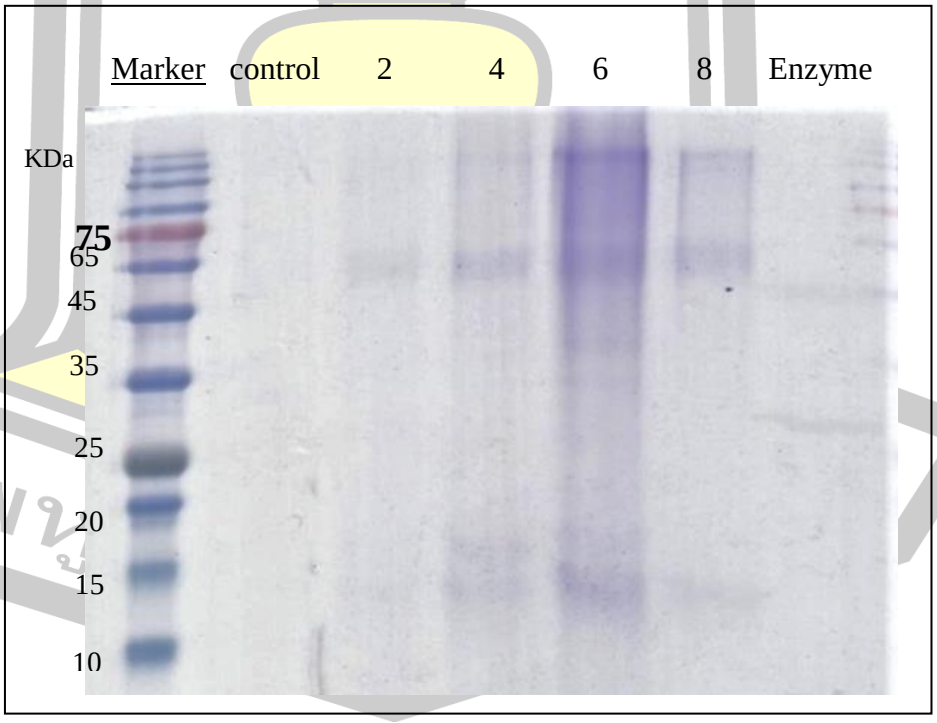
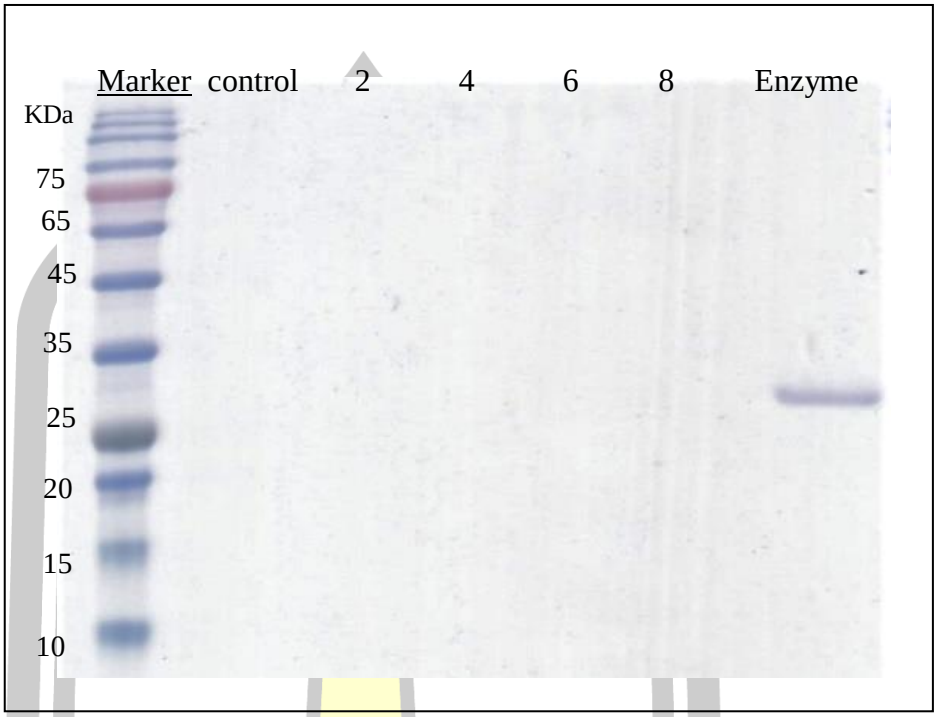



**Figure 4.10** The effect of extraction method (hours of water extraction) on SDS-PAGE profiles of extracts from Nangtui strains.




**igure 4.11** The effect of extraction method (hours of water extraction) on SDS-PAGE profiles of extracts from Eri strains.

( 7) The microstructure of silk cocoons after extraction. SEM and expanded image sizes at 200x and 1,200x showed that the silk fibers were progressively shrinking and that the size of the silk was smaller at 2-4 hrs. After that, it was shrinking more than before and continued to fracture, until becoming almost completely broken. (Figure 4.12-4.15).

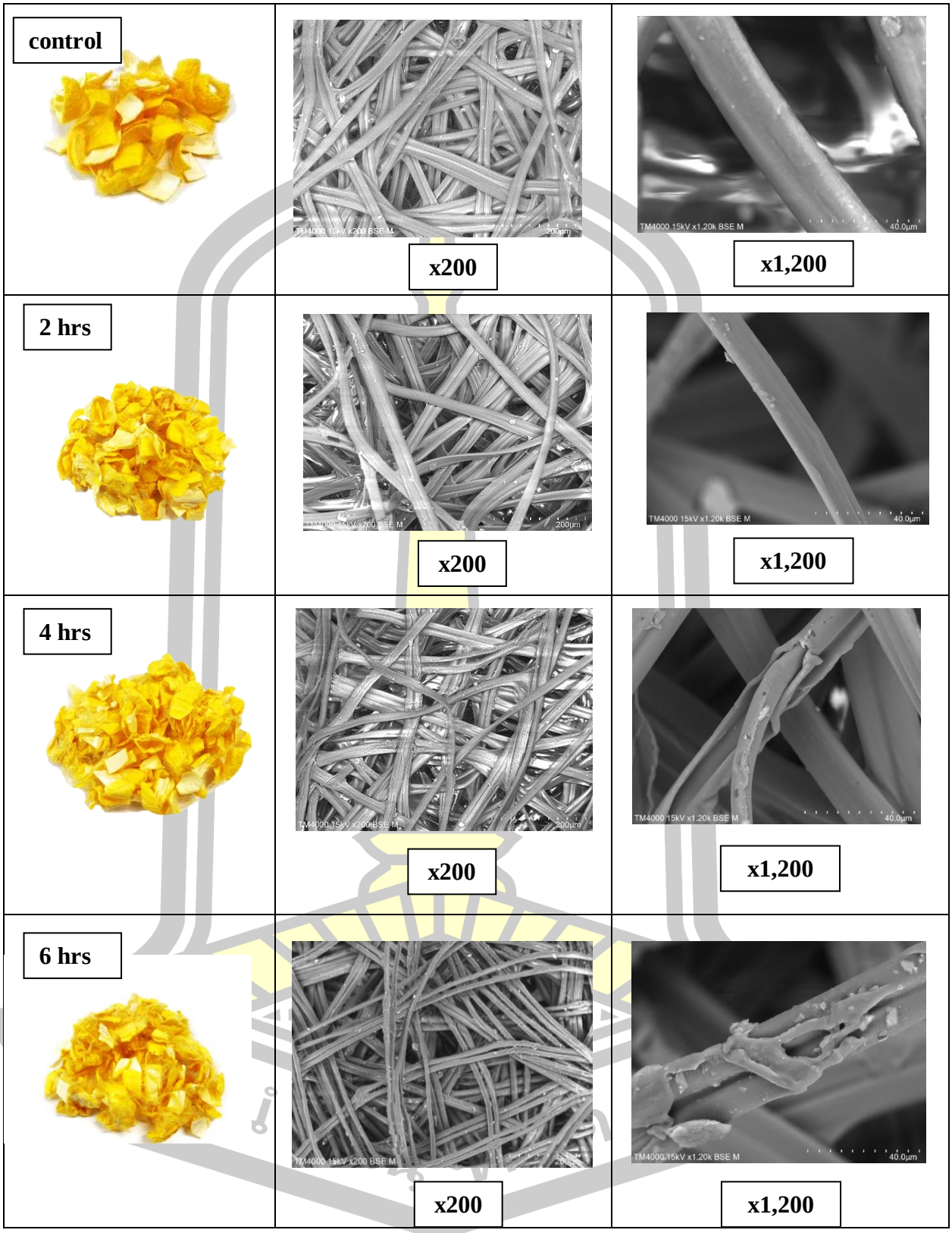



**Figure 4.12** The effect of extraction method on the microstructure of Leaungsaraburi strains**.**

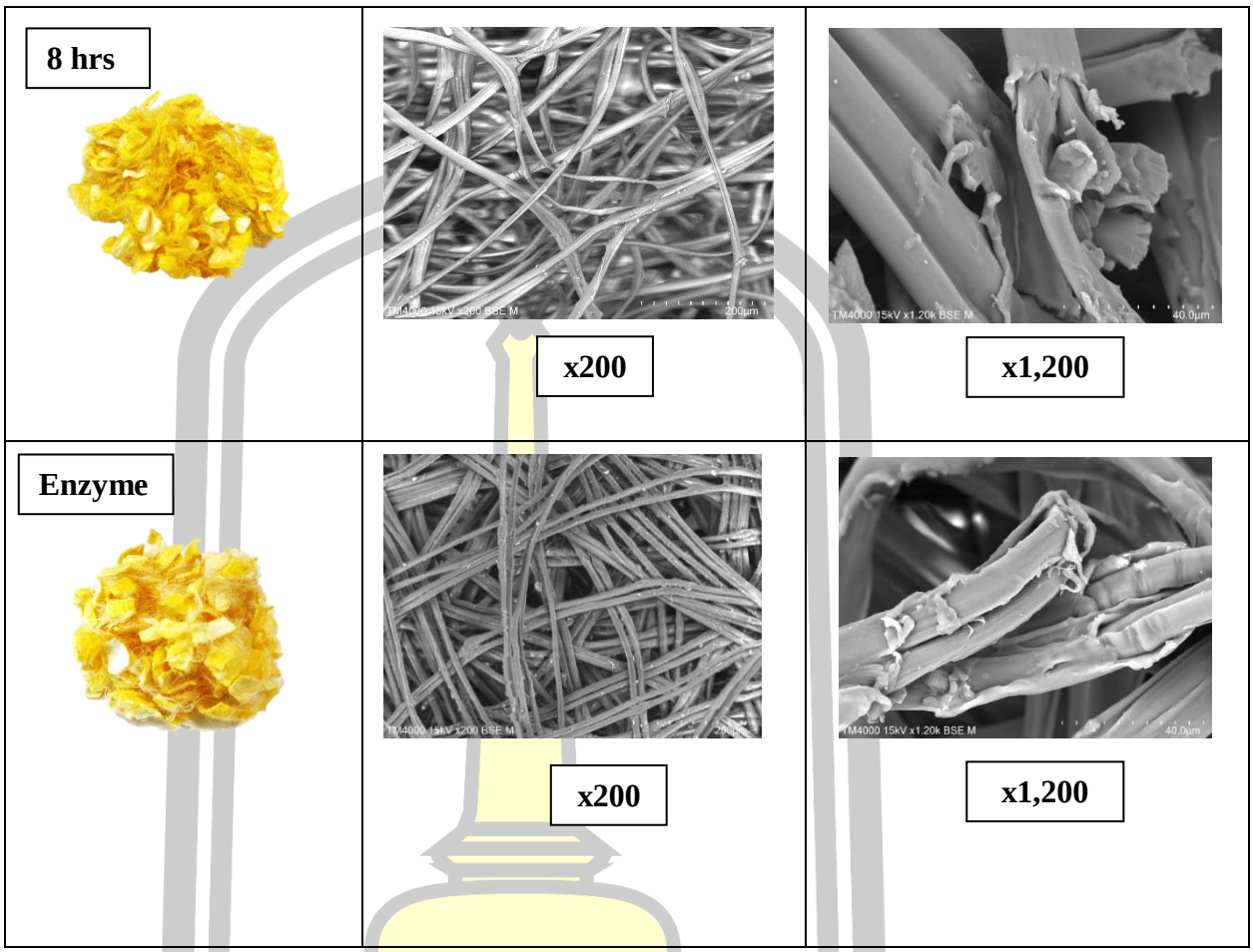



**Figure 4.12** The effect of extraction method on the microstructure of Leaungsaraburi strains. (continued)

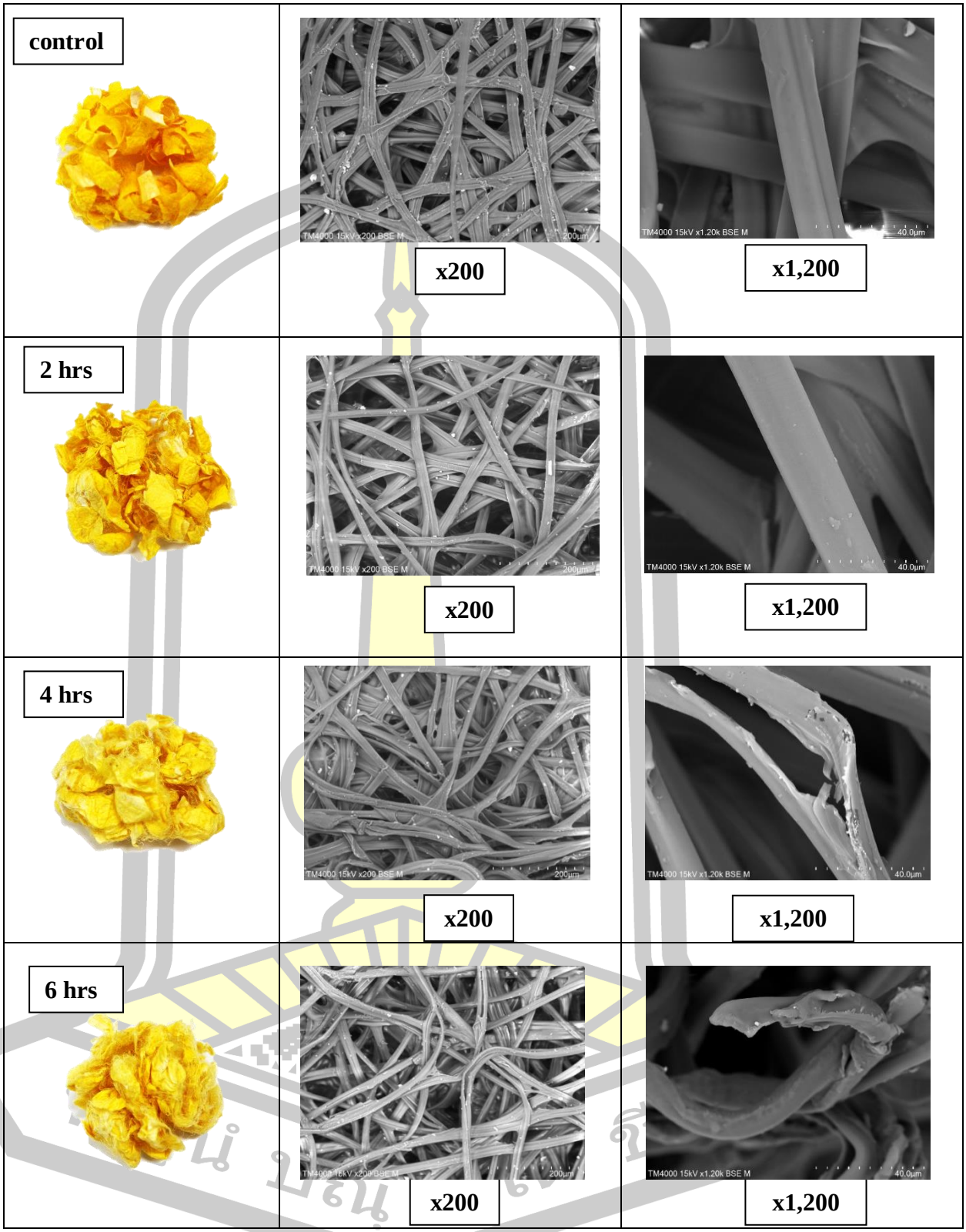



**Figure 4.13** The effect of extraction method on the microstructure of Nangsew strains.

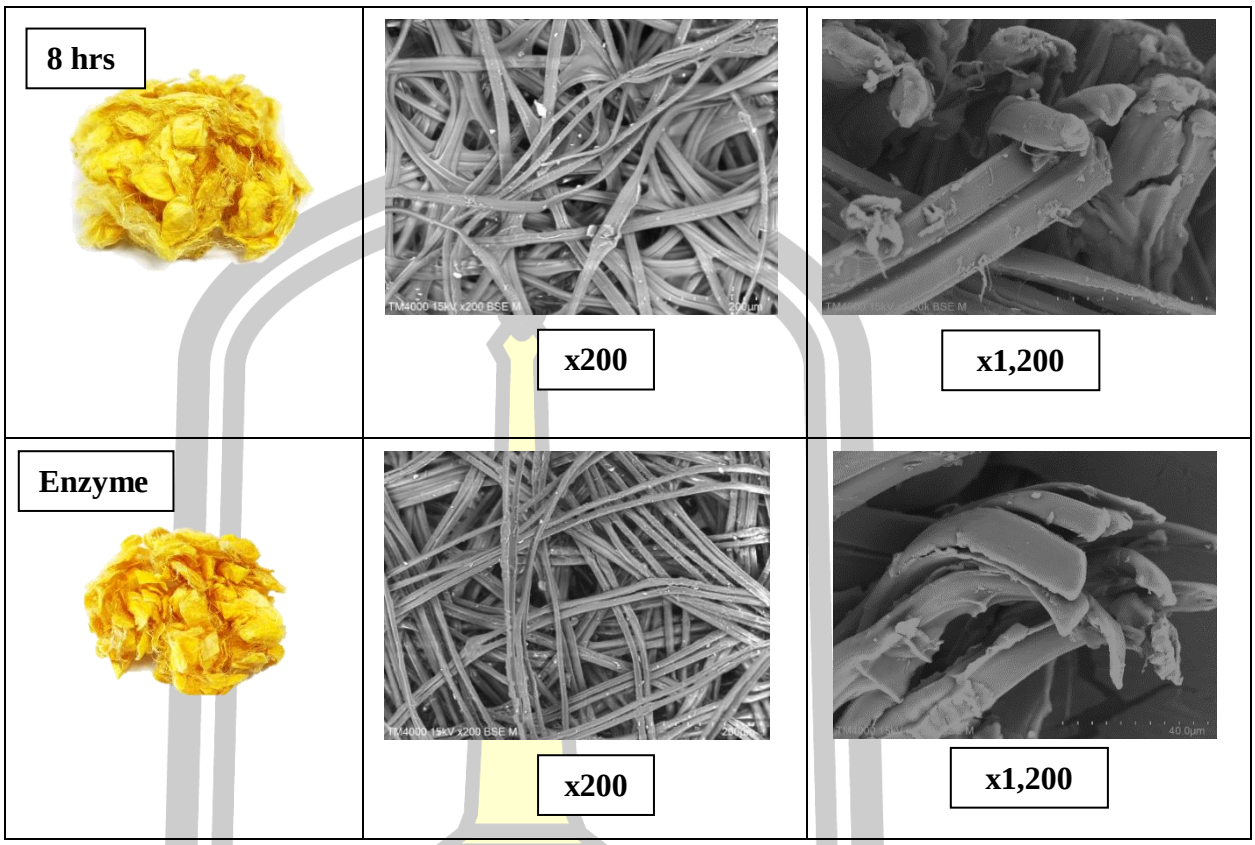



**Figure 4.13** The effect of extraction method on the microstructure of Nangsew strains. (continued)

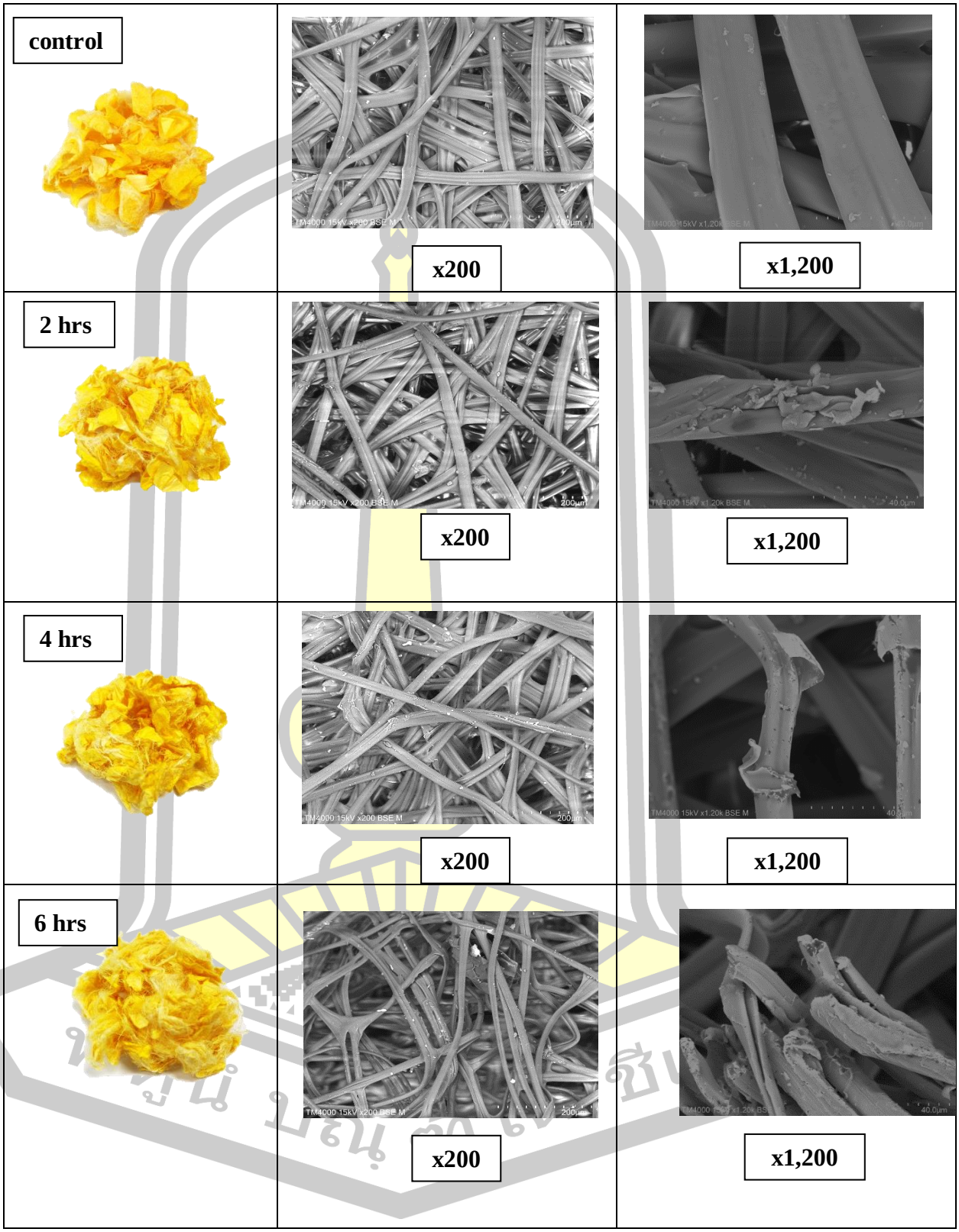



**Figure 4.14** The effect of extraction method on the microstructure of Nangtui strains.

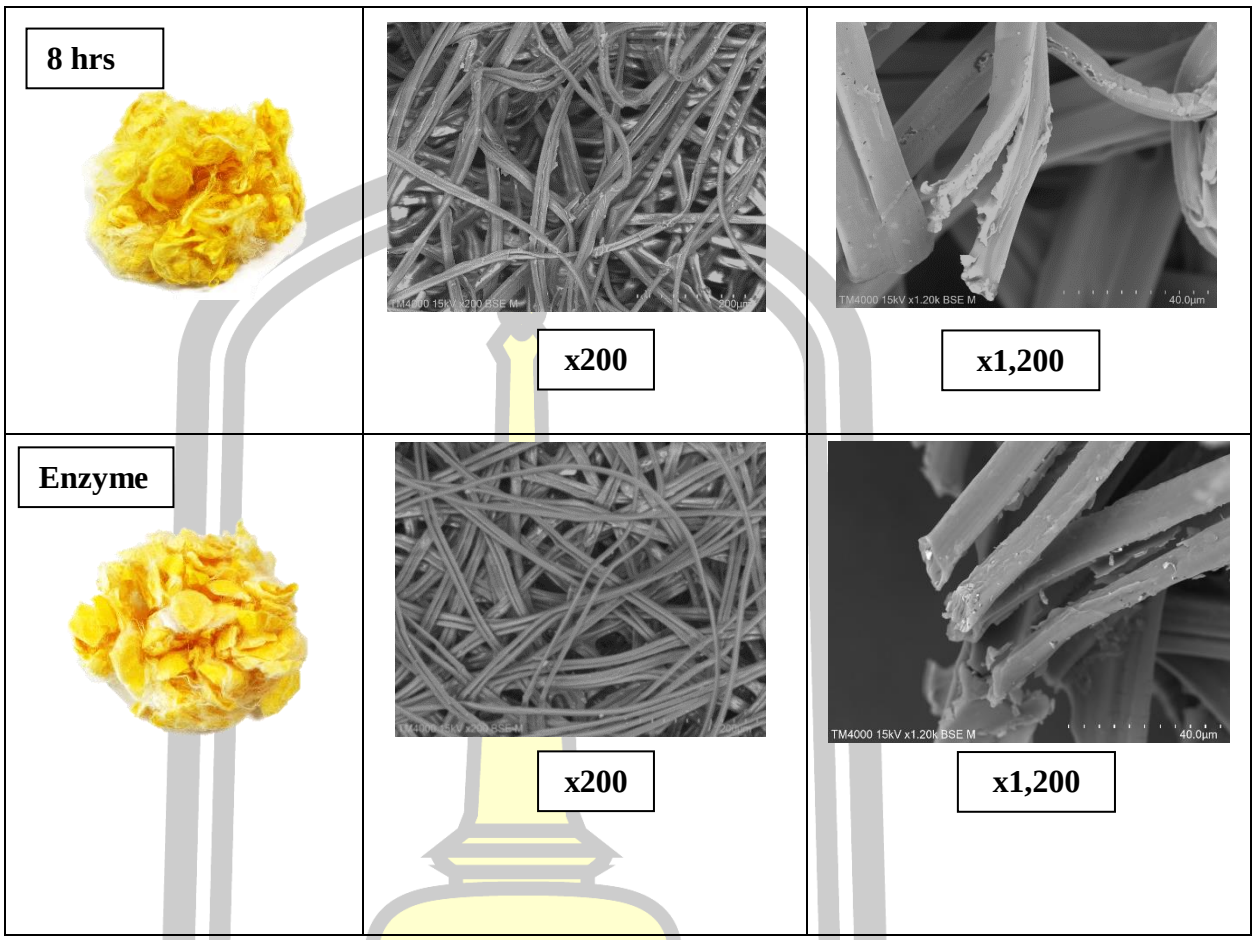



**Figure 4.14** The effect of extraction method on the microstructure of Nangtui strains. (continued)

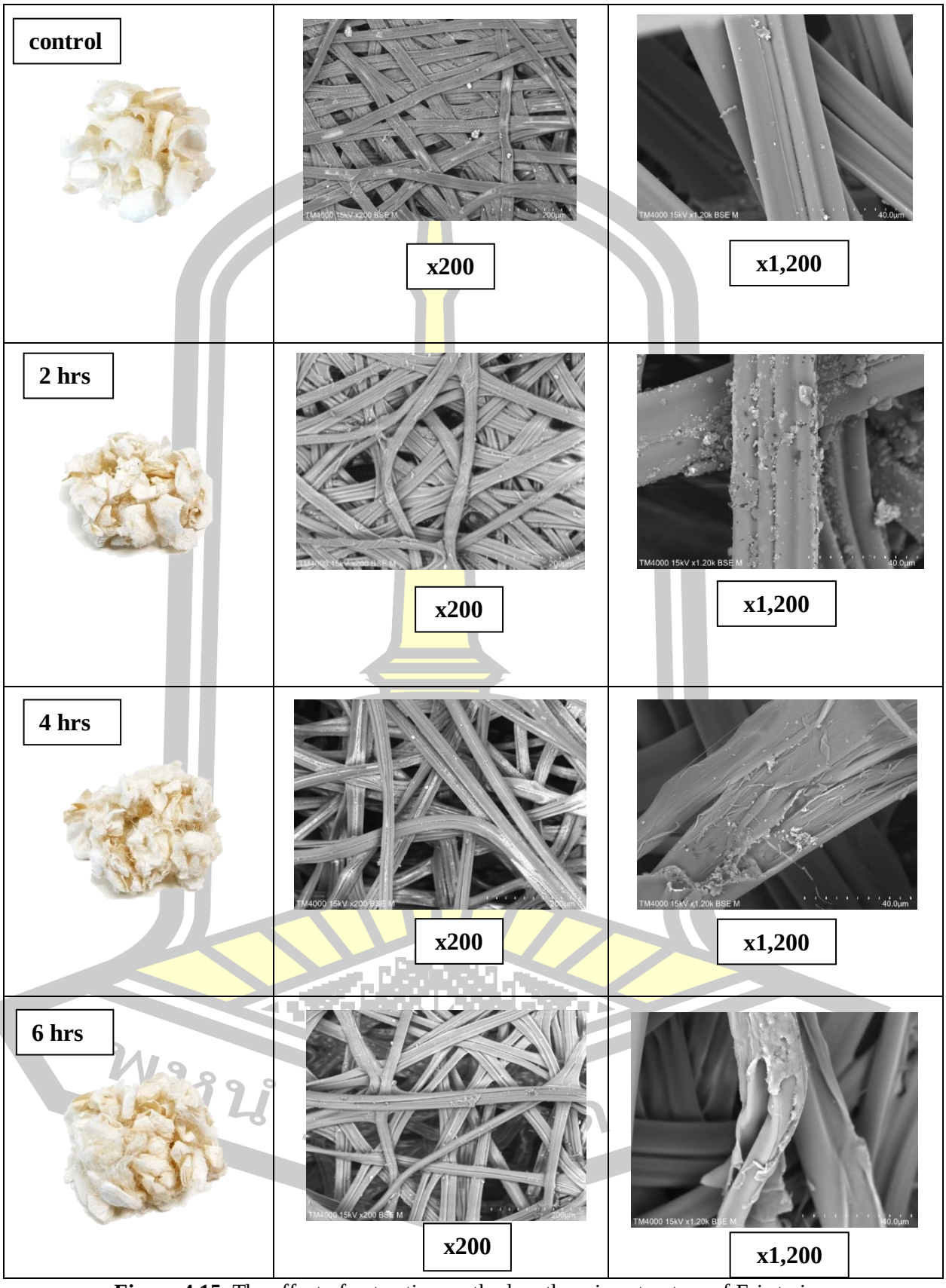



**Figure 4.15** The effect of extraction method on the microstructure of Eri strains.

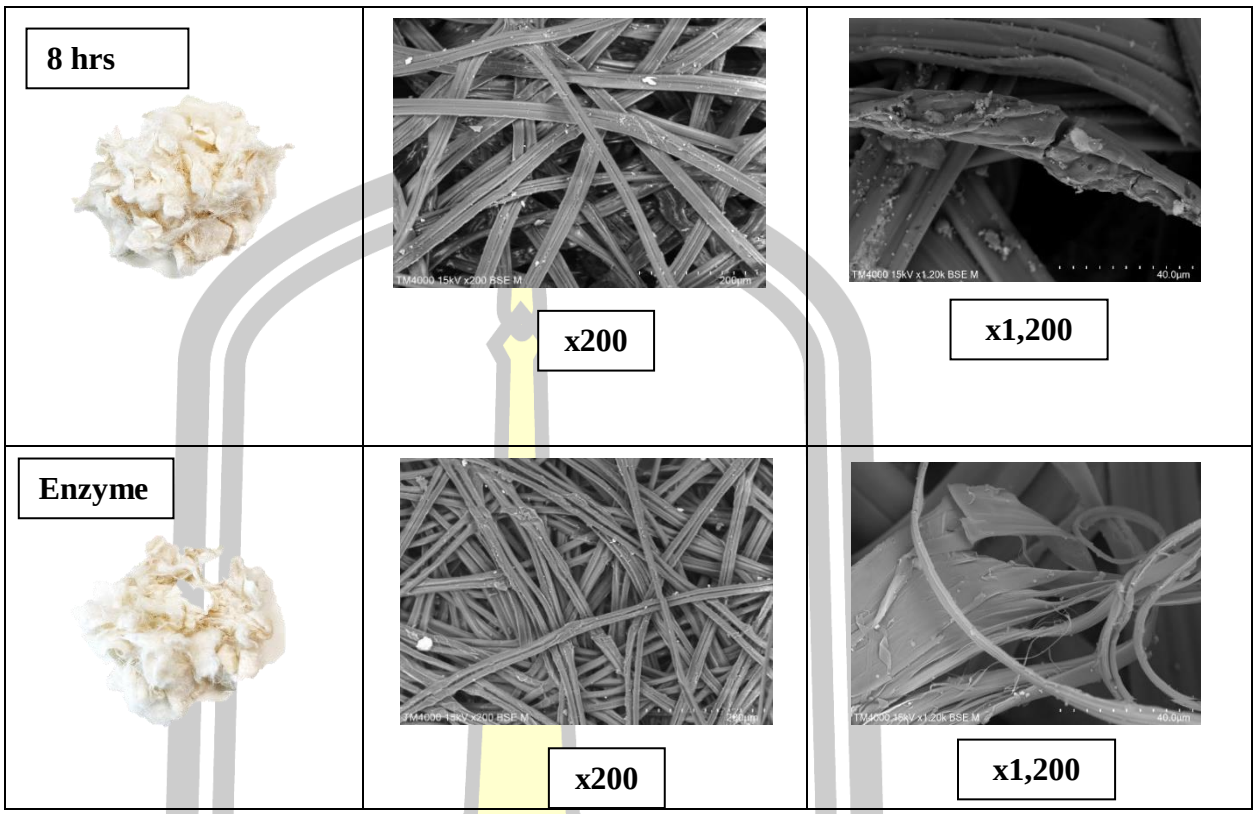



**Figure 4.15** The effect of extraction method on the microstructure of Eri strains. (continued)

Considering all the above experimental results, the water extraction method at 6 hours for each of the strains gave the best overall value. Of the various strains and when comparing the statistics, it was clear that the Nangtui strain was the best of all the strains (Table 4.19). When considering the enzyme extraction method, it was found that Nangtui is the best of the strains as well (Table 4.20). Therefore, the decision was made to use Nangtui for product development.

**Table 4.19** The effect of water extraction at 6 hrs on biological properties of SPE in Thai silk strains.

| Thai silk strains | Total phenolic content (mg GAE/gDW) | Total flavonoid content (mg RE/gDW) | DPPH radical-scavenging activity (% inhibition) | $ABTS^{+\bullet}$ (% inhibition) | FRAP assay (mg $FeSO_4$/ g DW) | Anti-AGEs formation activity (% inhibition) | Inhibitory activity against enzyme α-amylase (% inhibition) | Inhibitory activity against enzyme α-glucosidase (% inhibition) |
|---|---|---|---|---|---|---|---|---|
| Leaungsaraburi | $63.24 \pm 1.12^{c}$ | $43.27 \pm 1.19^{c}$ | $16.33 \pm 0.03^{c}$ | $23.22 \pm 0.20^{c}$ | $1.55 \pm 0.14^{c}$ | $16.33 \pm 0.03^{c}$ | $23.22 \pm 0.20^{c}$ | $4.55 \pm 0.14^{c}$ |
| Nangsew | $78.89 \pm 1.82^{b}$ | $47.29 \pm 1.11^{b}$ | $20.90 \pm 0.07^{b}$ | $23.94 \pm 0.24^{b}$ | $5.01 \pm 0.22^{b}$ | $21.90 \pm 0.07^{b}$ | $24.94 \pm 0.24^{b}$ | $5.51 \pm 0.02^{b}$ |
| Nangtui | $87.92 \pm 1.08^{a}$ | $49.10 \pm 1.07^{a}$ | $24.40 \pm 0.10^{a}$ | $30.13 \pm 0.12^{a}$ | $5.11 \pm 0.06^{a}$ | $25.07 \pm 0.73^{a}$ | $29.40 \pm 0.13^{a}$ | $10.58 \pm 0.39^{a}$ |
| Eri | $32.06 \pm 1.14^{d}$ | $12.67 \pm 1.13^{d}$ | $12.36 \pm 0.19^{d}$ | $4.88 \pm 0.17^{d}$ | $3.45 \pm 0.22^{d}$ | $9.30 \pm 0.73^{d}$ | $7.91 \pm 0.30^{d}$ | $3.63 \pm 0.35^{d}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column were significantly different at the level $p < 0.05$.

**Table 4.20** The effect of enzyme extraction on biological properties of SPE in Thai silk strains.

| Thai silk strains | Total phenolic content (mg GAE/gDW) | Total flavonoid content (mg RE/gDW) | DPPH radical-scavenging activity (% inhibition) | $ABTS^{+\bullet}$ (% inhibition) | FRAP assay (mg $FeSO_4$/ g DW) | Anti-AGEs formation activity (% inhibition) | Inhibitory activity against enzyme α-amylase (% inhibition) | Inhibitory activity against enzyme α-glucosidase (% inhibition) |
|---|---|---|---|---|---|---|---|---|
| Leaungsaraburi | $69.42 \pm 1.01^{c}$ | $48.64 \pm 1.16^{c}$ | $17.07 \pm 0.11^{a}$ | $24.62 \pm 0.18^{c}$ | $1.70 \pm 0.02^{c}$ | $18.07 \pm 0.11^{c}$ | $24.62 \pm 0.18^{c}$ | $5.07 \pm 0.02^{c}$ |
| Nangsew | $81.25 \pm 1.16^{b}$ | $50.38 \pm 1.19^{b}$ | $21.27 \pm 0.14^{a}$ | $24.51 \pm 0.19^{b}$ | $5.78 \pm 0.42^{b}$ | $22.27 \pm 0.14^{b}$ | $25.51 \pm 0.19^{b}$ | $6.78 \pm 0.02^{b}$ |
| Nangtui | $87.21 \pm 1.03^{a}$ | $51.87 \pm 1.27^{a}$ | $25.87 \pm 0.23^{a}$ | $31.09 \pm 0.09^{a}$ | $6.10 \pm 0.02^{a}$ | $26.11 \pm 0.69^{a}$ | $30.67 \pm 0.37^{a}$ | $11.26 \pm 0.36^{a}$ |
| Eri | $40.09 \pm 1.21^{d}$ | $14.09 \pm 1.40^{d}$ | $13.87 \pm 0.20^{a}$ | $5.32 \pm 0.24^{d}$ | $3.61 \pm 0.10^{d}$ | $10.68 \pm 0.77^{d}$ | $8.60 \pm 0.38^{d}$ | $4.84 \pm 0.36^{d}$ |

Values are expressed as mean ± standard deviation (n = 3). Means with different letters in the column were significantly different at the level $p < 0.05$.

## 4.3 Product development of SPE and assessment of its stability for chemical and biological properties of the developed products

4.3.1 Justification of funding for product development

According to the toxicity assessment by NR assay (Pocasap et al. 2018). The samples tested did not show toxicity to the cells studied in Cancer cells in the breast (MCF-7), lung (HCT116) and in normal cells that are not cancerous cells (Vero) as Figure 4.16.

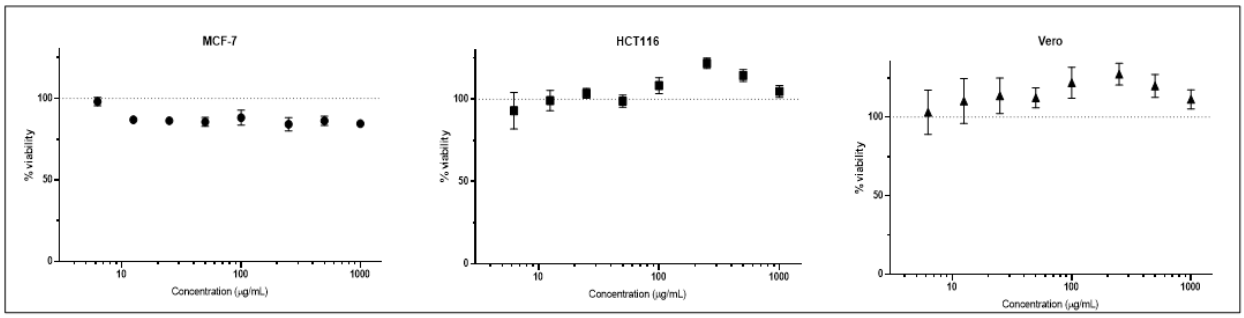



**Figure 4.16** The effect of Cell lines and culture and Cell cytotoxicity by NR assay on silk protein extract.

However, the major concern of the product is its bitter taste. Subjectively, we had tried the extract together with someone from the company (Siam Natural Product, Co. ltd). It was quite obvious that water extracts provided greater taste with lesser bitterness than the enzyme extract. More importantly, the operating costs of enzyme treatment would be rather more expensive for industry. It can be summarized in Table 4.21.

**Table 4.21** Details of operating cost for SPE.

| Method | Ingredient cost (Bath) | Operating cost (Bath) | Total/125 ml (Bath) |
|---|---|---|---|
| Water extraction (Silk cocoon+ Water) | 5+0.11 **(5.11)** | 5 | 30 |
| Enzyme extraction (Silk cocoon+ Water + enzyme) | 5+0.11+76 **(81.11)** | 10 | 150 |

4.3.2 Formulation of silk protein drink (SPD) development from SPE.

By using the mixture design program studies, two factors were significant: the amount of silk protein extract (90-100%) and of honey (0-10%) to develop drink products. When inputting the program, there are seven formulas (Table 4.22), pasteurization at 80°C for 15 min (Petruzzi et al. 2017) and testing for sensory evaluation by 30 panelists using the 9-point hedonic scale.

**Table 4.22** The formulas of sensory tests of SPD.

| Formula | SPE (%) (X1) | Honey (%) (X2) |
|---|---|---|
| 1 | 100.00 | 0.00 |
| 2 | 93.33 | 6.67 |
| 3 | 90.00 | 10.00 |
| 4 | 92.50 | 7.50 |
| 5 | 96.66 | 3.37 |
| 6 | 97.50 | 2.50 |
| 7 | 95.00 | 5.00 |

The sensory evaluations results showed that the appearance was neither ‘like’ to ‘like slightly’ (5.50-6.50), color was ‘dislike slightly’ to ‘like slightly’ (4.93-6.20), odor was ‘dislike slightly’ to ‘like moderately’ (4.73-7.20), taste was ‘dislike slightly’ to ‘like moderately’ (4.20-7.73) and overall acceptance is in the range ‘dislike slightly’ to ‘like moderately’ (4.21-7.63) (Table 4.23).

**Table 4.23** Sensory evaluations of SPD.

| Formula | X1 | X2 | Appearance$^{ns}$ | Color$^{ns}$ | Odor | Taste | Overall acceptance |
|---|---|---|---|---|---|---|---|
| 1 | 100 | 0 | 5.50±0.73 | 5.63±0.54 | 5.11±0.43$^{e}$ | 4.20±0.61$^{e}$ | 4.21±0.75$^{f}$ |
| 2 | 93.33 | 6.67 | 6.20±0.75 | 5.93±0.61 | 5.90±0.61$^{c}$ | 5.83±0.53$^{c}$ | 6.10±0.61$^{c}$ |
| 3 | 90 | 10 | 5.80±0.43 | 6.17±0.49 | 6.30±0.53$^{b}$ | 6.53±0.51$^{b}$ | 6.66±0.49$^{b}$ |
| 4 | 92.50 | 7.50 | 6.50±0.51 | 6.20±0.73 | 7.20±0.41$^{a}$ | 7.73±0.48$^{a}$ | 7.63±0.51$^{a}$ |
| 5 | 96.67 | 3.33 | 5.91±0.61 | 5.93±0.64 | 5.20±0.55$^{e}$ | 4.70±0.59$^{d}$ | 4.91±0.46$^{e}$ |
| 6 | 97.50 | 2.50 | 5.62±0.50 | 5.20±0.71 | 4.73±0.64$^{f}$ | 4.00±0.91$^{e}$ | 4.32±0.45$^{f}$ |
| 7 | 95.00 | 5.00 | 5.84±0.41 | 5.80±0.55 | 5.60±0.56$^{d}$ | 5.53±0.51$^{c}$ | 5.40±0.62$^{d}$ |

Values are expressed as mean ± standard deviation (n = 30). Means with different letters in the same column were significantly different at the level $p < 0.05$. Refer to Table 4.22 for ingredients X1 and X2.

The appearance, color, odor, taste and overall acceptance results were used to create regressions to find the best model with the software program Design-Expert 7 and to explain the relationship of this results with the studied factors, namely, the amount of silk protein extract (X1) and honey (X2). The regression analysis showed that the model relating the quality values and factors suitable for the products by considering P-value less than 0.05, show that the main hypothesis is rejected and conclude that there is at least one parameter that is not zero. In addition, the R-squared ($R^2$) and adjusted R-squared (Adj. $R^2$) values are the highest value and the closest approach to 1, which shows that the regression data is appropriate.

**Table 4.24** Mathematical model of sensory evaluations of SPD

| Parameters | Predicted model equations [a] | $R^2$ | Adj. $R^2$ | Model P-value |
|---|---|---|---|---|
| Appearance | Y1= 5.55(X1)+6.54(X2) | 0.62 | 0.24 | 0.22 |
| Color | Y2= 5.32(X1)+6.52(X2)-1.16 (X1) (X2) | 0.87 | 0.75 | 0.21 |
| Odor | Y3= 4.73(X1)+6.70(X2) | 0.61 | 0.54 | 0.03* |
| Taste | Y4= 3.83(X1)+7.14(X2) | 0.71 | 0.65 | 0.01* |
| Overall acceptance | Y5= 3.95(X1)+7.22(X2) | 0.77 | 0.72 | 0.00* |

Regression equation : $Yi = \beta X1+\beta X2 +\beta X1X2$ , Y1-5 = sensory evaluations β = The coefficient of variables in the regression equation : X1 = amount of silk protein extract, X2= amount of honey, R2 = Decision coefficient and *The factors studied have a significant effect on the response value calculated from the model at the level 95% ($p<0.05$).

The regression analysis shows that the relationship model between the factors studied in odor can explain the relationship due to the model having a P-value of less than 0.05, including $R^2$ and Adj. $R^2$ of 0.61 and 0.54, similar to the factors studied for taste and overall acceptance, the relationship can be explained due to the P-value model being less than 0.05, including $R^2$ and Adj. $R^2$ is 0.71, 0.65 and 0.77, 0.72 respectively, which has the highest value and the closest approximation to 1. It shows that the models of odor, taste and overall acceptance are able to explain the relation of amount of silk protein extract and honey at 95%. The model of the relationship between studied factors, appearance and color cannot explain the relationship due to the model having a P-value greater than 0.05, including $R^2$ and Adj. $R^2$ as low as 0.62, 0.24 and 0.87, 0.75 respectively (Table 4.24).

Prediction of optimum conditions for the amount of silk protein extract and honey in order to have good sensory evaluations. The regression analysis shows that the model of the relationship between the factors studied at odor, taste and overall acceptance can explain the relationship of the amount of silk protein extract and honey at 95%. In selecting the appropriate amount of ingredients, use the criteria considered in Table 4.26.

**Table 4.25** The conditions used for the selection of suitable amount of SPE and honey for formulation in SPD.

| Name | Goal |
|---|---|
| SPE | maximize |
| Honey | maximize |
| Appearance | none |
| Color | none |
| Odor | maximize |
| Taste | maximize |
| Overall acceptance | maximize |

**Table 4.26** Amount of silk protein extract and honey for formulation in SPD that is predicted using mathematical models of sensory evaluations.

| Parameters | Predicted values | |
|---|---|---|
| SPE | 92.39 | |
| Honey | 7.61 | |
| Odor | 6.98 | |
| Taste | 7.04 | |
| Overall acceptance | 7.13 | |
| **Desirability** | 0.78 | **Selected** |

4.3.4 Analysis of chemical and biological properties in SPD.

The suitable formulas of silk protein drink product are the amount of silk protein extract and honey; they were 92.39% and 7.61 % respectively (Figure 4.17). The chemical and biological properties higher than silk protein extract are shown in Table 4.27.

**Figure 4.17** The silk protein drink.

**Table 4.27** The chemical and biological properties in SPD and SPE.

| Parameter | SPD | SPE |
|---|---|---|
| Chemical properties | | |
| Protein (mg/g) | 6.32 | 2.13 |
| pH | 5.14 | 6.62 |
| Biological properties | | |
| Total phenolic content (mg GAE/g) | 100.18 | 87.92 |
| Total flavonoid content(mg RE/g) | 55.93 | 49.10 |
| DPPH radical-scavenging activity (% inhibition) | 30.17 | 24.40 |
| $ABTS^{+\bullet}$ (% inhibition) | 47.79 | 30.13 |
| Anti-AGEs formation activity (% inhibition) | 28.82 | 25.07 |
| Inhibitory activity against enzyme α-amylase (% inhibition) | 32.89 | 29.40 |
| Inhibitory activity against enzyme α-glucosidase (% inhibition) | 13.84 | 10.58 |

# CHAPTER 5

## Conclusions

The objectives of the present study included as follows:

1. To investigate the physical and chemical properties of silk cocoons from different strains.

2. To investigate the effects of extraction method on the physical, chemical and biological properties in silk protein extract and to optimize the extraction methods of silk protein extract.

3. To develop the process and product of functional foods from silk protein extracts.

The findings of the present research can be concluded as follows:

5.1 The physical and chemical properties of silk cocoon extracts varied among different strains.

5.2 The chosen extraction method for silk protein involved extraction with distilled water at 100°C for 6 hours; this procedure provided the highest values of protein content, amino acids, total phenolic content (TPC), total flavonoid content (TFC), DPPH radical-scavenging activity, ABTS radical scavenging capacity assay, FRAP assay, anti-glycation, α-amylase and α-glucosidase inhibitory activities when compared to other extraction procedures. The results showed that longer extraction time provided higher contents of all parameters when extraction times of 2-6 hrs. However, these values started to decline again after 6 hours' extraction. The enzymatic extraction gave the highest values of all parameters for all strains studied.

5.3 Two extraction methods studied have provided different effects on silk protein extracts. The enzyme extract received a lower sensory score than did water extraction because of an undesirable bitter taste; furthermore, the enzyme process is costly. Although both extraction methods have advantages and drawbacks, water extraction was selected. The development of SPD was successfully produced using a specific mixture. The most acceptable formula consists of 92.4% of silk protein extract and 7.6% of honey.

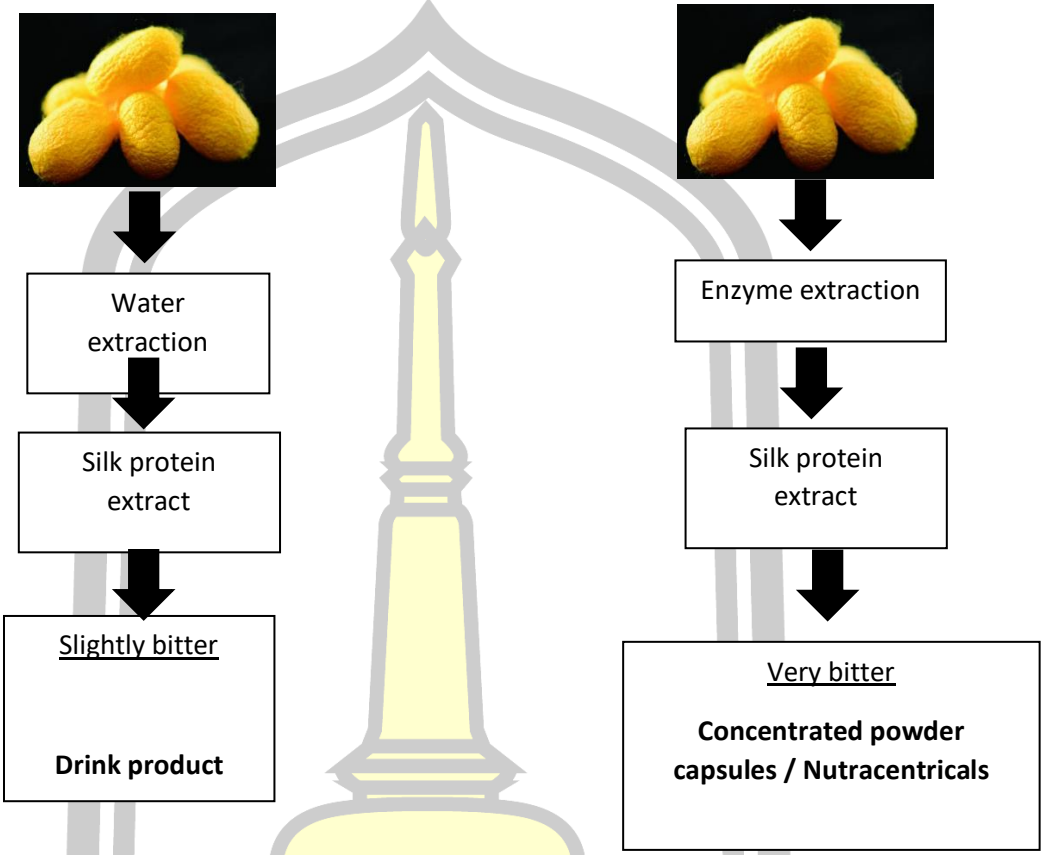



**Figure 5.18** Graphic information of using a silk protein extract for a function food product.

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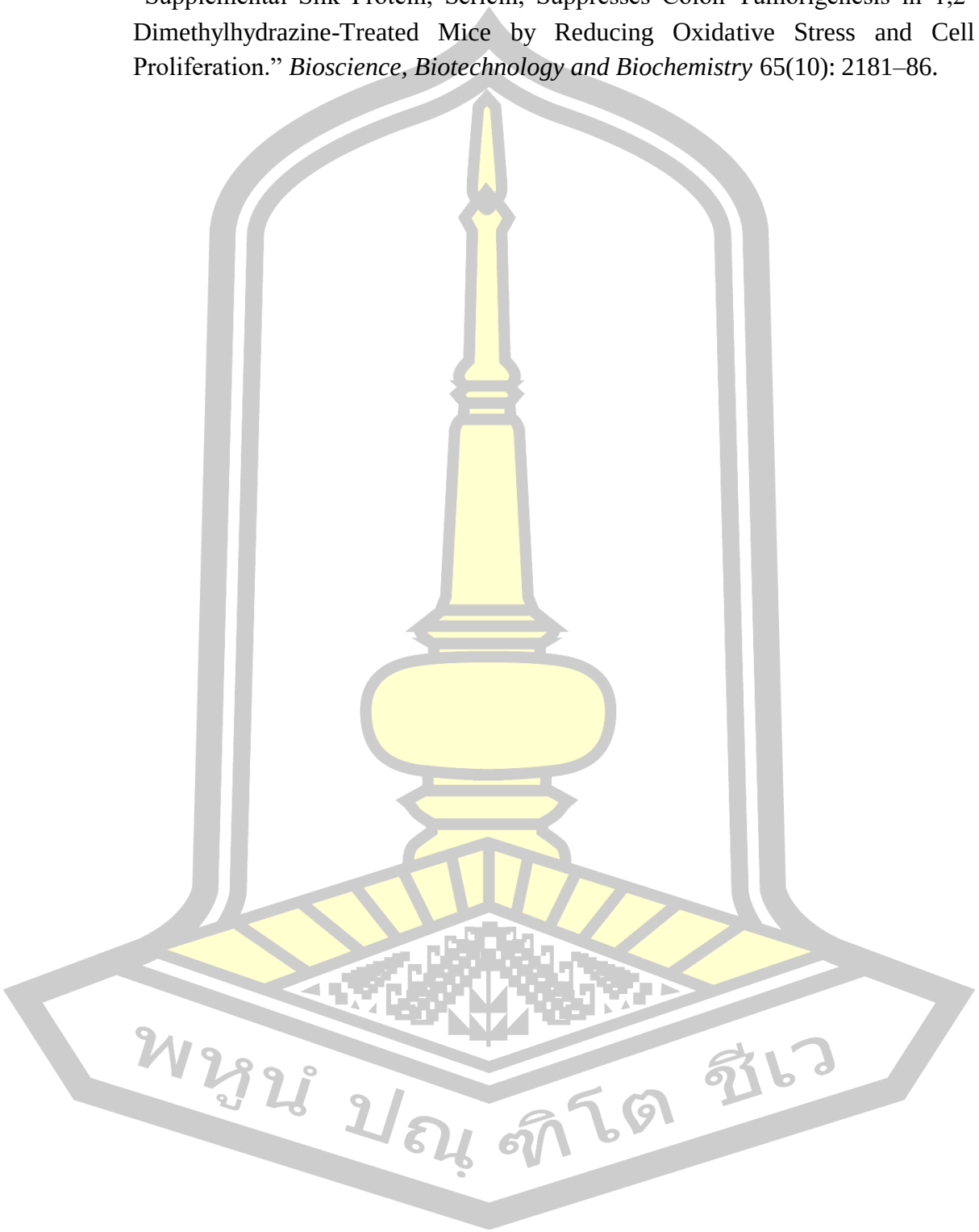

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