EXTRACTION AND CHARACTERIZATION OF PROTEIN FROM TEA SEED (*Camellia oleifera* Abel) MEAL

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

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EXTRACTION AND CHARACTERIZATION OF PROTEIN FROM TEA SEED (*Camellia oleifera* Abel) MEAL. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 104 PP.

EXTRACTION/TEA SEED MEAL/PROTEIN/CHARACTERIZATION/ FUNCTIONAL PROPERTIES

Seed meal of *Camellia oleifera* Abel is a byproduct after tea oil extraction. It is normally used as organic fertilizer with low economic value. The objective of this study was to isolate and characterize proteins from defatted seed meal of *C.oleifera* Abel. The optimal extraction condition was using deionized water at pH 7 as an extractant with the ratio of meal to water of 1:20, at 40^{o}C for 60 min. Protein recovery was 50.4%. The proteins were isolated using size exclusion, followed by anion exchange chromatography. The isolated protein showed light yellow appearance with relatively low phenolic content of 10.7%. Proteomic analysis including SDS-PAGE, 2-dimensional gel electrophoresis and LC-MS/MS were employed. SDS-PAGE analysis showed 6 major proteins in the tea seed meal and 5 major proteins in the tea seed protein isolated (TSPI). Two-dimensional gel electrophoresis revealed 12 major protein spots displaying molecular mass of 21-28 kDa with isoelectric points from 3.6-10. Protein identification by LC-MS/MS indicated peptide homology with G-type lectin S-receptor-like serine / threonine-protein kinase RLK-like from *Solanum lycopersicum*, 11s globulin-like protein from *Actinidia chinensis* and Argonaute protein

group from *Theobroma cacao*. The FT-IR spectra of purified tea seed protein exhibited glycoprotein characteristics. The TSPI showed good ABTS radical scavenging ability and metal chelation. In addition, isolated tea seed protein exhibited good functional properties as compared with casein. Emulsifying activity of isolated tea seed protein was higher than that of casein at pH 3 and 5 ($p < 0.05$). The foaming ability of isolated tea seed protein was lower than that of casein, except for pH 5. These results demonstrated that tea seed protein extracted from tea seed meal could be a potential source for protein recovery to be used as a functional protein ingredient.

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LIST OF ABBREVIATION

ABTS	=	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
ANS	=	8-anilino-1-naphthalenesulfonic acid
AOAC	=	Association of Official Chemists
Asp	=	Aspartic acid
BHA	=	Butylated hydroxyanisole
BHT	=	Butylated hydroxytoluene
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
cm^{-1}	=	Reciprocal centimeter
CID	=	Collision-induced dissociation
DI	=	Deionized water
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
DTSM	=	Defatted tea seed meal
DTT	=	Dithiotheitol
DEAE	=	Diethylaminoethyl
EAI	=	Emulsifying activity index
ESI	=	Emulsifying stability index

LIST OF ABBREVIATION (Continued)

ESI	=	Electrospray ionization
EDTA	=	Ethylenediaminetetraacetic acid
Fe	=	Iron
FA	=	Formic acid
FI	=	Fluorescence intensity
FRAP	=	Ferric reducing antioxidant power
FT-IR	=	Fourier transform – infrared spectroscopy
Glu	=	Glutamic acid
GSH-PX	=	Glutathione peroxidase
H	=	Hour
H_o	=	Surface hydrophobicity
HAT	=	Hydrogen atom transfer
HDRB	=	Heat-stabilized defatted rice bran
HDL	=	High density lipoprotein
IAA	=	Iodoacetamide
IEF	=	Isoelectric focusing electrophoresis
IPG	=	Immobilized pH gradient
kDa	=	Kilodalton
LC	=	Liquid chromatography

LIST OF ABBREVIATION (Continued)

LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
LDL	=	Low density- lipoprotein cholesterol
LOP	=	Lipidperoxides products
M	=	Molar
M	=	Marker
Mg	=	Milligram
mL	=	Milliliter
MS	=	Msaa spectroscopy
MALDI-TOF	=	Matrix assisted laser desorption/ionization-time of flight
mM	=	Millimolar (10^{-3}mol l^{-1})
µl	=	Microliter (10^{-6}l)
min	=	Minute
m/z	=	Mass per charge ratio
NSI	=	Nitrogen soluble index
NCBI	=	National Center for Biotechnology nonredundant
PAGE	=	Polyacrylamide gel electrophoresis
PC	=	Phenolic content
pI	=	Isolelectric point
PMF	=	mass finger printing

LIST OF ABBREVIATION (Continued)

PVPP	=	Polyvinyl-polypyrrolidone
RPI	=	Rapeseed protein isolate
SDS	=	Sodium dodecyl sulfate
SOD	=	Superoxide dismutase
SFE	=	Supercritical fluid extraction
TCA	=	Trichloroacetic acid
TEAC	=	Trolox Equivalent Antioxidant Capacity
TGh	=	Total cholesterols
TG	=	Triglycerides
TPZE	=	2, 4, 6-tripyridyl-s-triazine
Tris	=	Tris(hydroxymethyl)aminomethane
TSP	=	Tea seed protein
TXB2	=	Thromoboxane B2
WGPI	=	Wheat germ protein isolate
V	=	Volt

CHAPTER I

INTRODUCTION

1.1 Introduction

In recently, the use plant proteins as an alternative to animal proteins in the food industry has been arisen due to its nutritive values, bioactivity and different functional properties (Alonso et al., 2006; Arcan and Yemeniciogu, 2010). Proteins from oilseeds have been widely used as a functional protein ingredients e.g. soybean, sunflower (Salgado et al., 2012), canola (Manamperi et al., 2011) and rapeseed (Yoshie-Stark et al., 2006).

Camellia oleifera Abel is an evergreen shrub or small tree in the Camellia family, distributed in East and Southeast Asia. China is the world's largest *C.oleifera* Abel production (Fu and Zhou, 2003). It has been widely cultivated in four provinces of China including, Guizhou, Hunan, Jiangxi and Guangxi. The seed of *C.oleifera* Abel is an important woody oil material. The oil of *C.oleifera* Abel has been used extensively for producing cooking oil which has similar fatty acid profile with olive oil (Long and Wang, 2008). In addition, it is also traditionally applied as a medicine for stomachache and burning injury in China (Chen, Qin and Peng, 1998). The meal of *C.oleifera* Abel. is a by-product of oil production with about 2 million ton per year in China (Shen et al., 2010). It is usually used as organic fertilizer with low economic value (Chen et al., 2010). However, the defatted tea seed meal represent not only an

inexpensive resource but also rich bioactive compounds, such as proteins, phenolic compounds, saponis, etc (Wang and Wei, 1990). Zhang et al., (1992) evaluated the chemical composition of defatted tea seed meal and reported that it contained about 16.7% protein. They also found that it contained higher level of lysine, sulfur-containing amino acids as compared to corn and wheat. Therefore, it may be a good source of plant protein.

A larger number of methods have been used to isolate protein from oilseeds, based on their solubility behavior (Horax et al., 2010). Alkaline extraction is a common and simple method for plant protein extraction. It can result in relatively high yield (Shen et al., 2008). Ding et al., (2010) used alkaline extraction followed by acid precipitation for extraction of protein from tea seed meal with protein recovery of 57.8%. However, the tea seed contains large amounts of polyphenol compounds (Chen et al., 2010), which are readily oxidized and interact with protein by hydrogen bonding, during the alkaline processing. This can cause discoloration of the extracted protein (brown color) and unpleasant bitterness (Xu and Diosady, 2002). In contrast, extraction of protein in the acidic and neutral pH might reduce oxidation of phenolic compounds. Thus far, there is no study concerning the optimum conditions of protein extraction from tea seed meal to obtain high protein with low phenolic compounds.

Plant tissues have a relatively low content of soluble proteins (Saravanan and Rose, 2004) and some different plant-specific cellular components such as polysaccharides, lipids, phenolic compounds and secondary metabolites (Fröhlich and Lindermayr, 2011), which make it more difficult for protein identification. Hu et al.,

(2005) reported that some storage proteins have been found in *Camellia oleifera* seed such as *oleosin, lipidbodybrane, caleosin, steroleosin, globulin, glutelin* and *albumin.* However, proteome information available for *Camellia oleifera* seed are still limited. In recently, a combination of separation techniques and mass spectroscopy (MS) are the most important tools in protein identification. Esteve et al., (2012) used SDS-PAGE to separate the proteins from olive seed and pulp and applied LC-MS/MS to identify these proteins. A large number of compounds have been identified: 61 in the seed (only four reported in current literature) and 231 in the pulp (56 described so far). However, there is no research employed these techniques to identify proteins in the tea seed meal.

In order to prolong the storage stability of food, the synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) were applied in food industry. However, their undesirable side effects are liver damage and carcinogenesis (Soubra et al., 2007). Therefore, a natural antioxidant has been extensively studied to replace synthetic antioxidants such as flavones, polysaccharides, polyphenols and dietary proteins, (Li et al., 2013). Nowadays, many researchers reported that proteins isolated from various plant sources, such as *Curcuma comosa* (Boonmee et al., 2011), *Solanum torvum* seeds (Sivapriya and Srinivas, 2007), curry leaves (Ningappa and Srinivas, 2008) and *Terminalia chebula* fruit (Srivastava et al., 2012) also showed good antioxidant activity. Some antioxidants from tea seed meal have been reported thus far are polysaccharides (Jin and Ning, 2012) and flavonoid glycosides (Chen et al., 2010). However, there is no study concerning the antioxidant

activity of protein isolated from tea seed meal.

The objectives of this study were to optimize the protein extraction process from meal of tea seed at the neutral region (pH7) with respect to temperature, extraction time and solvent / meal ratio. The information of proteomes of purified tea seed protein was analyzed. Additionally, characterization, functional properties, and antioxidant activity of tea seed protein were also elucidated

1.2 Research objectives

The objectives of this research were:

1) To optimize the protein extraction process from tea seed (*Camellia oleifera* Abel) meal at the neutral region (pH 7) with respect to temperature, extraction time and solvent / meal ratio.
2) To evaluate of proteomes of protein isolated from tea seed (*Camellia oleifera* Abel) meal.
3) To characterize structure, functional properties and antioxidant activity of protein isolated from tea seed (*Camellia oleifera* Abel) meal.

1.3 Research hypotheses

High protein recovery of tea seed meal would be obtained under the optimal extraction condition. Isolation of tea seed protein could be achieved through chromatographic technique, resulting in the removal of phenolic compounds and brown pigments. Proteins isolated from tea seed meal could be a novel source and

exhibit some functional properties for food application.

1.4 Scope of the study

The optimum condition of protein extraction from tea seed (*Camellia oleifera* Abel) meal were studied. Tea seed meal protein was isolated using Hi-Trap desalting and ion exchange chromatography. Tea seed meal protein isolate was separated on two-dimensional gel electrophoresis and identified using LC-MS/MS. Characteristics of tea seed meal protein isolate was studied, including secondary structure and surface characteristics. Functional properties, namely solubility, emulsifying and foaming properties were investigated. Antioxidant activity of tea seed meal protein isolate were tested based on ABTS, FRAP and metal chelating ability.

CHAPTER II

LITERATURE REVIEWS

2.1 Camellia oleifera Abel

Camellia oleifera Abel is an evergreen shrub in the camellia family. It flowers from October to April and seeds ripen in September. *Camellia oleifera* taxonomy as determined by Linnaeus is as follows (Integrated Taxonomic Information System, 2006):

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Ericales

Family Theaceae

Genus *Camellia*

Species *C. oleifera*

(Integrated Toxonomic Information System 2006. http:// www.itis.gov)

In the genus, some species of *Camellia* are usually used for decoration such as: *Camellia chrysantha* (Golden Camellia), *Camellia rusticana* (Snow Camellia) and *Camellia sasanqua* (Christmas Camellia). *Camellia sinensis* is well known and widely planted in order to make popular commercial tea drinks. However, *Camellia oleifera* is the most important material used for producing tea seed oil in China (The

American Camellia Society, 2006; The international Camellia Society, 2006; Weihrauch and Teter, 1994).

Camellia oleifera Abel plant can grow 4.5 to 6 m, and its color is brown bark (Figure 2.1). It can grow on barren area without fertilizer. The first time fruits need 8 years after planting, and it can remain highly productive for 80 year (Lee and Gioia, 2009).The leaves are alternately arranged, serrated, and 3-17 cm long by 2-4 cm wide. The flowers are white. The fruit are round, usually 2-5 seeds hiding in the fruit. The seeds are triangular in shape with diameter of 15-20 cm (Figure 2.2) (Gilman and Waston, 1993).

Figure 2.1 Plant of *camellia oleifera* Abel. Source: http:// www.nipic.com

Camellia oleifera Abel is also known as oil-seed *Camellia* or tea oil *Camellia*. They are distributed in their homeland of China. After the oil extraction, a large amount of seed cake is remained, which is recently used for animal feed, fertilizers and the development of a natural insecticide (Hu et al., 2005).

Camellia oleifera Abel was cultivated in China about 100 B.C., and is mainly distributed in Guizhou, Hunan, Guangxi, Jiangxi, and other southern provinces of

Figure 2.2 Flower (A), fruits (B), seeds (C) and seed cake (D) of *Camellia oleifera* Abel obtained after tea oil extraction. Photographs are taken from Tongren by Tian (2011).

China. Guizhou province is situated at Yunnan-Guizhou Plateau, which is the northwest parts with higher altitude than southeast. The cultivars of *Camellia oleifera* Abel are broadly distributed in parts of Guizhou with average altitude 249-2341 m, the east longitude 103°35′ to 103°48′ and north latitude 22°28′ to 23°01′. In the past years, local people use seeds of *C.oleifera* to produce the tea oil and use this oil as their primary cooking oil. In addition, it is also considered an edible oil in other countries such as Taiwan, Japan, India and Indonesia (Ravichandran and Dhandapani, 1992; Sahari, Atii and Hamedi, 2004; Tokue, Kataoka and Tanimura, 1989). During

the process of the oil extraction, abundance of tea seed cakes are produced (Yan et al., 2011). Therefore, the amount of seed cake was disposed. However the seed cake of *C.oleifera* is not only an inexpensive and residual resource but also contains large amounts of active compounds (Chen et al., 2010). Thus, the *C. oleifera* might have significant value in food industry

2.2 Tea oil of *Camellia oleifera* Abel

The tea oil (*Camellia oleifera* Abel) is the main edible oil from woody plant in China (Yu, Ren and Tan, 1999). It has bright yellowish and remains liquid even at refrigerated temperatures, with high smoke point of 251.67^{o}C, thus it is suitable for high-temperature cooking condition. Two primary methods for the commercial oil processing are mechanical pressing and solvent extraction (Zhou, Liu, Zhu and Zhou, 2006). The former method is free of solvents and inexpensive, while the latter is more efficient. Supercritical fluid extraction (SFE) has recently been suggested for oil extracting, requiring expensive equipment (Rajaei, Barzegar and Yamin., 2005). The tea oil is applied as adjuvant medicine for burning injuries and stomachache in China. Chen et al., (1998) indicated the tea oil could markedly reduce serum total cholesterols (TGh), triglycerides (TG) and low density-lipoprotein cholesterol (LDL) and high density lipoprotein 3 (HDL 3) in the blood of the rats. It was also found that the tea seed oil could inhibit atherosclerosis as a consequence of lowering liver and blood lipids by decreasing Thromoboxane B2 (TXB2) and lipidperoxides products (LOP), while increasing antioxidative enzymes including superoxide dismutase (SOD)

and Glutathione peroxidase (GSH-PX) activities (Chen et al., 1996). Thus, it can prevent cardiovascular disease. Furthermore, it has stronger antioxidant compounds and could prevent degenerative disease (Lee and Yen, 2006). Zhang and Zhou (1995) found that oil has ability to reduce the liver reactive oxygen species (ROS) in the rat. Due to high content of antioxidants, tea oil is usually used for emollient skin, preventing skin aging. In addition, the tea oil has a large amount of vitamins A, B, and E, and contains nutritonal and health protection functions because of many unsaturated fatty acids (Liu, Lee and Sun, 1979). Deng et al., (1993) also reported that the tea seed oil, which contained a high content of monounsaturated fatty acids, exerted a beneficial effect on the prevention of cardiovascular diseases. The fatty acids are mainly composed of oleic and linoleic acid, which are similar to olive oil as shown in Table 2.1.

Table 2.1 Composition of fatty acids of camellia oil (%)

Fatty acid	**Tea seed oil**	**Olive oil**
Palmitic	8.03 - 11.73	7.6- 13.0
Stearic	1.05 - 1.83	1.0 -3.0
Oleic	75.08 - 81.91	65.8 - 84.9
Linoleic	8.05 - 10.51	3.3 - 17.7
Linolenic	0.51 - 0.87	0.3 - 1.3

Source: (Fu and Zhou, 2003)

Lee and Gioia, (2009) reported that tea seed oil has high content of healthy omega-9 fatty acids but very low content of omega-6 and saturated fatty acids.

Therefore, it is used in Chinese medicine to benefit human health.

2.3 Tea seed (*Camellia oleifera* Abel) meal

Tea seed meal is composed of the remaining grounded fruit and shell after oil extraction (Gao et al., 2011). Its color is purple-brown and has poor palatability and spicy. It constitutes about 65% of tea seeds (Shi, 2001). There is abundant quantity of tea seed meal in China with approximately 400,000 tons per year. It can be distinguished in 2 forms as bulk and powder due to the different methods of processing. The nutritional content varies greatly with breed, producing area, collecting time, and processing techniques. The meal of tea seed represents not only an inexpensive resource but also rich bioactive compounds, such as proteins, phenolic compounds, saponis, etc (Wang and Wei, 1990), which have health-promoting effect related to theirs protective effects on oxidative stress-induced diseases such as cancer and cardiovascular diseases (Yeh and Yen, 2003).

2.4 Tea saponin (*Camellia oleifera* Abel)

Saponin is a group of glycosides widely distributed in higher plants. It is a natural surfactant with emulsifying, dispersion and wetting ability. The saponin dissolves in water to form colloidal solutions that foam upon shaking (Tyler, Brady and Robbers, 1981). In addition, it has many bioactivities, for example antimicrobial, anti-inflammatory, antioxidant, antiallergic (Sparg et al., 2004). Chen et al., (1998) reported saponins of tea seed could decrease the cholesterols, triglycerides and low

density-lipoproteins (LDL) in rats. In addition, it has gastro protective effects (Morikawa et al., 2006).

Bruneton, (1995) reported that saponin, according to their aglycone skeleton, can be classified into two groups. The first group is just found in the monocotyledonous angiosperms, which consists of the steroidal saponins. The second group is composed of the triterpenoid saponins, which are most common and occur mainly in the dicotyledonous angiosperms.

Tea saponin is a new commercial source of saponin which is belonged to saponin family, a kind of petacylic triterpenoid (Yan, Wu, Zhao and Jiang, 2011). Due to its functional properties and numerous pharmacological properties, it is widely used in food, drinks, medicines, hair care products and so on. Thus, saponin is a very important ingredient in tea seed cake.

2.5 Tea polysaccharides

In the past years, medicinal plant polysaccharides have been widely studied for their chemical properties and biological activities (Chen et al., 2008), including antitumor, immunostimulation and antioxidation (Wasser, 2002; Li et al., 2003). According to the dictionary of Chinese's Materia Medica records, the whole plant of *C.oleifera* Abel has some biological activity. Jin and Ning, (2012) reported the polysaccharides from meal of *C.oleifera* Abel, which exhibited a good antioxidant and antitumor. Tea seed polysaccharides also have ability to prolong thrombosis formation and reduce blood glucose (Tian, Qiu and Shi, 2004).

2.6 Phenolic compounds

Phenolic compounds are considered as one of the main classes of secondary metabolites that can be divided into non-flavonoids and flavonoids. In addition, it also plays an important role in reducing oxidative stress, reportedly exhibits anti-carcinogenic, anti-mutagenic, antioxidant activities (Borges et al., 2005). Recently, researchers disclosed that the seeds of *C.oleifera* Abel. contained large amounts of active compounds, such saponins and flavonoids (Luo and Li, 2003). Liu et al., (2005) reported the main of phenolic compounds in tea seed meal were kaemeferol glycosides. Luo and Li, (2003) isolated seven compounds from tea seed, kaemferol-3-O-[2-O--D-xylopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranos ide,kaemferol-3-O-[2-O-α-L-rhamnopyranosyl-6-O-β-D-xylopyranosyl]-β-D-glucopy ran-oside,kaemferol-3-O-α-L-rhamnopyranosyl(1→6)β-D-glucopyranoside,kaemferol -3-O-[2-O-β-D-glucopy-ranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside, dimethyl terephthalate, p-hydroxybenzoic acid and kaemferol. Among these chemicals, kaemferol was a strong antioxidant compound that might be comparable to Trolox, a standard antioxidant for the DPPH radical scavenging assay (Park et. al., 2006). Gao et al., (2011) reported other two new kaempferol acetylated glycosides identified in tea seed meal: kaempferol-3-O-[4""-O-acetyl-a-L-rhamnopyranosyl-(1→6)]-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside,kaempferol-3-O-[4""-O-acetyl-a-L-rhamnopyranosyl-(1→6)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranoside. Thus, the high content of kaempferol derivatives suggested that the seed meal of *C. oleifera* may provide a good source of bioactive compounds.

2.7 Plant proteins

People absorb proteins from two main sources: animals and plants. Milk protein is the most commonly consumed protein in all major types (Fox and Mcsweeney, 1998). As for plant proteins, soy protein was wieldy applied in food industry because of its functional properties (Endres, 2001). Animal proteins are associated with high fat and cholesterol content. Thus, plant proteins have gained more attention in the food industry because of its nutritional value and neutraceutical properties.

Kanazawa et al., (1995) indicated soy protein could reduce the level of cholesterols, good for liver cirrhosis triglycerides and low density-lipoprotein in the blood. Soybean can restrain activation of the blood cell, and reduce the contents of 5-hydroxytryptophan from blood cell which is the thrombus (Shun, 2001). Rowlands et al., (2001) reported the soy protein isolate could restrain the activities of carcinogen, and reduce the rate of generation of the liver cancer in rats. Li, Cai and Xia (2001) reported the protein extraction from tea has the capability in protecting biological cells against mutagenesis caused by irradiation. Up to now, proteins from other sources have also been investigated about their functional properties and potential food applications. For examples, corn protein (Myers, Hojillaevangelista, and Johnson, 1994), wheat protein (Hettiarachchy, Griffin and Gnanasambandam, 1996) and rice protein (Morita et al., 1996). However, little information is available about tea seed meal protein.

2.8 Functional properties of proteins

Protein functionality is defined as physical and chemical properties which affect the behavior of proteins in food system during processing, storage, preparation and consumption (Damodaran, 1997). The physical and chemical properties affecting the functions of proteins depend on their size, shape, amino acid composition and sequences, net charge and distribution of charges, the ratio of hydrophobicity to hydrophilicity, secondary, tertiary and quaternary structure, molecular flexibility / rigidity and ability to interact with other components (Damodaran, 1997).

The functional properties of protein mainly included: (1) the sensory and motion perception, such as smell, taste and texture; (2) hydration capacity, dispersibility and solubility; (3) the surface activity properties, such as foaming, foam stability, emulsification, emulsion stability, oil absorption, water holding capacity (4) rheological properties, mainly including gelation and viscosity; (5) other properties, such as adhesion and dough forming ability.

In these functional properties, proteins solubility is the most important because of its significant influence on other functional properties. It is usually the first functional property determined during development and testing of new protein ingredients (Zayas and Joseph, 1997). Xu and Diosady (1994) used two phase solvent ($CH_3OH/NH_3/H_2O$-hexane) to extract protein from Chinese rapeseed. Compared with soybean protein isolation, the precipitated part gave high value for all properties (emulsion properties, foaming properties, NSI and fat absorption) except for nitrogen soluble index (NSI), while the soluble part showed high NSI and fat absorption but

the emulsifying properties is poor. These results indicated functional properties of rapeseed protein isolate (RPI) that was suitable for incorporation into foods with functional properties equal or superior to soybean protein.

Hettiarachchy, Griffin and Gnanasambandam, (1996) reported the protein isolates from defatted wheat germ had nitrogen solubility more than 70% at pH 6.0, and showed better emulsifying properties than bovine serum albumin (BSA). The foaming capacity of wheat germ protein isolate (WGPI) was similar to that of egg white. However, WGPI was inferior to egg white in term of foaming stability. A high nitrogen solubility, favorable emulsifying properties, foaming capacity, and hydrophobicity made WGPI a useful ingredient for several food products, including processed cereal and baked foods and beverages.

Wu et al., (2005) extracted proteins from dried and fresh tea leaves at alkaline solution and compared with functional properties. The oil absorption, emulsion capacity and gelation of protein from dried tea leaves was better than that extracted from fresh tea, but the solubility, water absorption, emulsion stability, foaming capacity and foaming stability of protein from fresh tea was better than that extracted from dried sample.

2.9 Overview of protein extraction methology

There were various methods for plant protein extraction. Methods often employed are discussed below.

2.9.1 Alkaline extraction

Plant proteins could be produced from various methods, such as, alkaline extraction, enzymatic treatments, physical processes and combination of physical processes and enzymatic treatments. Alkaline solution has been widely used for plant protein extraction because of its effectiveness. Proteins from plants are difficult to dissolve in water because of their hydrophobic nature and disulfide bonds between protein molecules. High concentration of alkaline can lead to the breakdown of hydrogen bonds. Then, it can increase surface charge of protein molecules and enhance protein-water interactions. Guo, Pang and Wang (2005) used 0.1 M NaOH solution for rice protein extraction and obtained 55% protein. Other researchers used similar alkali approach but obtained 90% extraction of rice protein (Sun & Tian, 2003). Lu et al., (2011) used 0.3 M NaOH solution to extract protein from green tea and extract 82% protein. Ding et al., (2010) used one method based on alkaline extraction followed by acid precipitation for extraction protein from *Camellia oleifera* seed meal with the protein extraction of 57.8%. Alkaline conditions might increase extractability of oilseed meal protein (Schwass and Finley, 1984). However disadvantage of alkaline extraction was that it promotes oxidation of phenolic compounds readily and increases brownish appearance and unpleasant bitterness (Xu and Diosady, 2002). In addition, exposing protein to extreme alkaline conditions might cause degradation of protein, and potential toxicity, such as formation of lysinolanine, resulting in the loss of nutritional value (Shewry and Miflin, 1985).

2.9.2 Enzyme extraction

To avoid protein denaturation during alkaline extraction, enzyme is another feasible method for protein extraction. Ansharullah, Hourigan and Chesterman, (1997) reported that carbohydrases (cellulose, hemicellulase, pectinase and viscozyme L) can hydrolyze a-1, 4 linkage of polysaccharide and indirectly increases the protein recovery by liberating polysaccharide-bound protein. Other than that, proteases change the protein to be more soluble for extraction by hydrolyzing protein into peptides (Tang, Hettiarachchy, and Shellhammer, 2002). Celluase and Alcalase have been used to increase soybean protein extraction. (Rosenthal et al., 2001). Shen et al., (2008) reported the extraction of proteins from tea leave pulps using various enzymes (Neutrase, Alcalase, Protamex and Flavourzyme). The use of an enzyme alone or only one enzyme appeared to be less effective in tea protein with less than 20% protein recovery. . However, a combination of two enzymes (Alcalase and Protamex at w/w=1:3) could produce much higher recovery of 47.8%. But extraction recovery was lower than that extracted by alkaline method, 56.4%. Li et al., (2011) reported that protein was extracted from pruned tea leaves by Alcalase, pectinase, and cellulose. When the alcalase, pectinase, and cellulose was used at 480, 600, 400IU/g, respectively. Protein extraction capacity significantly increased. Combination of two enzymes, cellulose and alcalase, for tea leave protein extraction yielded 35% protein recovery, which was lower than that extracted by Alkaline alone. Although the enzyme method resulted in a slight lower protein yield than that of the alkali method, its mild extraction condition and low environment impact make it preferable for tea

protein extraction.

2.9.3 Physical-assisted extraction

Physical methods can disrupt cell wall, thus providing a suitable environment for enzymatic catalysis or increasing protein solubility. Recently, enthusiasm has been put on physical processing-assisted extraction of protein. Tang, Hettiarachchy and Shellhammer (2002) evaluated the effects of physical processing and enzyme treatments on extractability of protein from heat-stabilized defatted rice bran (HDRB). The use of freeze-thaw, sonication, high-speed blending, and high-pressure methods alone extracted 12, 15, 16 and 11% protein, respectively. Rice bran sonication with 100% output of 750 W, and extracted with water, amylase, and amylase in combination with protease P resulted in 13.5%, 33.9%, and 57.8% protein, respectively. Similar results were reported when using high-speed blending or high pressure treatment followed by water, amylase, protease and amylase in combination with protease.

Ma and Zhang (2006) reported protein extraction from defatted rice bran using ultrasound-assisted method. The sample was treated under these conditions: extraction time of 80 min, ultrasonic power was 600 W at pH 9. The protein extraction 48.3% protein yields, which was higher than alkaline method. The ultrasound-assisted method is much easier to be conducted and higher protein recovery is higher than alkaline method. Extraction of spirulina proteins using ultrasound at 900 W with phosphate buffer, pH 7.0 yielded 18.8% protein. Ultrasound-assisted method showed 4 times higher yield of protein extraction than without supersonic (Hao, Wang & Hu,

2007). These results suggested that a physical process in combination with other methods can be effective in plant protein extraction.

2.10 Plant proteomics

In past decades, proteomic techniques have been improved. A large number of studies about plant proteomics have been carried out, however it still lags behind other material proteomics (e.g. human, bacterial). Because of plant tissue has a relatively low protein content compared to bacterial or animal tissues. In addition, the cell wall and vacuole of plant normally are associated with some substances, such as polysaccharides, lipids, and polyphenols interfering with protein analysis. For this reason, available data of plant proteome are relatively limited. Nowadays, many methods for protein isolation from plant tissues have been developed, involving the use of acetone, trichloroacetic acid (TCA) with ammonium acetate precipitation (Carpentier et al., 2008; Ashoub, Berberuch and Beckhaus, 2011) or protein extraction under alkaline condition followed by acid precipitation (Shen et al., 2008). But low abundant proteins remain difficult to analyze (Boschetti et al., 2009).

Extraction and separation of proteins from a complex biological sample is very difficult task. The main challenge is to find the most efficient methods to resolve different samples. Electrophoretic and chromatographic methods are often applied in the protein separation. The multi-dimensional techniques where the dimensions are depended on different separation mechanisms (e.g. affinity, ion exchange, partition, adsorption, size exclusion) may offer higher selectivity and improve the change of

resolving a complex protein mixture. One dimensional (1-D) or two dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) is often used for protein separation (Rabilloud et al., 2009). Several varieties of liquid chromatography (LC) are widely used in proteomics (Zhang et al., 2010).

A combination of separation techniques and mass spectrometry (MS) are the most important tool in protein identification. Two ionization techniques are mainly used in the proteomic research: the electrospray ionization (ESI) (Fenn et al., 1989) and the MALDI (Karas and Hillenkamp, 1988). Both methods are also referred to as 'soft' ionization methods. The sample for MALDI is mixed in a large quantity of matrix. The matrix chromophore absorbs and distributes the energy of a laser, producing a plasma, vaporizing and ionizing the sample. In ESI, the sample solution is sprayed across a high potential difference (a few kilovolts) from a needle into an orifice in the interface. Although, the way of ionization of sample is different, both methods are useful for protein identification.

2.11 Gel electrophoresis

Raymond and Weintraub (1959) reported that a gel could be formed by two organic monomers, acrylamide and N, N'- methylenbisacrylamide (crosslinking agent). Denaturing agents (sodium dodecyl sulfate, SDS) are widely used in protein separation. SDS is a negatively charged detergent masking their native charges. Therefore, proteins separated by SDS-PAGE migrate according to their molecular weight regardless of charge. However, SDS-PAGE is insufficient in terms of

separation for large-scale proteome research. In order to increase the ability of the protein separation, other separation technique was used before the SDS electrophoresis. Therefore the 2-D SDS-PAGE was developed (Kenrick and Margolis, 1970). 2-D SDS-PAGE is a high resolution technique which is a combination of two separation mechanisms. Isoelectric focusing (IEF) is the first dimension of the protein separation according to its charge, following the SDS-PAGE that is carried out in 90 degrees direction to the first one. Nowadays, immobilized pH gradient (IPG) was developed and the resolution, reproducibility and loading capacity of IEF dimension was enhanced (Görg, Weiss and Dunn, 2004). Harris et al., (2007) reported various approaches of protein visualization. Various methods of protein staining were applied for visualization of protein in gel such as: Coomassie Brilliant Blue and silver staining. Higher sensitivity and broader linear dynamic range of the detection can be achieved with fluorescent dyes. The statistic evaluation of the protein spots could be provided by a computer 2-D image analysis. High quality and reproducible 2-D gels are required to examine patterns and spot intensities to assess the difference of the protein expression between samples. Software is designed for automated data processing, correction by user is usually necessary (Penque, 2009)

2.12 Different approaches for protein digestion

An efficient protein digestion is an important step for successful identification and characterization of proteins by MS. Several specific proteases such as trypsin, chymotrypsin, Endo Asp N and chemical agents (cyanogen bromide) are widely used

in the proteomic experiments. However, trypsin is the most frequently applied, which cleaves peptide bond predominantly at the carboxyl side of lysine and arginine residue. The digestion can be done directly in gel, although much higher concentration of the enzyme is required to be compared to an in-solution digestion. Temperature of $37^{O}C$ is usually chosen for digestion (Shevchenko et al., 1996). In order to speed up and enhance ability of protein digestion, several alternative techniques have been applied.

Modified trypsin can be used for the in-gel digestion of proteins instead of native trypsin (Shevchenko et al., 2006). Trypsin, modified by reductive methylation, decreases autolysis and shifts the optimum temperature of its catalytic activity to 50–60 ^{O}C. Therefore, the enzymatic cleavage can be carried out at a higher temperature and the digestion time can be significantly reduced from 16 h to 30 min without losing the efficiency of the protein digestion. Various water-miscible organic solvents are used for the in-solution digestion of proteins (Russell, Park and Russell, 2001). The rate of protein digestion in an organic-aqueous solvent system was comparable to the digestion in water alone. In addition, the tryptic digestion is more efficient in the presence of the organic solvent. The potential of this approach was illustrated by efficient digestion of proteins that are resistant or partially resistant to proteolysis. Fast and efficient proteolytic digestion using microwave technology has been also extensively explored (Sun et al., 2006). The first demonstration of this method was made in temperature controlled, single beam microwave applicator in 2002 (Pramanik et al., 2002). The rapid microwave-assisted digestion may be also applied using a domestic microwave oven. Other promising approaches include

ultrasonic-assisted protein enzymatic digestion (Rial-Otero et al., 2007) and proteolysis accelerated by infrared radiation (Bao et al., 2008). Researchers have shown that the in-solution, or the in-gel digestion using microwave, ultrasonic and infrared light energy, results both in high efficiency and accuracy in minutes, while the conventional method requires several hours. The proteolytic protein digestion in a microwave can be further enhanced in the presence of organic solvents (Lin et al., 2005).

An appliation of fast enzymatic digestion protocols was shown by various authors (Vaezzadeh et al., 2010; Santos et al., 2010). The digestion methods using microwave and ultrasonic energy were also utilized in plant proteomics (Hu and Owens, 2011). A quantitative analysis of glycated hemoglobin A1c was carried out using microwave-assisted trypsin and endoproteinase GluC digestion. (Vesper et al., 2005). Previous researchers have also shown that, even with complex mixtures, rapid enzymatic digestion procedure yields a higher number of peptides than the conventional method.

2.13 Mass spectrometry

Mass spectrometer is a technique that separates and identifies molecules based on mass and it has become an important tool for proteomics. A typical mass spectrometer contains three components: ion source, mass analyzer and detector. Molecules were ionized and converted into gas in an ion source prior to the separation by mass analyzer. Then mass spectrometer is connected to a computer with software

that analyzes the ion data according to their mass-to-charge ratio (m/z) and relative abundance. MS requires charged and gaseous molecules for analysis. This made it difficult to apply MS for large biomolecules including peptides and protein since large biomolecules with high polarity are not easily transferred into the gas phase. So some ionization techniques were developed such as Fast atom bombardment (Barber et al., 1981), plasma desorption (Macfarlane and Rorgerson, 1976) and thermospray (Blakley and Vestal, 1983). These techniques were the first ionization techniques that led to successful MS detection of large molecules. However, high quantities of a protein were required for the analysis. The development of two ionization methods, electrospray ionization (ESI) (Fenn et al., 1989) and matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) were important breakthrough in proteomics, which can successfully be used to transfer biomolecules into gas phase. Sensitivity of peptide and protein analysis by MS was significantly increased to femtomole level. Therefore, this allows polypeptides and proteins to be accessible to MS analysis.

2.14 Mass spectrometry for protein identification

So far, protein has been identified most frequently by two techniques: peptide mass finger printing (PMF) using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) and partial amino acid sequencing using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2.14.1 Peptide mass finger printing (PMF) using MALDI-TOF

Karas and Hillenkamp (1988) reported that MALDI is a soft ionization technique used in MS, allowing for analysis of large biomolecules that tend to be fragmented when conventional ionization methods are used.

The sample was mixed with organic molecules, called matrix, before allowing co-crystallized with one matrix. Matrix was used to protect the sample from being destroyed by direct laser beam during ionization. Matrix material must have a strong absorption at the laser wavelength. Hillenkamp, et al. (1991) reported that α-cyano-4-hydroxycinnamic acid was suitable for analysis of peptide and protein smaller than 10 kDa, whereas sinapinic was recommended for higher mass peptide. The crystals are irradiated with laser pulses under vacuum conditions. The matrix absorbs the most of the energy of the laser, which is partially vaporized and carries intact sample into gas phase. During expansion of the MALDI plume, protons are exchanged between analytes and matrix, resulting in formation of charged analytes (Karas, Gluckmann, and Schafer, 2000). MALDI ion source was often coupled with time-of-flight (TOF) mass analyzer. The ions generated in the ion source by laser pulses are initially accelerated in an electric field to the same kinetic energy and fly along the tube with different velocities, depending on their masses. The mass-to-charge ration (m/z) of those ions was measured from their flight time through a tube of specified length (Weickhardt, Moritz, and Grotemeyer, 1996). This effective approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. The

experimentally obtained masses are compared with the theoretical peptide masses in databases by means of mass search programs (Fabris et al., 1995). PMF significantly increased the speed of protein analysis. Analyzed protein, after proteolysis with a specific protease, produces a unique set of peptides according to the protein amino acids sequence. These peptides are then compared to the theoretical masses derived from a sequence database. The advantage of this approach is that only small amount of material is needed for analysis and very fast identification of proteins is possible. On the other hand, the protein sequence has to be present in the database to be identified by commonly used software (for example MASCOT) and the protein sample must be relatively homogeneous as protein mixture complicates the analysis. However, PMF is not suitable for protein mixture analysis because it is unclear that all peptide masses observed are originally from the same protein species. In addition, the measured masses of protein modification are different from those without modifications so masses of modified proteins may be wrong (Poutanen et al., 2001).

2.14.2 Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

LC-MS/MS is another feasible method for protein identification. LC-MS/MS was a superior platform for protein identification because of its sensitivity and reliability. The sample could be effectively concentrated 50-200 folds by LC before MS detection. The MS detection was concentration-dependent, therefore high concentration could be translated into the increase of MS signal. Consequently, it can dramatically increase real sensitivity in identifying proteins. Fragmentation spectra of sample were produced by tandem mass spectrometry (MS/MS) which contains two

analyzers. Interest ion was separated in the first mass analyzer and fragmentation in collision cell with an inert gas. During the collision, amide bond was cleaved to generate smaller fragments. Since a low energy collision-induced dissociation (CID) was a method widely used to produce peptide fragments. In order to sequence the peptide, products of fragmentation were analyzed in the second mass analysis. The fragmentation at amide bond with charge retention on the N- or C- terminal was designated as b- and y- ions, respectively. The sequence of peptides determined from the fragmentation in MS/MS is called *de novo* peptide sequencing. Then, peptide sequence can be used to search more information in a protein database. Simpson (2003) reported that proteins in a complex mixture can be identified from CID spectrum of a single peptide and matches one or more tandem mass spectra to peptide sequences in the same protein. Therefore, this method provides a high level of confidence in the identification process. Esteve et al., (2012) used LC-MS/MS to identify olive (*Olea europaea*) seed and pulp protein, which found that there were 61 proteins from the seed and 231 proteins from the pulp. Kwon (2004) applied LC-MS/MS to identify soluble proteins in Sauvignon Blanc wine. Twenty proteins were identified in wine, in which 1, 5, 12 and 2 proteins from fungi, grapes, yeast and bacteria, respectively. Proteins from the human gland were characterized by separation on two-dimensional gel electrophoresis and identified by LC-MS/MS. The blood-feeding proteins were detected in the distal-lateral lobes and/or medial lobes of the female glands. In addition, at least 15 glycoproteins were also found in female gland (Sor-Suwan et al., 2012).

2.15 Protein characteristics

2.15.1 Fourier transform infrared (FT-IR) spectroscopy

The three-dimensional structure of a protein is an impotent guide for its function (Creighton 1993). Protein structure is essential for correct function because it allows molecular recognition (Whisstock and Lesk 2003). Twenty different amino acids were used to make protein, each protein has a 3 dimensional native structure required for its function. FT-IR is an optical technique that provides information about the molecule vibration of any stable electronic state of molecule system (Cάrcamo et al., 2012). Each compound has a characteristic set of absorption bands in its infrared spectrum. When the frequency of IR is same as vibration frequency of a bond, then absorption of that IR radiation occur. Thus, the frequency of the vibrations can be associated with a particular bond type. FT-IR is a nondestructive technique for elucidating structure of biological system and can be used to monitor changes in the secondary structure of protein. Amide I and Amide II are characteristic bands of protein, which were found in the infrared spectra. The amide I band are characteristic frequencies in 1600-1690 cm^{-1}, which is attributed to the stretching vibrations of C=O bond of the amide, the amide II (1480-1575 cm^{-1}) are associated with the stretching vibrations of N-H bond. The FT-IR was applied to analyze the structure of polysaccharides in the glycroproteins extracted from leaves, flowers and seeds of green tea. (Wang et al., 2012). Li et al., (2013) used FTIR to identify structure of protein isolated from *Mustelus griseus* (Pietschmann) and found that it was a glycoprotein.

In addition, the intensities of IR absorption provide quantitative information about sample contents, depending on the nature of the molecular bond, structure and environment (Belbachir et al., 2009). Zhang et al., (2012) applied FT-IR to identify the change of phenolic content of Pu-erh tea in the different stages of fermentation. Thus, FT-IR not only identify structure of compounds, but also provide quantitative information of a compound.

2.15.2 Surface characteristics

The physicochemical characteristics of the food protein such as molecular size, surface hydrophobicity, net charge, steric hindrance and molecular flexibility have been found to greatly influence protein functional properties. (Sikorski, 2001).

Zeta potential is a key physical parameter that describes surface charge on proteins. The zeta potential of a protein can be used as an indicator of stability. The zeta potential indicates the degree of repulsion between adjacent similarly charged particles. The large net charge could be overcome various attractive forces (i.e., van der Waals, hydrophobic), leading to significant stabilizing electrostatic repulsive force between proteins and increasing protein solubility. Karaca, Low and Nickerson, (2011) reported that high solubility of faba bean protein isolate is attributed to its high surface charge (zeta potential). In addition, zeta potential is also correlated with isoelectric point (IEP) of protein because the isoelectric point (IEP) is the pH of a protein solution at which the zeta potential of protein is zero (Salgin, Salgin and Bahadir, 2012). Cheng et al., (2006) used zeta potential method to check IEP of collagen and the result close to that of isoelectric focusing electrophoresis (IEF) method.

Surface hydrophobicity greatly influences protein emulsifying properties (Sikorski, 2001). Due to the protein amphiphilic nature, it remains in the aqueous phase and is adsorbed at the surface of oil droplets at same time (Claesson, Blomberg and Poptoshev, 2004). A major requirement for protein adsorption at the oil-water interface is the presence of hydrophobic patches on its surface (McClements, 2004). Voutsinas et al., (1983) reported high correlation between surface hydrophobic and emulsifying activity. Aider et al., (2012) reported that the mustard protein isolate at neutral pH showed higher surface hydrophobicity, and emulsifying properties. In addition, Karaca et al., (2011) reported that the chickpea protein at the neutral pH, has high surface hydrophobicity and surface charge with high EAI, ESI and FS

2.16 Antioxidant activity of protein

In term of the effects in the human body, antioxidant can be defined as ‘‘a substance in food that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen, on normal physiological functions” (Huang, Ou, and Prior, 2005). In term of foods, antioxidants are a substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents autooxidation processes (Gutteridge and Halliwell, 1990; Mielnik, Aaby, and Skrede, 2003).

Several synthetic antioxidants are commonly used in food industry to retard lipid oxidation. However, these synthetic antioxidants have some disadvantages, such as 3-ter-butyl-4-hydroxyanisole (BHA) and 3, 5-di-tert-butyl-4-hydroxytoluene (BHT)

have toxicity at high doses (Wichi, 1988).

In recent years, interest in applied natural antioxidants, because of due to limitations on the use of synthetic antioxidants and enhanced public awareness of health issues. Protein is important components of foods and ingredients of food products. Protein usually acts as functional food ingredients, gelling agents and emulsifiers. In addition, protein can also act as antioxidant. Some protein extracts from plants show antioxidant ability. Turmerine (~ 28 kDa), an antioxidant protein, from *Curcuma longa* and antioxidant protein (~ 35 kDa) extracted from curry leaves have been reported (Ramadas and Srinicas, 2011; Ningappa and Srinivas, 2008). Srivastava et al., (2012) isolated an antioxidant protein (~ 16 kDa) from *Terminalia chebula* fruit. It exhibited significant radical scavenging in DPPH, NO, H_2O_2 and ABTS assays and good metal chelating ability. Proteins from other materials also exhibited antioxidant activity. Whey protein concentrate acts as an antioxidant in cooked beef (Shantha and Decker, 1995). Soy and whey proteins inhibited lipid oxidation in cooked pork patties containing 2% protein (Pena-Ramos and Xiong, 2003). Therefore, protein might be as potential antioxidant additives used in food. Protein can inhibit the lipid oxidation of food according to its different antioxidant mechanisms such as biologically designed mechanisms (e.g. antioxidant enzymes and iron-binding proteins) or nonspecific mechanisms (e.g. scavenging of free radicals and reactive oxygen species) (Elias, Kellerby and Decker, 2008). Overall, the antioxidant activity of proteins is mainly due to interactions between their ability to inactivate reactive oxygen species, chelate prooxidative transition metals, scavenge free radicals,

and reduction of hydroperoxides.

2.17 Antioxidant Assays

Antioxidant capacity assays can be divided into two types based on the chemical reaction mechanisms: hydrogen atom transfer (HAT) reaction and single electron transfer (SET) (Huang., Ou and Prior, 2005). The HAT assays determine the hydrogen atom donating capacity, while the SET assay indicated the reducing power of antioxidant (Benzie and Strain, 1999). Food antioxidants normally involve radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors.

2.17.1 2, 2'-Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS assay, also known as TEAC (Trolox Equivalent Antioxidant Capacity) assay, when calibrated relative to Trolox. It is one of spectrophotometric methods to determine the antioxidant activity of solution. The pre-formed radical monocation of 2,2'-azinobis(3- ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) is generated by ABTS oxidation with potassium persulfate and is reduced in the presence of antioxidants. The reaction is pH-independent and is not affected by ionic strength. The concentration of antioxidants is linearly proportional to a decrease of the $ABTS^{\cdot+}$ concentration. The advantage of the ABTS assay is soluble in both aqueous and organic solvents. Therefore, it is usually used in many research laboratories to study both water-soluble and lipid-soluble antioxidants, pure compounds, food extracts. (Re et al., 1999). For example, the radical scavenging activity assay using

ABTS has shown in zein (Kong and Xiong, 2006) and potato (Wang and xiong, 2005) proteins. In addition, the ABTS radical cation is more reactive than the DPPH radical (Miller et al., 1993). Because of its operational convenience, it is a popular and routine test for antioxidant assay. However, ABTS also has some major disadvantages. The high extinction coefficient of $ABTS^{\bullet+}$ limits the useful antioxidant concentration range that can be analyzed accurately to about 1.5–70 μM final concentrations. Antioxidant concentrations outside this range require too much or too little $ABTS^{\bullet+}$ for accurate optical measurements (Apak et al., 2013).

2.17.2 Ferric reducing antioxidant power assay (FRAP)

Benzie and Strain (1999) developed the FRAP to measure reducing power in plasma. Subsequently, this method was used to measure reducing capacity of various substrates (Nilsson et al., 2005) and pure compounds (Nenadis, Lazaridou and Tsimidou, 2007). The FRAP determined the ability of a compound to reduce a ferric salt, Fe(III) $(TPTZ)_2CI_3$ (TPTZ= 2,4,6-tripyridyls- triazine) which is used as an oxidant, to the blue-colored ferrous complex (Fe^{2+}-TPTZ) at low pH. (Pérez-Jiménez et al., 2008). The values of FRAP are estimated by measuring the absorbance at 593 nm. However, the FRAP assay cannot detect compound that act by radical quenching. This may lead to underestimation of antioxidant activity of some complex matrices. The assay measures only the reducing capability based upon the ferric ion in the environment of a polar solvent, which has poor relationship to the radical quenching process mediated by most antioxidants via the HAT mechanism in a lipid (non-polar) substrate (Prior et al., 2005). The advantage of the FRAP assay is that it is a simple,

rapid, inexpensive and robust assay, and it does not need specialized equipment. It can be performed using automated, semiautomatic, or manual methods. (Prior, Wu and Schaich, 2005). However, a disadvantage of the FRAP assay is its inability to detect other small molecular weight thiols and sulfur containing molecules (Carlsen et al., 2010).

2.17.3 Metal chelating assay by using the ferrozine reagent

Transition metals, such as iron and copper are important promoters of lipid oxidation. (Akoh and Min, 2002). Chelators or sequestering agents can inhibit the activity of the metals. Stookey, (1970) developed the ferrozine (monosodium salt hydrate of 3-[2-pyridinyl]-5, 6-diphenyl-1, 2, 4-iazine-p,p'-disulfonic acid) reagent for measurement of iron-chelating capacity. It can specifically react with ferrous ions to form a stable, magenta-colored solution. The complex (ferrozine-Fe^{2+} chromophore) has an absorptive peak at 562 nm so that it can be measured spectrophotometrically. (Viollier et al., 2000). The major advantages of ferrozine are the high molar absorptivity of the ferrous ferrozine complex (28,000), its water solubility, and stability over the pH range of 4–9 (Stookey, 1970). However, some ion (Fe^{3+}) could interfere this method (Sun, 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

Tea seed meal, a byproduct from tea oil extraction, was obtained from Yuping County, Guizhou province, China. Samples were ground using a milling machine (Satake co., Hiroshima, Japan) and passed through to a 60-mesh sieve. Ground samples were further defatted using petroleum ether with a ratio of powder to solvent of 1:10, at 40°C, for 2 h. Fat extraction was repeated twice. Defatted samples were left in a fume hood for 4h. It was then vacuum-packed, and kept at -20°C throughout the study.

3.1.2 Chemicals

2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from BioChemika (Buchs, Switzerland). Diethylaminoethyl (DEAE) and Hi-trap™ desalting columns were purchased from GE Healthcare (Uppsala, Sweden). 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox). Other chemicals and reagents used were of analytical grade.

3.1.3 Proximate composition of defatted tea seed meal (DTSM)

Moisture content, crude fat, crude fiber, ash and crude protein of DTSM were determined according to the standard methods of analysis (AOAC, 2000).

3.1.3.1 Moisture

Drying to constant weight in an oven at 110°C for 24h was applied to determine moisture content of DTSM. One g of DTSM was placed into a pre-weighed can and dried in an oven at 110°C for 24 h. After removing the samples from the oven, they were placed in a desiccator to cool and then reweighed. The moisture content of the samples was calculated as:

$$\text{Moisture } (\%) = \frac{\text{Sample weight (g)-dried sample weight (g)}}{\text{Sample weight (g)}} \times 100.$$

3.1.3.2 Crude fat

Crude fat of DTSM was determined by Soxhlet extraction using petroleum ether on a Soxtec™ 2050 auto fat extraction system. Briefly, one g of the sample was put into a Soxhlet extraction thimble and 80 ml of petroleum ether were added. The thimble was moved to the Soxtec System. Extraction was performed for 4 h. Subsequently, the solvent was evaporated off, the extraction cup was removed and placed into an oven at 110°C. After 1 hour the drying cup was removed from the oven and left to cool in a desiccator. Fat content was calculated as :

$$\text{Fat } (\%) = \frac{\text{After extraction cup wight (g)-Before extraction cup weight (g)}}{\text{Sample weight (g)}} \times 100.$$

3.1.3.3 Crude fiber

Crude fiber content of the DTSM was analyzed by digesting the samples in 1.25 % H_2SO_4 followed by similar treatment with 1.25 % NaOH per methods detailed in AOAC (2000).

3.1.3.4 Ash

The ash content of DTSM was determined by dry ashing. Approximately one g of sample was placed in a porcelain crucible in a muffle furnace at 600°C for 3h. After ashing, the samples were removed from the furnace, cooled to room temperature in a desiccator and then reweighed. The ash content was calculated as:

$$\text{Ash (\%)} = \frac{\text{Ash wight (g)}}{\text{Sample weight (g)}} \times 100.$$

3.1.3.5 Crude protein

Crude protein content of DTSM was determined by Kjeldahl analysis. Briefly, one g of sample was weighed into a Kjeldahl digestion tube, $CuSO_4$:K_2SO_4 (1:10) and 15 ml H_2SO_4 were added. The tube was then placed into the digestion block. After digestion the tubes were removed from the block and left to cool inside a fume cupboard for at least 15 min. Then, the tubes were distilled using Gerhardt distiller system Vapodest 30 and the titration using HCl. The protein content of the samples was calculated as:

$$\text{Crude protein (\%)} = \frac{V \times 0.014 \times N}{m} \times F \times 100.$$

V= the volume of HCl (ml)

N= concentration of HCl (mol)

M= sample weight (g)

F= 6.5

3.2 Protein solubility

One g defatted sample was added 20 ml of 50 mM phosphate buffer at various pHs (2-12). Suspensions were stirred at room temperature for 30 min and centrifuged at 10,000 × rpm for 15 min. Supernatant was collected and protein content was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

3.3 Protein extraction

One g of DTSM was added to deionized water at various ratios of meal to water specified below. The pH of the slurry was adjusted to pH 7.0 using 1 N NaOH and the mixture was shaken continuously for a period of set time and at controlled temperature in a water bath. The effect of extraction temperature was carried out at 30, 35, 40, 45, 50 and 55°C at a ratio of meal to water of 1:20; and extraction time of 2 h; the extraction time was studied at 0.5, 1, 2, 3, 4 and 5 h at meal to water ratio of 1:20, at extraction temperature of 40°C; Lastly, the extraction ratio of 1:10, 1:15, 1:20, 1:25 and 1:30 was studied using the extraction time of 1 h, at 40 °C.

After each extraction condition, sample was centrifuged at 10,000×rpm, 4°C for 15 min (Legend^{TM} MΛCH 1.6R, Thermo Electron LED GmbH, Lengensellbold, Germany). The supernatant was collected and the amount of protein was determined by the Bradford (1976), and the protein recovery (%) was calculated.

$$\text{Protein recovery (\%)} = \frac{\text{Protein content in supernatant (mg)}}{\text{Total protein(\% crude protein} \times \text{one g sample)}} \times 100\%.$$

3.4 Phenolic content (PC)

Phenolic content of the extracted solution was determined using the method described by Waterhouse (2005). An aliquot (20 µl) of the diluted extract was mixed with 1,580 µl deionized water and 100 µl of Folin-Ciocalteau reagent and allowed to incubate at room temperature for 5 min. Three hundred µl of 20% (w/v) sodium carbonate (Na_2CO_3) was added to the mixture followed by incubation for 120 min at room temperature and the absorbance was measured at 765 nm. A calibration curve of gallic acid was prepared (50-1000 mg/L). The results were expressed as mg/L of gallic acid equivalents.

3.5 Isolation of tea seed protein (TSP)

Tea seed protein was extracted using the optimal extraction condition determined from 3.3. The extracted solution was applied onto a Hi-Trap™ Desalting column (5ml) and eluted with deionized water manually to separate large and small molecules. The protein and phenolic content of each fraction were determined by Bradford and Folin-Ciocalteau, respectively. The fraction of highest protein content and a relatively low phenolic content were collected. Subsequently, the collected samples were loaded onto a DEAE–Sephacel ion exchange column (2.6 × 7 cm), equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The elution was performed using an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) with a linear gradient of 0-2.0 M NaCl at a flow rate of 1 ml/min. The eluates were monitored at 280 nm and 420 nm for protein and brown pigment, respectively. Fractions with highest protein

content (determined by Bradford) was pooled and lyophilized and referred to as tea seed protein isolate (TSPI). Protein content and total phenolic contents were determined as described above.

3.6 Characterization of tea seed protein (TSP)

3.6.1 IR spectroscopy

IR spectroscopy was used to study the functional groups. The infrared spectra of TSP were recorded with TENSOR 27 Fourier Transform Infrared Spectrometer (BRUKER OPTICS, Kowloon Bay, Hong Kong). Same weight of each samples (crude TSP, after Hi-Trip desalting column and after DEAE-Sephacel column) without potassium bromide (KBr) was placed into FT-IR and measurement was performed at 4000-400 cm^{-1} at a data acquisition rate of $2cm^{-1}$ per point in the mode of transmittance. All samples were analyzed in triplicate.

3.6.2 Surface charge (Zeta potential)

The surface charge of TSP was determined using Zetasizer Nano ZS (Malvern Instruments Limited, Worcestershire, UK) with automatic titrator unit (MPT-2). The freshly prepared protein solution (crude TSP and TSPI) with concentration of 0.5 mg/ml was filtered through 0.45µl polyvinylidene fluoride (PVDF) filter before the measurement. Protein solution was titrated from pH 2 to 10 using 0.1 M HCl or 0.1 M NaOH by automatic titrator (MPT-2) under constant stirring at 25°C.

3.6.3 Surface hydrophobicity (H_O)

Surface hydrophobicity (H_O) was measured using the fluorescence probe

8-anilino-1-naphthalene sulfonate (ANS) according to the method of Kato and Nakai (1980) with some modification. Stock solutions of protein (0.05% w/v) were prepared in 0.01M phosphate buffer (pH 3.0, 5.0, 7.0 and 9.0) and centrifuged at 10000× rpm for 20 min (Legend^{TM} MACH 1.6R, Thermo Electron LED GmbH, Lengensellbold, Germany). Protein concentration in the supernatants was determined by the method of Bradford (1976). Each supernatant was serially diluted with the respective buffer to obtain protein concentrations ranging from 0.001 to 0.02% (w/v). Ten µl of 8.0 mM of ANS solution were added to 2 ml of each dilution and incubated in the dark for 10 min. Subsequently, fluorescence intensity (FI) of the mixture was monitored at 374 nm (excitation) and 485 nm (emission) using Luminescence Spectrometer LS 50B (Perkin Elmer, Waltham, MA, USA). The initial slope of the FI versus concentration (%w/v) plot was used as an index of Ho.

3.6.4 SDS-PAGE

SDS-PAGE was performed using Laemmli (1970) with slight modifications. Protein (20 µg) was loaded onto 15% running gel and 4% streaking gel. Gel was run at a constant voltage at 120V, stained with 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid. Protein molecular weight makers (Bio-Rad Laboratories, Hercules, CA).

3.6.5 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed according to 2D system (GE Healthcare, Buckinghamshire, UK) The protein sample (400 µg) was treated using 2-D Clean-Up kit (GE Healthcare, city UK). Protein pellets were solubilized in

250 µl sample solubilization solution (8 M urea, 50 mM DTT, 4 % CHAPS, 0.2 % 3/10 Bio-lyte Ampholyte, 0.002 % Bromophenol Blue) and then loaded on an IPG strip (pI 3–10, 13 cm, GE Healthcare, Buckinghamshire, UK) to perform isoelectric focusing (IEF) separation. Following 15-h rehydration, the strips were focused using Ettan IPGphor III (GE Healthcare, Buckinghamshire ,UK) according to the manufacturer's instruction. The focused IPG strips were then incubated in 20 ml SDS equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris, pH 8.8, 30 % glycerol, 0.002 % Bromophenol blue) containing 100 mg DTT for 15 min and for another 15 min in 10 ml equilibration buffer containing 250 mg iodoacetamide. The equilibrated strips were applied to the surface of vertical 12 % SDS-polyacrylamide gels and proteins separated in the second dimension using the SE600 Vertical Electrophoresis System (GE Healthcare, Buckinghamshire ,UK) The gels were fixed in 40% ethanol and 10% acetic acid for 30min, then stained with 0.12% Coomassie Brilliant Bule G250 and 10% ammonium sulfate in 10% o-phosphoric acid and 20% methanol for 1h, and finally detained with successive wash of deionized water.

3.6.6 Protein identification by LC-MS/MS

Protein spots were cut from the 2-DE gel with a clean scalpel blade. The gel pieces were subjected to in-gel digestion using a method developed by Jaresitthikunchai et al. (2009). The gel pieces were dehydrated with 100% acetonitrile (ACN), reduced with 10mM dithiotheitol (DTT) for 1 h at 56°C and alkylated with 15 mM iodoacetamide (IAA) in dark at room temperature for 1h. To perform in-gel digestion of proteins, sequencing grade trypsin (Promega, Mannheim, Germany) was

added, followed by incubation at room temperature for 20 min, and further incubated at 37°C for 16h. Tryptic peptides were resupended in 0.1% formic acid (FA) and centrifuged at 10,000× rpm at room temperature for 10min, the supernatants were collected. The supernatant was injected in to a NanoAcquity system (Water Corp., Milford, MA) equipped with a Symmetry C_{18} Trap column (5μm, 180 μm x 20 mm) and a BEH130 C_{18} analytical reversed phase column (1.7μm, 100μm x 100 mm) (Water Corp., Milford, MA). The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 μl / min for 1min. Water and acetonitrile with 0.1% formic acid were used as eluent A and B, respectively. The elution was performed using a 15-min linear gradient of 15-50% of eluent B at a flow rate of 600 nl/min. The column temperature was maintained at 35°C. Analysis of tryptic peptides was performed using a SYNAPT^{TM} HDMS mass spectrometer (UPLC/ESI-SYNAPT-HDMS) (Water Corp, Manchester, UK) and then the generated spectral data were submitted to database search using the Mascot software (http://www.matrixscience.com/search_form_select.html) against the National Center for Biotechnology nonredundant (NCBInr) protein database. Database interrogation was; taxonomy (Viridiplantae); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+). Proteins considered as identified proteins had at least one peptide with an individual ions score> 50 indicate identiy or extensive homology ($p<0.05$). The data was searched.

3.7 Antioxidant activity of TSP

3.7.1 ABTS radical scavenging assay

ABTS free radical scavenging activity of each sample was determined according to the method described by Zielinska, Wiczkowski and Piskula (2008) with slight modifications. The radical cation $ABTS^{\cdot+}$ was generated by persulfate oxidation of ABTS. A mixture (1:1, v/v) of 7.0 mM ABTS and 2.5 mM potassium persulfate was allowed to stand overnight at room temperature in the dark to form radical cation $ABTS^{\cdot+}$. A working solution was diluted with 80% ethanol to reach absorbance of 0.7 ± 0.02 at 734 nm (constant initial absorbance values must be used for standard and samples). Twenty µl of each sample was mixed with 1.48 mL of the working solution, and a decrease of absorbance was measured immediately at 734 nm after 6 min at 30 °C in the dark. The control was prepared using 80% ethanol without $ABTS^{\cdot+}$ solution. Trolox standard solution in 80% ethanol (0.3 – 1.5 mM) was prepared and assayed under the same condition. The results were expressed as mM Trolox equivalents.

3.7.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to the method reported by Yang and Zhai (2009) with slight modifications. One hundred µl of each extract were added to 1.9 mL of freshly prepared FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ in 40 mM HCl, and 1 part of 20.0 mM $FeCl_3 \cdot 6H_2O$ solution), and the reaction mixture was incubated at 37 °C for 30 min. An increase in absorbance at 593 nm was measured. Trolox was used to establish the calibration curves and results were expressed as mM Trolox equivalents.

3.7.3 Metal chelating assay

The ability of protein to chelate to ferrous ions (Fe^{2+}) was assessed using the method reported by Decker & Welch (1990) with some modifications. One hundred µl of protein solution were mixed with 2400 µl of deionized water, 50 µl of 2 mM $FeCl_2$ and 100 µl of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine). The mixture was incubated at room temperature for 20 min in the dark. The color of the ferrous iron–ferrozine complex was measured at 562 nm. Results were expressed as mM ethylenediaminetetraacetic acid (EDTA) equivalents.

3.8 Functional properties of tea seed protein

3.8.1 Emulsifying properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined using the turbidimetric methods (Pearce & Kinsella, 1978). Stock solutions of protein (1% w/v) were prepared at various pHs (3, 5, 7, and 9) using 0.1 M NaOH or 0.1 M HCl and centrifuged at 10000 rpm for 20 min (Legend™ MACH 1.6R, Thermo Electron LED GmbH, Lengensellbold, Germany). Protein concentration in the supernatant was determined by the method of Bradford (1976). Protein content at pH 3 was about 0.45 mg/ml, thus, protein contents at other pHs (pH 5, 7, 9) were diluted to obtain the same concentration of 0.45 mg/ml. To do the dilution, DI water and 0.1 M NaOH or 0.1 M HCl were added with the controlled volume to attain the set pH and protein content of 0.45 mg/ml. Soybean oil (2 ml) mixed with 6 ml of each protein solution (0.45mg/ml). The mixture was homogenized

using a homogenizer (IKA Works Asia, Bhd, Malaysia) at 10,000 rpm for 1 min to produce emulsion. Emulsion (50 µl) was pipetted from the bottom of container at 0 and 10 min and mixed with 5 ml of 0.1% SDS. Absorbance of emulsion was measured at 500 nm. EAI and ESI were calculated by the following equations:

$$\text{EAI } (m^2/g)=\frac{2\times T\times A_0\times \text{dilution factor}}{c\times \emptyset\times 10,000}$$

$$\text{ESI (min)} = \frac{A_0}{A_0-A_{10}}\times 10,$$

Where, T=2.303, dilution factor = 100, c is weight of protein per volume (g/ml), $\emptyset$ is the oil volumetric fraction (0.25), A_0 is the absorbance at 0 min and A_{10} is the absorbance at 10min.

3.8.2 Foaming properties

Foaming capacity (FC) and foaming stability (FS) were determined using the method reported by Ogunwolu et al., (2009) with some modifications. Ten mL of 1% (w/v) of protein solution at various pHs (3, 5, 7 and 9) using 0.1 M NaOH or 0.1 M HCl to adjust. Then, protein solutions were homogenized at 10,000 × rpm for 2min. The total sample volume was taken at 0 min for foam capacity and up to 30 min for foam stability. Foam capacity and foam stability were then calculated:

$$\text{FC}(\%)=\frac{\text{Total volume after homogenization-Total volume before homogenization}}{\text{Total volume before homogenization}}\times 100,$$

$$\text{FS}(\%)=\frac{\text{Total volume after standing for 30 min-Total volume before homogenization}}{\text{Total volume before homogenization}}\times 100.$$

3.9 Statistical analyses

Experiments were repeated twice. All data were analyzed using the SPSS package (SPSS 17.0 for window, SPSS Inc, Chicago, IL, USA). Data were statistically analyzed by one-way analysis of variance (ANOVA). The comparison among the means was conducted using Duncan's multiple range test (DMRT) at $p< 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Proximate composition of DTSM

Crude protein of DTSM was 10.5±0.07%. Similar result was observed by Ding et al., (2010). Relatively high content of crude fiber (17.1±0.021%) was found in DTSM. DTSM contained relatively low crude fat content of 0.38±0.004% as this was a sample obtained after fat extraction. In addition, moisture and ash content was 5.8±0.004% and 2.3±0.067%, respectively. Base on proximate analysis, the carbohydrate of DTSM is 63.92%. Tea seed meal contains a large number of starch about 30~50% (The China State Forestry Bureau, 2009). .Proximate composition indicated that DTSM was a rich source of protein that could be recovered and utilized as more value added products.

4.2 protein solubility

The protein solubility profiles of DTSM at various pHs (2-12) were shown in Figure.4.1. The solubility profile showed the typical U-shaped pattern. It exhibited the lowest solubility of 20% ($p < 0.05$) at pH 3. However, in a more acidic condition at pH 2, solubility of DTSM increased to 45%. The maximum solubility of 68% was found at pH 12 ($p < 0.05$). Vani and Zayas, (1995) reported that a large number of the plant proteins have isoelectric pH at 4.0–5.0. The minimum solubility of protein

normally found at around the isoelectric point (pI), because of zero net charge on the protein (Damodaran, 1997). At low or high pH, a protein becomes either positively or negatively charged, resulting in partial unfoldings and improving protein solubility (Kaur, 2007). This solubility pattern was similar that of tea seed meal studied by Wu et al. (2008). From the profile of solubility, DTSM displayed good solubility in both acid and alkaline pH regions.

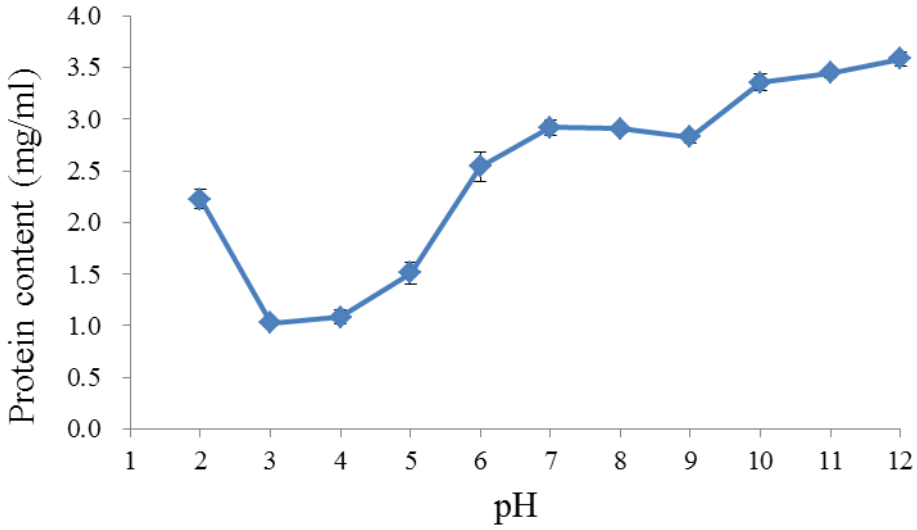

Figure 4.1 Solubility of proteins of DTSM at different pH values

4.3 Protein extraction

Phenolic compounds in DTSM readily oxidized under alkaline condition, causing undesirable brown color. Quinones, the oxidation products of phenolic compounds, can react with reactive groups of protein, promoting phenolic-protein linkages, which negatively affect protein functional properties (Saeed and Cheryan, 1989). Thus, pH 7 was chosen as the extraction medium in order to minimize such negative effect. For the effect of temperature, the highest recovery was found at 40 ^{o}C

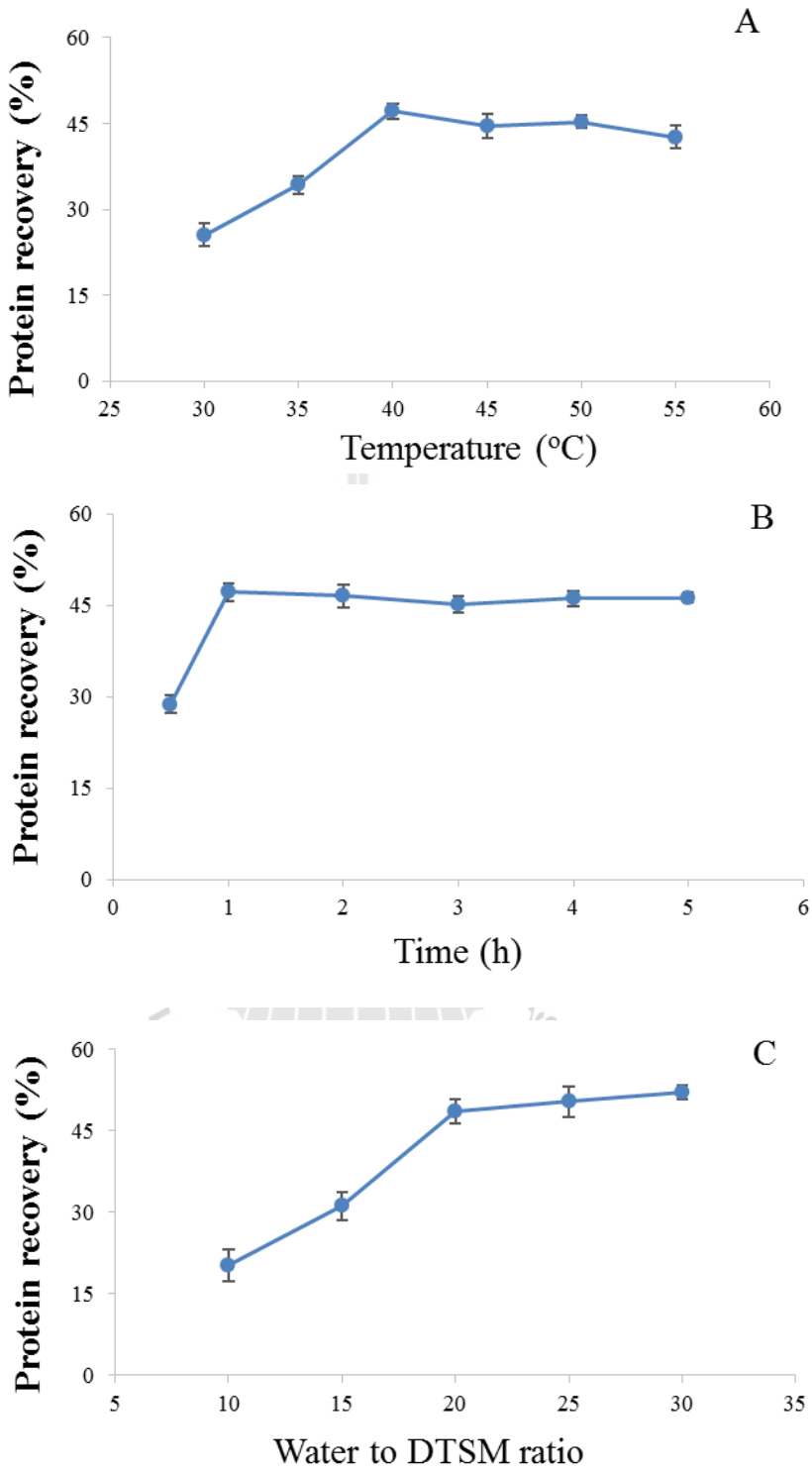

Figure 4.2 Effect temperature (A), time (B) and water to DTSM ratio (C) on protein extraction of DTSM

with protein yield of 47.1% ($p < 0.05$, Fig. 4.2 A). Yield decreased at temperature above 40 ^{o}C ($p<0.05$). This could be due to thermal denaturation. Therefore, 40^{o}C was chosen as the optimal temperature for the extraction.

The most effective extraction time was 1 h (Fig. 4.2 B), where the protein recovery was the highest at 47% ($p < 0.05$). The protein recovery did not further increase as extraction time was prolonged. The effect of extraction ratio was also investigated (Fig. 4.2 C). The protein recovery increased linearly with volume-weight ratio to up to a ratio of 20:1, and remained constant at higher volume-weight ratios. At low volume-weight ratio, the extraction capacity was limited by the limited amount of extraction medium. When the volume-weight ratio increased, sufficient extraction medium solubilized and extracted proteins from DTSM, leading to an increase in protein recovery. However, a relatively high solvent ration could increase the cost of production and create difficulty in the solvent removal. Thus, the ratio of 20:1 was chosen for protein extraction in this study.

The optimum conditions found in this study are extraction DTSM with deionized water at pH 7 with the ratio of water to DTSM of 20:1, at 40^{o}C for 1 h. The protein recovery and phenolic contents at the optimum and alkaline condition were shown in Table 4.1. A higher recovery rate of protein was found at alkaline condition (pH 12). At pH12, the hydrogen bonds of protein are likely broken down and proteins possess negative charges, leading to more interactions with water and the improvement in protein extraction. However, a relatively high content of phenolic compounds was observed at alkaline condition. Phenolic compounds reported in

oilseeds may exist as free, esterified, etherified or insoluble forms (Alu'datt et al., 2013). Xu & Diosady, (2002) stated that proteins interact with phenolic compounds by hydrogen bonding via hydroxyl groups of phenolic compounds and carbonyl groups of the peptide bonds, or by the "oxidation to quinines" that bind with reactive groups of proteins. Alkaline condition can destroy some hydrogen bonds between proteins and phenolic compounds, inducing more extracted phenolic compounds (Krygier et al., 1982). Exposing protein to alkaline conditions may also cause some undesirable side reactions (i.e., denaturation and hydrolysis of proteins) and causes discoloration of protein (brown color) (Wang et al., 1999). For this reason, extraction at pH 7 would be more appropriate.

Table 4.1 Protein recovery and phenolic contents (PC) of extracted DTSM at the optimum and alkaline condition. Values are expressed as the mean±S.D. (n=2) ($p < 0.05$).

Condition	Protein recovery (%)	Phenolic content (%)
Alkaline condition (pH 12)	68.3±2.2	2.2±0.1
Optimum condition (pH 7)	50.4±1.5	1.5±0.2

4.4 Isolation of tea seed protein

Desalting column was applied to separate low molecular species from proteins, especially phenolic compounds. After desalting column, fraction 3 yielded the highest protein content with approximately 68.4% protein yield with a relatively low phenolic content (Figure 4.3A, Table 4.2). Fraction 3 was further purified using anion exchange

chromatography (DEAE-Sephacel). The separation pattern is shown in Figure 4.3B. There were two peaks showing absorbance at 280nm, peak a and b. The fraction b exhibited high OD_{280}, indicating that the fraction might contain either high protein content or high phenolic content. The relatively high browning index (OD_{420}) was also found in fraction b, suggesting polymerization of phenolic compounds. After Bradford and Folin–Ciocalteau analyses. It was found that fraction b showed the approximately 26.7% protein yield and the lowest phenolic content ($p<0.05$). compared with other.

Table 4.2 Changes of protein and phenolic content during isolation of tea seed proteins

Steps	**Total**		**Protein Yield (%)**	**Phenolic Remaining (%)**
	Protein (mg)	**Phenolic (mg)**		
Crude	26.5	9.75	100	100
Hi-Trap desalting	18.1	1.73	68.3	17.3
DEAE-Sephacel	7.09	1.05	26.7	10.7

samples (crude TSP and the sample after desalting column). Xu & Diosady (1994) isolated proteins from the rapeseed with high protein content, low phytates and with desirable functional properties for food application. However, using of these proteins was limited because of dark color and unpleasant bitter taste. Phenolic compounds were a major cause of dark color and undesirable taste. Thus, phenolic compounds should be removed. Polyvinyl-polypyrrolidone (PVPP) is generally used to remove phenolic compounds from plant extract (Charmont et al., 2005). Numerous

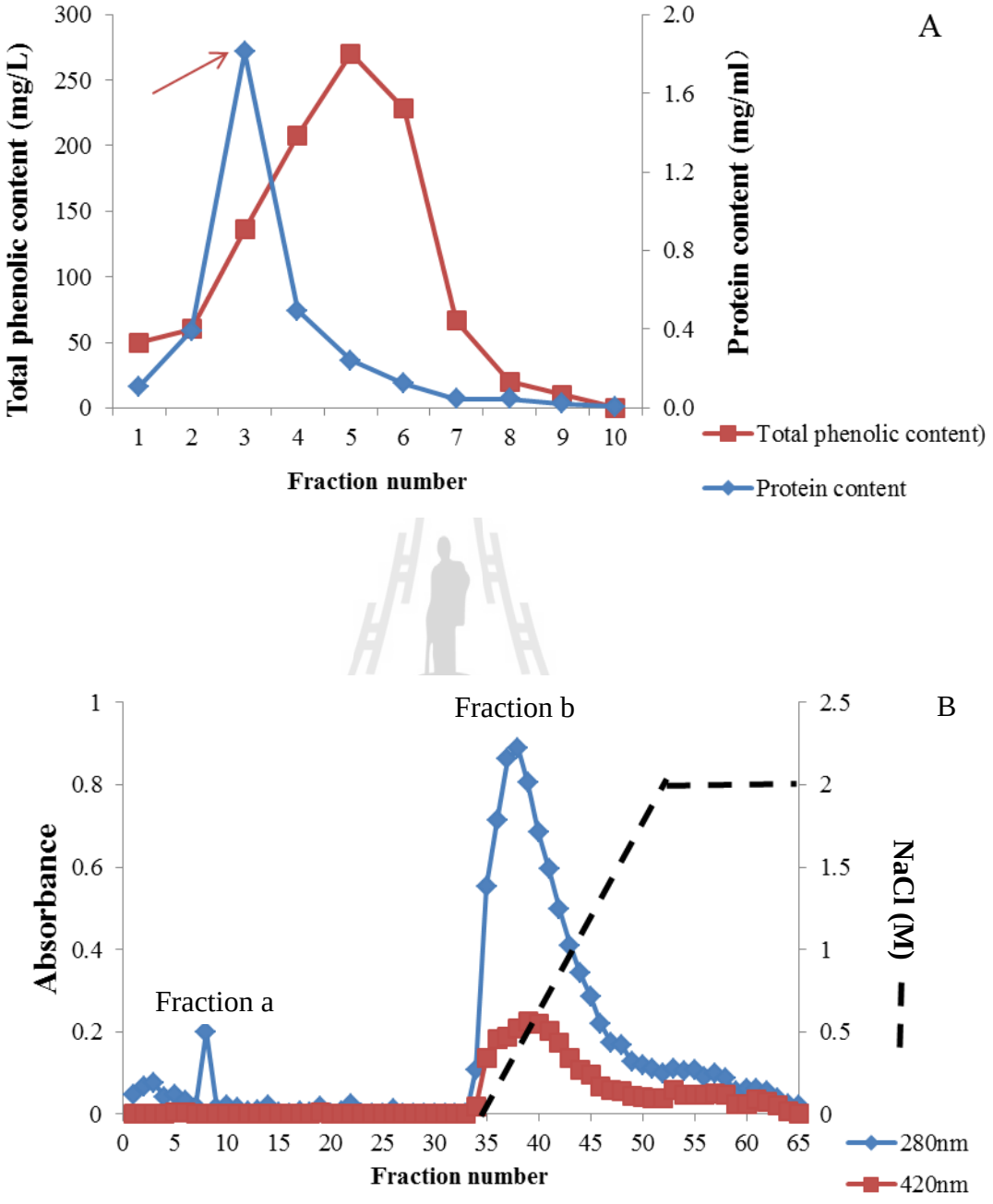

Figure 4.3 Chromatograms of tea seed protein obtained from Hi-Trip desalting column (A) and DEAE-Sephacel column (B)

researchers have reported using PVPP to remove phenolic compounds from tea extract (Yi et al., 2001), rapeseed extract (Xu & Diosady, 2002) and plant leaf extract (McCown et al.,1968). However, the use of PVPP is limited for complex of "protein-phenolic" because it lowers protein yield. Weisz et al., (2010) reported the use of adsorption and ion exchange chromatography to remove polyphenols from crude sunflower extracts in order to produce high-quality protein isolate. Thus, our results suggested that 2-step chromatography isolation was effective to remove about 90% phenolic compounds.

4.5 Characterization of tea seed protein (TSP)

4.5.1 IR spectroscopy

The IR spectra of crude TSP, the sample obtained after desalting column, and the sample obtained after DEAE-Sephacel column are shown in Figure 4.4A. The strong and broad absorption around 3348 cm^{-1} indicates O-H stretching vibration, absorption within the range of 2800-3000 cm^{-1} was for C-H stretching vibrations, and a strong extensive absorption in the region of the 1000-1200 cm^{-1} was attributed to C-O-C glycosidic band vibrations. All these absorption bands were characteristic absorption peaks of glycosides (Chen et al., 2010). Furthermore, the appearance of bands within the range of 1600-1700 cm^{-1}, corresponds to amide group (EI-Bahy, 2005). After desalting column, the IR spectra of crude TSP and the sample after desalting column were basically indistinguishable except for some differences in the intensity. The intensity of bands within the 3600–3200 cm^{-1}, 3000–2800 cm^{-1},

1200–1000 cm^{-1}, 1530-1700 cm^{-1} and 1180-1300cm^{-1} decreased. These ranges of absorption were characteristic absorption of glycosides and aclyamino. Belbachir et al., (2009) reported the intensities of IR absorption could provide quantitative information about sample. Thus, some phenolic compounds, glycosides and some small peptides were removed by desalting column.

After DEAE-Sephacel column (Figure 4.4B), the intensity of bands (3600–3200 cm^{-1}, 3000–2800 cm^{-1}, 2000-1000 cm^{-1} and 1000-500 cm^{-1}) were decreased, some new bands were also appeared. The peak at 3348.26 cm^{-1}, indicating hydroxyl (-OH) stretching vibration, 2945.55cm^{-1} assigning to C-H group weak vibration and one strong band at 1039.07cm^{-1} indicating pyranose ring (C-O-C) stretching vibrations, were noticed (Chen et al., 2008). All these bands were characteristic of polysaccharide (Lconomidou et al., 2000). The strong absorption at 1627.98 and 1553.92 cm^{-1}, corresponding to the stretching vibration for C=O of the amide group and bending vibration of the N-H bond, respectively, showed the existence of amide I and amide II bands (Kong and Yu, 2007). There is also an amide III band at 1295.66 cm^{-1}, contributing from C-N stretching and N-H deformation (EI-Bahy, 2005). Some new peaks were observed at 3188 cm^{-1} corresponding to N-H stretching of amide A band (Krimm and Bandekar, 1986). A weak band at 1739.08 cm^{-1} corresponded to C-O stretching vibration of carbonylic products (Wang, Mao and Wei, 2012). The band at 1220.64 cm^{-1} corresponded to phenolic compounds (Yang and Wang, 2008), indicating some bound phenolic compounds decreased after DEAE-Sephacel chromatography. Furthermore, some new characteristic absorption

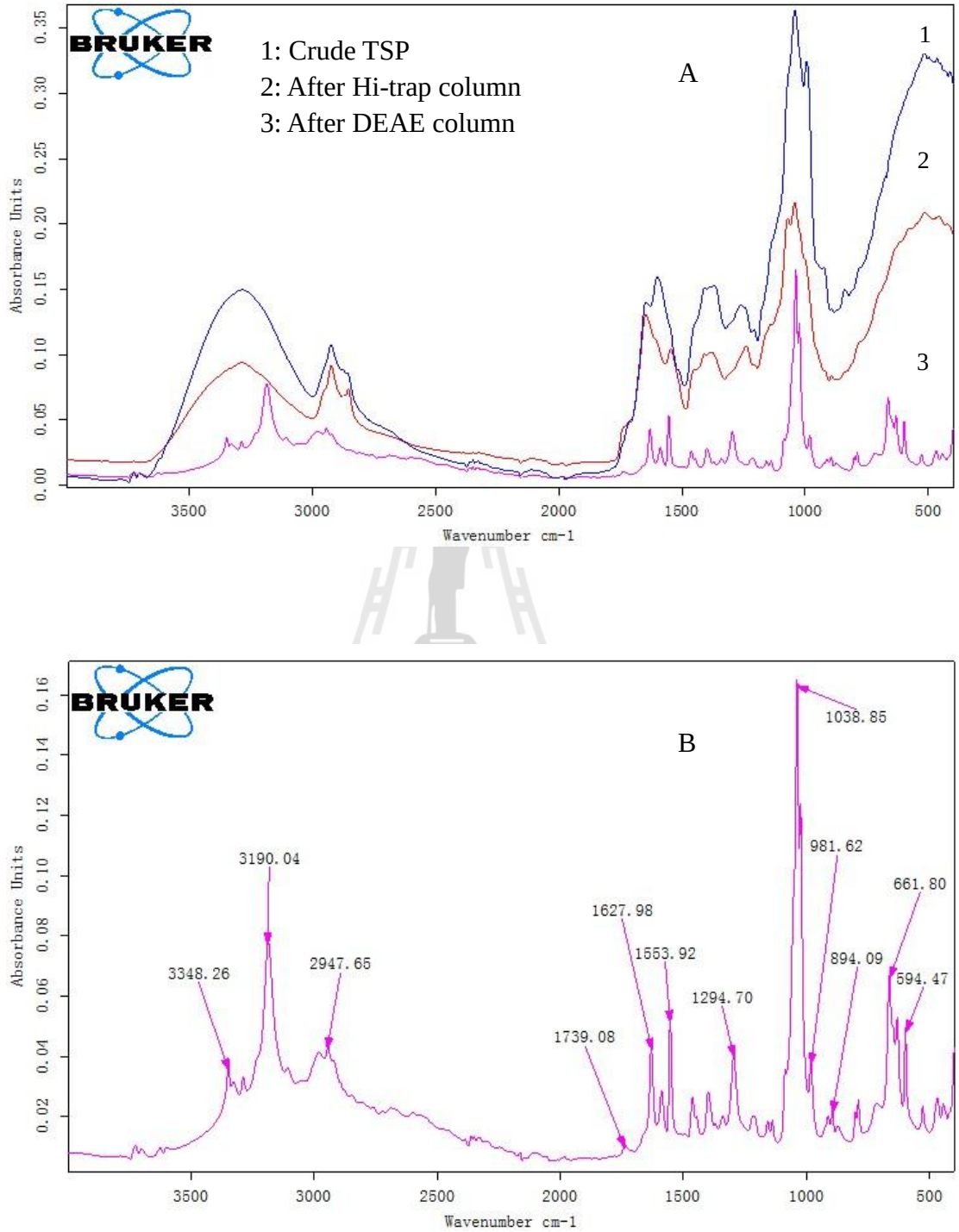

Figure 4.4 IR spectra of Crude, Hi-Trap desalting and DEAE-Sephacel (A), IR spectra of DEAE-Sephacel (B)

was also observed within the 500-1000 cm^{-1}. The characteristic absorption at 894.04 cm^{-1} corresponding to C-H vibration indicated the β-configuration of the sugar units (Barker, Bourne and Stacey, 1954). In addition, a strong band within the 590-670 cm^{-1} might be due to the presence of phosphate group (Kumar et al., 2014). Therefore, after DEAE-Sephacel column, some substances were further removed (i.e., phenolic compound, glycosides and small peptides). In addition, the IR spectra of isolated TSP suggested the characteristic of glycoprotein, which is in agreement with the report of Li et al., (2013). However, the more details of structure of isolated TSP need further studied.

4.5.2 Surface characteristics

The physicochemical characteristics of the food protein, such as molecular size, surface hydrophobicity, net charge, steric hindrance and molecular flexibility have been found to greatly influence their functional properties. (Sikorski, 2001). The surface hydrophobicity and net surface charge were important factors for protein functional properties (Schwenke, 2001). Proteins are amphiphilic molecules, that allow them to remain in the aqueous phase and adsorb at the surface of oil droplets at same time (Claesson, Blomberg and Poptoshev, 2004). Protein could be adsorbed at the oil-water interface depending on its surface hydrophobicity. In addition, the net charge of the protein should be large enough to overcome various attractive forces (e.g. van der Waals), leading to significant stabilizing electrostatic repulsive forces between oil droplets (McClements, 2004).

Surface charge (Zeta potential) of crude TSP and TSPI at each pH were shown

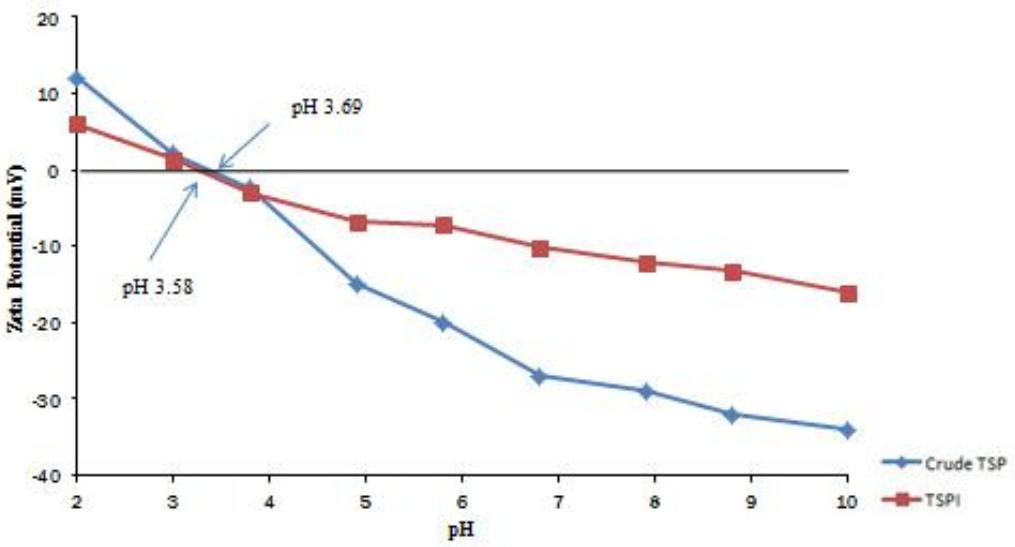

Figure 4.5 Zeta potential of crude TSP and isolated TSPI at various pHs

in Figure 4.5. Zero charge of crude extract and TSPI were found at acidic region of 3.69 and 3.58, respectively, corresponding to the results of solubility curve (Figure 4.1). The isoelectric point (IEP) was the pH of a protein solution at which the net charge or zeta potential of protein was zero (Salgin et al., 2012). The IEP of crude TSP and TSPI was at pH 3.69 and 3.58, respectively. In general, isoelectric point of biomolecules changed with pH environment (Salgin et al., 2012). Zeta potential value of the TSPI as a function of pH was lower than those of the crude TSP. After 2-step isolation techniques, some substances, i.e., phenolic, polysaccharide and small peptides, were removed, which could change ionic environment of the protein. Protein solubility was significantly affected by the net charge on the protein molecules. Protein showed the lowest solubility at zeta potential near zero (Zayas and Joseph, 1997). At pH 3, the protein of tea seed exhibited the lowest solubility ($p < 0.05$), and zeta potential was close to zero, limiting inter-molecular repulsive forces (Patil et al.,

2007). At neutral pH (7.0), the zeta potential was far away from zero with more negatively charged surface. Thus, the solubility of TSP increased.

Surface hydrophobicity of TSPI varied with pH between 3 and 9 ($p < 0.05$). The surface hydrophobicity decreased with an increase in pH (Figure 4.6). Surface hydrophobicity of TSPI is negatively related to its solubility. At pH 3, the TSPI exhibited the highest surface hydrophobicity, suggesting the unfolding of protein. Solubility of protein depends on large charge frequency and balance of hydrophobicity and hydrophilic (Nakai, 1983). Thus, protein with low hydrophobicity usually exhibits high solubility. At high pH, protein possesses negatively charges which resulted in the replusive forces with negatively charged ANS. This would partly be a reason of lower Ho at alkaine pHs.

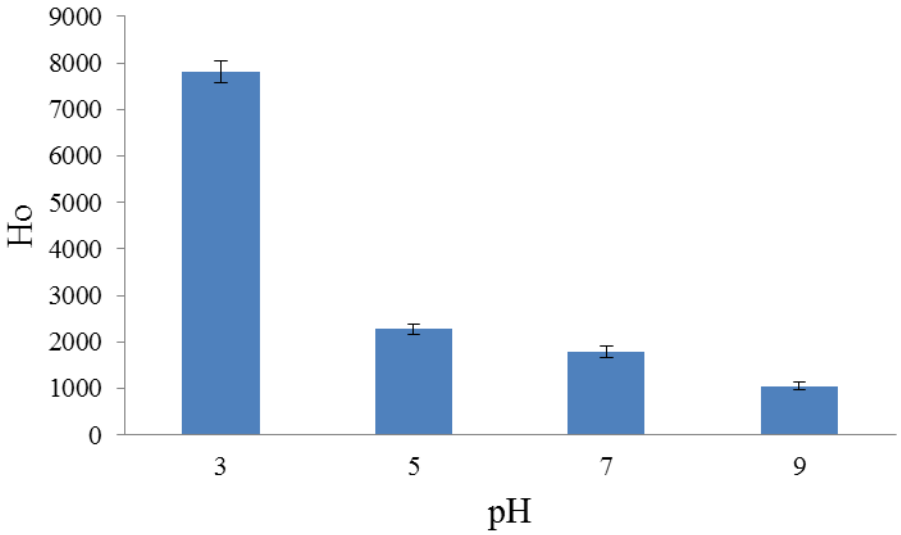

Figure 4.6 surface hydrophobicity of TSPI at various pHs

4.5.3 SDS-PAGE

SDS-PAGE analysis of all protein samples were shown in Figure 4.7. Crude

TSP contained various proteins with molecular mass ranging from 21 to 43 kDa. After removal of phenolic compounds by Hi-Trap desalting column, the same major bands were obtained, indicating that desalting column (Hi-Trap) did not remove any proteins from crude extract. After anion exchange chromatography, TSPI showed intense protein bands at 33, 31, 28, 23 and 14 kDa. The SDS-PAGE patterns of tea seed protein was close to that reported by Li et al., (2013), except for 14 kDa band.

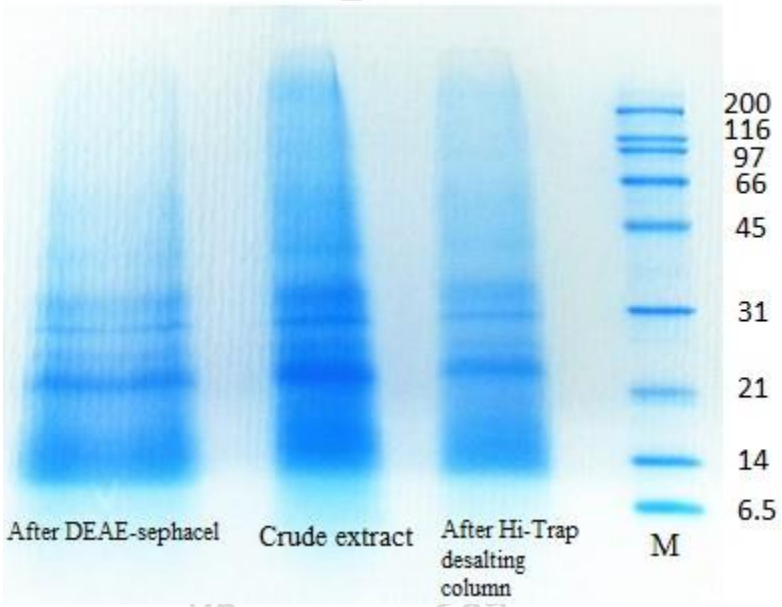

Figure 4.7 SDS-PAGE patterns of tea seed proteins at various stages of isolation. M=protein markers

4.5.4 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of the sample after Hi-trap deslating and the sample after DEAE-Sephacl are shown in Figure 4.8A and B. Patterns of these 2 samples were comparable except for spot 3. It did not appear in the sample after DEAE-Sephacel. It could be removed by DEAE-Sephacel. Two-dimensional gel electrphoresis showed evidence of many proteins in the TSP. At least, twelve major

sopts were detecred in the TSPI with molecular mass ranging from 21-28 kDa with pI ranging of 3.6-10. Spots 1-8 show the same molecular weight about 21 kDa and a wide range of pIs. This indicated that TSPI have possibly been modified via glycosylation. Because the IR spectra of TSPI showed some functional groups of sugar moieties. These twelve spots were further identified by LC-MS/MS.

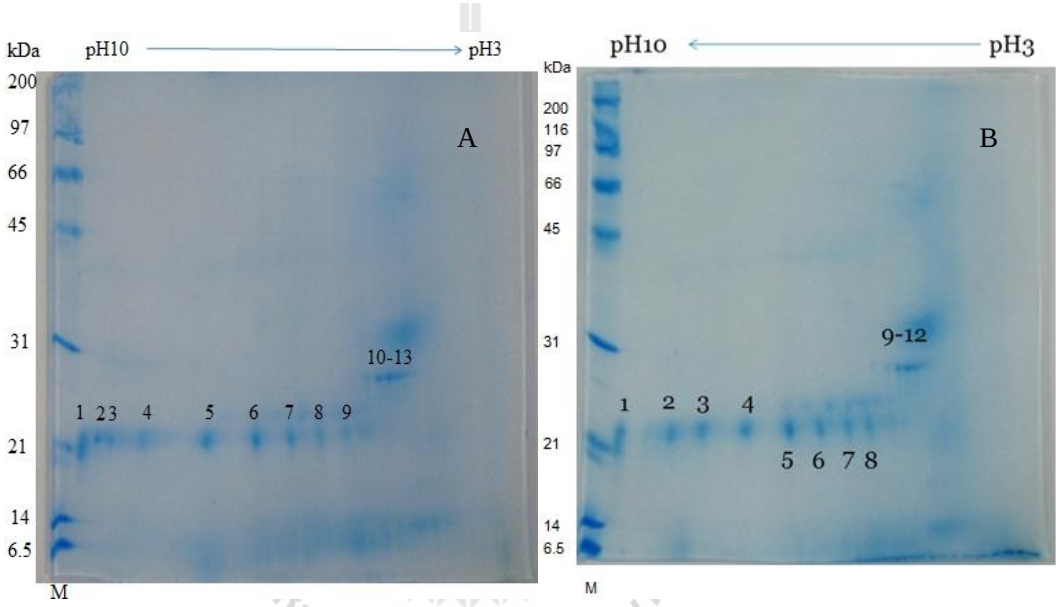

Figure 4.8 Two-dimensional gel electrophoresis of sample after Hi-trap deslating (A) and TSPI (B). Separation in the second dimension was performed using 12% SDS-PAGE. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top.

4.5.5 Protein identification by LC-MS/MS

Twelve major spots were analyzed by LC-MS/MS. The data obtained from LC-MS/MS were searched in NCBI databases. Spot numbers indicated in Table 4 correspond with numbers in Figure 4.8B. The NCBI database contains a large number of nucleotides, expressed sequence tag (EST) and protein sequences which is the ideal

for protein database search. A taxonomy restriction is also useful to reduce false-positive identifications. The whole NCBI protein database currently includes over 53 million entries, so the search was performed only on the Viridiplantae (green plants). Some storage proteins were found in *Camellia oleifera* seed such as oleosin, lipidbodybrane, caleosin, steroleosin, globulin, glutelin and albumin (Hu et al., 2005). However, the proteome information available for *Camellia oleifera* Abel is still limited. In the study, the combination of a protein separation by two-dimensional electrophoresis with the analysis of hydrolyzed samples by LC-MS/MS has permitted the identification of a munber of *Camellia oleifera* protein. Several spots identified as the same protein by LC-MS/MS were considered to be isomers. These proteins could be attributed by homology with other plant species such as *Theobroma cacao* (cocao tree), *Solanum lycopersicum* (tomato), *Acyinidia chinensis* (kiwi firut) and so on. Among the identified proteins, 11s globuline-like protien. and G-type lectin S-receptor-like serin/threonine-protein kinase RLK-like were the first time identified in tea seed protein. One putative protein (SELMODRAFT-166300) was also identified.

Table 4.3 A list of major protein spots of tea seed meal protein

Spot No.	Accession number	Protein score	Database MW (Da) / PI	Matching peptide (%)	Protein decription (species)	Peptide fragment
1	gi|508780241	61	89,591 / 9.07	1%	Argonaute protein group (*Theobroma cacao*)	SNLYLIRIMIFR
2	gi|460366084	53	94,551 / 6.18	0%	G-type lectin S-receptor-like serin/threonine-protein kinase RLK-like (*Solanum lycopersicum*)	KDGVLSPR
3	gi|302760929	54	66,305 / 4.81	1%	Hypothetical protein SELMODRAFT-166300 (*Selaginella moellendorffii*)	IASVMNER
4		58				
6		53				
7	gi|460366084	59	94,551 / 6.18	0%	G-type lectin S-receptor-like serin/threonine-protein kinase RLK-like (*Solanum lycopersicum*)	KDGVLSPR
8	gi|548852648	63	22,572 / 6.49	5%	Hypothetical protein AMTR_s00027p00144540 (*Amborella tirchopoda*)	EGGEMGGGER
9	gi|82469932	53	31,644 / 9.48	3%	11s globulin-like protein (*Actinidia chinensis*)	FFLAGNPQR
10		55				
11		54				
12		55				

4.6 Antioxidant of TSP

4.6.1 ABTS

ABTS assay was an indirect method illustrating the capacity of the ABTS radical cation to abstract a hydrogen atom or an electron from the compound studied (Prior, Wu and Schaich, 2005). ABTS scavenging ability of all proteins were shown in Table 5. The content of phenolic decreased in the order of crude TSP > the sample after Hi-trap > the TSPI. The protein obtained after the Hi-trap desalting column exhibited the lowest ABTS radical scavenging. This could be because some low molecular weight substances, such as free phenolic compounds, small peptides and amino acids were removed. These compounds likely contribute to antioxidant properties (Chen et al., 2008). TSPI exhibited the highest ABTS radical scavenging activity despite of its lowest phenolic content ($p < 0.05$). The IR spectra of TSPI indicated the characteristic of glycoprotein. Glycoproteins have been reported to exhibit high antioxidant activity, including that isolated from *Gardenia jasminoides* Ellis (gardenia) and *Salvia miltiorrhiza* Bunge (Lee et al., 2006; Duan et al., 2009). The ABTS radical scavenging activity of TSPI may also be attributed to the hydroxyl groups of TSPI, which could supply hydrogen atoms. The COOH, C=O, -S-, -O- function groups in the molecules can also confer a radical scavenging effect (Leung et al., 2009). In addition, the high antioxidant activity of TSPI may also be due to the higher frequency and /or proper positioning of antioxidant amino acids in their sequences (Arcan & Yemenicioglu, 2007).

4.6.2 FRAP

The FRAP values of proteins obtained from various stages of isolation are shown in Table 4.4. The FRAP of the TSPI was the lowest. Reducing ability of the antioxidants was due to their hydrogen donating ability (Shimada et al.,1992) .The TSPI showed hydrogen-donating ability based on ABTS assay better than other samples, but it exhibited the lowest FRAP value. This is could be due to its solubility. Because the FRAP assay was carried out at an acidic condition (pH 3.6), which is close to pI of TSPI. This could lead to protein precipitation, decreasing its reducing ability. However, the crude TSP and the sample after Hi-Trap contained relatively high phenolic content and exhibited high FRAP value. This result was different from other plant proteins, such as Chinese yam reported by (Duan, 2010). The protein of Chinese yam exhibited relatively high FRAP ability, this maybe depend on its good solubility in various pHs.

4.6.3 Metal chelating ability

The metal chelating ability of TSP obtained from various stages of isolation is shown in Table 4.4. The metal chelating ability of TSPI was the highest. It could be due to the functional groups of -OH, -COOH, C=O, which contributed to the chelating ability. Yuan et al (2005) reported that the compounds with structure containing two or more of the following functional groups: -OH, -SH, -COOH, C=O, -O- can show metal chelation activity. It is also possible that the partial purification helped unmasking the antioxidant activity of proteins. In addition, peptides or amino acid residues of TSPI, influenced the metal chelating ability (Decker et al., 1990). This is

the first report demonstrating antioxidant activity of protein isolated from tea seed meal of *Camellia oleifera* Abel. Mechanisms of TSPI antioxidant activity and its application in food deserve further investigation.

Table 4.4 Antioxidant ability of proteins extracted from tea seed meal subjected to chromatographic separation.

Sample	ABTS (mM Trolox)	FRAP (mM Trolox)	Metal chelating (mM EDTA)	PC (mg/ml)
Crude TSP	0.71±0.01	0.39±0.01	0.05±0.01	0.21
Hi-trap isolated TSP	0.43±0.02	0.24±0.02	0.02±0.01	0.07
DEAE isolated TSP	0.85±0.03	0.09±0.01	0.84±0.04	0.03

Note: All tests were based on the same protein content of 1 mg/ml.

4.7 Functional properties of TSP

4.7.1 Emulsifying properties of TSP

Emulsifying properties of food proteins are usually described by: emulsion activity (EAI) and emulsion stability (ESI). The former describes the ability of a protein to form an emulsion (Hill, 1996), whereas the latter reflects the ability of protein to impart strength to emulsion for resistance to stress (Liu et al., 2008). The emulsifying property of TSPI under various pHs are shown in Figure 4.9. The highest emulsifying activity (EAI) was found at pH 3 ($p < 0.05$). When pH was shifted from pH 3 to 9, the solubility of TSPI increased, but EAI decreased. A decrease in surface hydrophobicity at pH 9 would be the main cause of the reduction of EAI. This would

limit the movement of proteins to the interface. When emulsifying properties of TSPI were compared with casein, casein had the lowest emulsifying activity at pH 5 and the highest emulsifying activity at pH 7 ($p < 0.05$). This was in agreement with Chandi et al. (2007). Low EAI of casein at pH 5 was because it is close to its isoelectric point at pH 4.6. At pI, the net charge of protein is zero, and protein undergoes precipitation. Protein adsorption at the oil-water interface which is a diffusion controlled process is, thus, low. However, TSPI at pH 3, pH near its pI (~pH 3.6), exhibited good EAI and higher than that of casein. This is maybe depend on its high surface hydrophobicity and relatively low net charge; these factors would increase the movement of protein to the interface.

However, the ESI showed different trend. The lowest ESI was found in pH 3 ($p < 0.05$), and the increased towards alkaline pHs. The highest ESI of TSPI was observed at pH 7 ($p < 0.05$). The ESI of casein was higher than that of TSPI in all pHs studied, except for at pH 5. TSPI exhibited high surface hydrophobicity at pH 3, but its net charge was relatively low, leading to poor repulsive force. Thus, protein would associate via hydrophobic interactions, causing the low ESI. However, net charge of proteins increased with an increase in pH. This, in turn, increased repulsive force between proteins and thickness of hydration layers, resulting in an increase of ESI. At pH 9, ESI of TSPI was decreased, presumably due to protein denaturation.

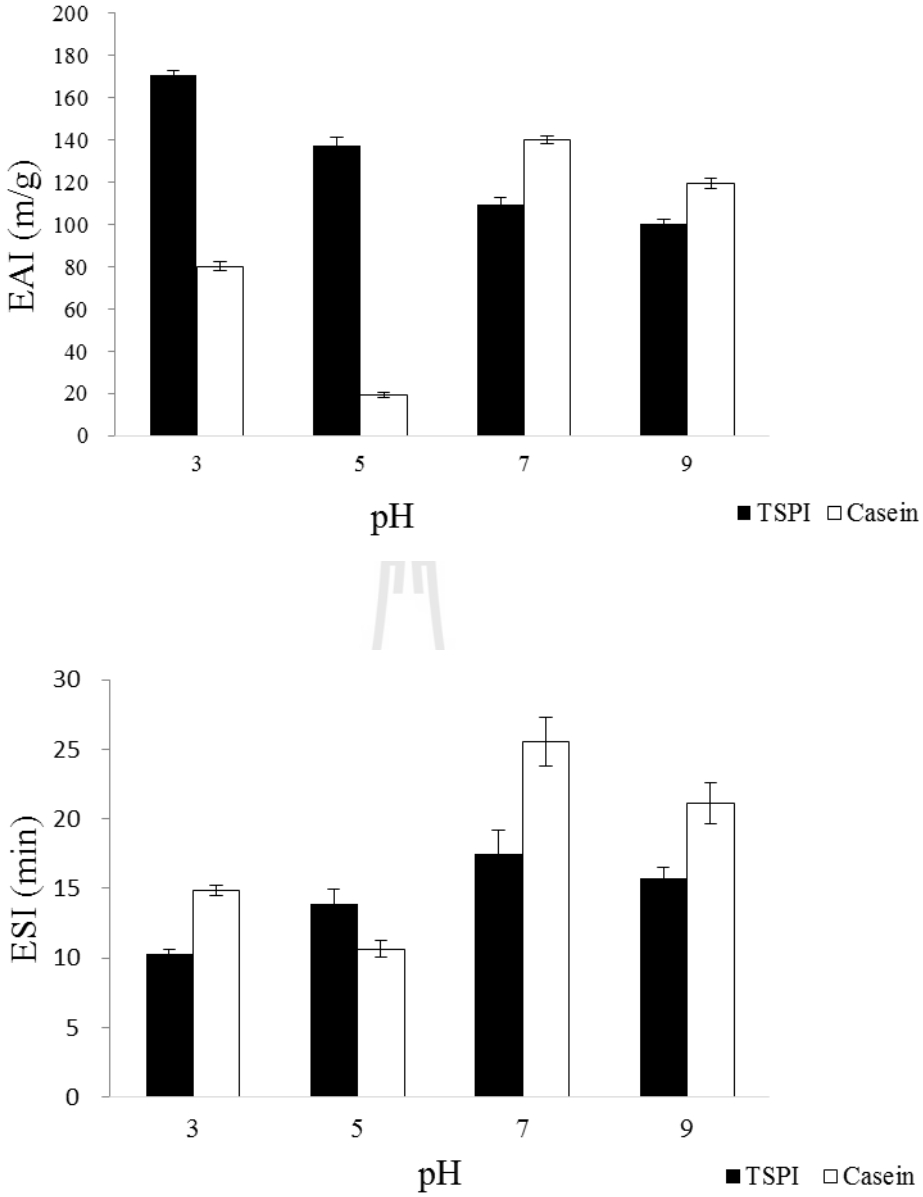

Figure 4.9 Emulsion activity (A) and emulsion stability of TSPI (B) at various pHs. Experiments were carried out at protein concentration of 0.1% (w/v) at 25^{o}C

4.7.2 Foaming properties

The effect of pH on foaming properties of TSPI was studied and compared with casein as shown in Figure 4.10. The lowest FC and FS of TSPI and casein was

found at pH 3 and pH 5 ($p < 0.05$), which was close to their respective pIs. Because protein exhibits poor solubility at its pI, the movement to air interface is limited. In addition, only the soluble protein fractions would be involved in the foam formation, and since the concentration of the soluble fraction is very low at its pI, FC would be less (Damodaran, 1997). Then, the foaming properties of both samples were increased with increased pH, especially at pH7. These results were likely due to an increase in the net charge of the protein, allowing the protein to spread to the air-water interface very quickly, encapsulating air particles and, thus, increasing foam formation. At pH 9, FC and FS of both samples decreased. FC and FS of TSPI was also lower than those of casein. This might be due to the repulsion of negatively charged tea seed protein.

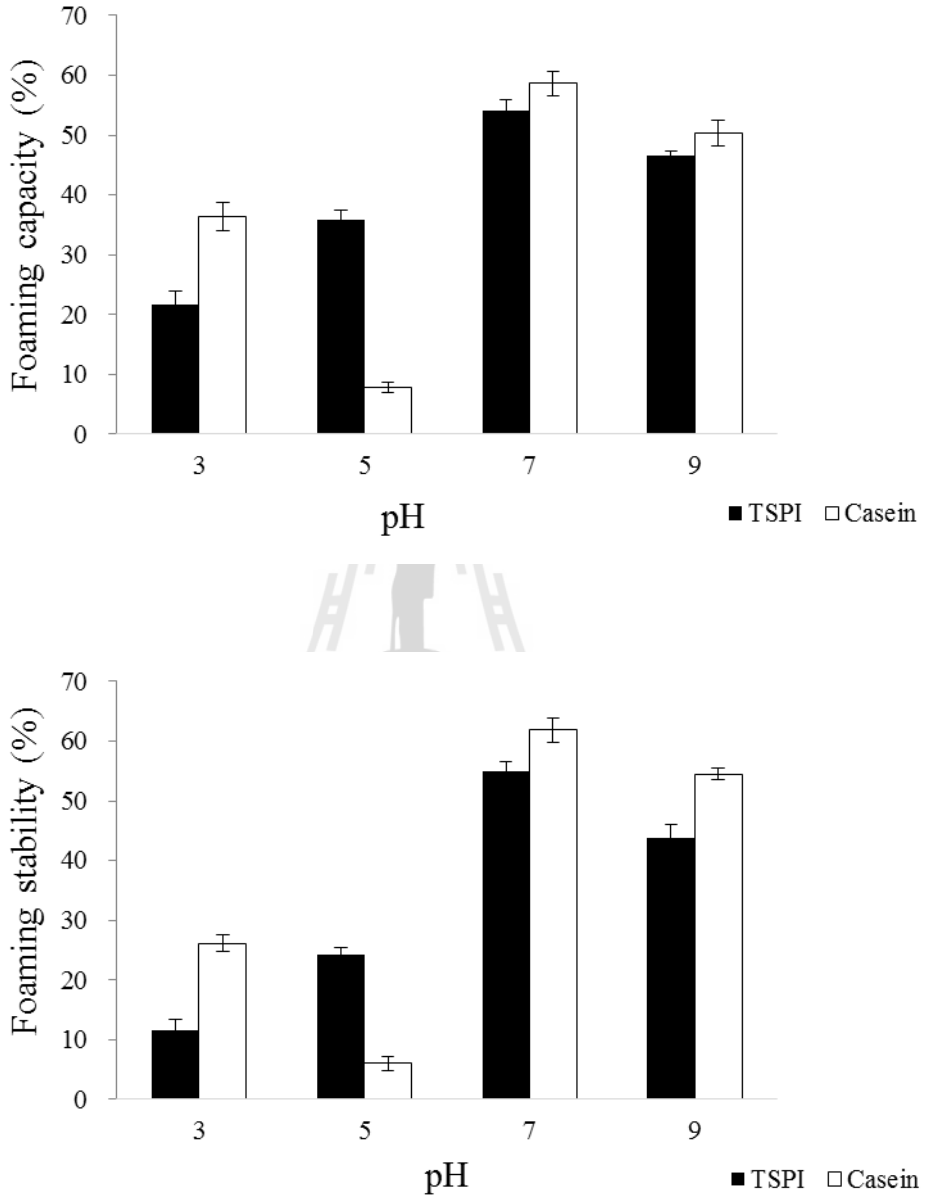

Figure 4.10 Foaming capacity (A) and stability (B) of TSPI and casein at various pHs. Experiments were carried out at protein concentration of 0.1% (w/v) at 25^{o}C .

CHAPTER V

CONCLUSIONS

This study showed the optimum condition for extraction and isolation of proteins from tea seed meal. Optimum extraction was found to be using deionized water at pH 7 as an extractant with the ratio of meal to water of 1:20, at 40^{o}C for 1 h. Protein recovery was found to be 50.4% with a relatively lower phenolic content. Desalting column and ion exchange chromatography were applied to remove phenolic compound. After 2-step isolation techniques, protein yield of 26.7 % was obtained with low phenolic content and light yellowish appearance. FT-IR spectra of TSP showed that some polysaccharides, phenolic compounds and small peptides were removed after 2-step isolation. In addition, FT-IR spectra indicated that TSPI exhibited a characteristic of glycoprotein. Surface charge (Zeta potential) of TSPI was lower than those of the crude TSP. The isoelectric point of crude TSP and TSPI was found to be at 3.69 and 3.58, respectively. The TSPI exhibited high value of surface hydrophobicity at acidic region (pH 3 and 5). Molecular mass and isoelectric properties of TSPI varied from 21-28 kDa, with pI ranging of 3.6-10. Protein identification revealed similarity to 11s globuline-like protein from *Actinidia chinensis*, G-type lectin S-receptor-like serin/threonine-protien kinase RLK-like from *Solanum lycopersicum* and one putative protein form *Selaginella moellendorffii*. It is likely that these are new proteins.

The TSPI possessed free radical scavenging property based on ABTS assay

and metal chelating ability. In addition, TSPI exhibited good emulsifying properties. Emulsifying activity of isolated TSPI was higher than that of casein ($p < 0.05$) at pH 3 and 5. However, the foaming properties of TSPI were lower than casein, expect for pH 5, which was pI of casein. Thus, the TSPI could be a novel functional protein ingredient.

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