การทำให้บริสุทธิ์บางส่วนและลักษณะสมบัติของหน่วยย่อยของไกลซินินจากถั่วเหลืองไทย Glycine max พันธุ์ ส.จ.5

นางสาว ฉันทลักษณ์ อาจหาญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญามหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545
ISBN 974-17-1135-2

PARTIAL PURIFICATION AND CHARACTERIZATION OF GLYCININ SUBUNITS FROM THAI SOYBEAN Glycine max VARIETY SOJO5

Miss Chantalak Arjhan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry

Department of Biochemistry
Faculty of science
Chulalongkorn University

Academic Year 2002
ISBN 974-17-1135-2

Thesis Title	PARTIAL PURIFICATION AND CHARACTERIZATION OF GLYCININ SUBUNITS FROM THAI SOYBEAN Glycine max VARIETY SOJO 5
By	Miss Chantalak Arjhan
Field of study	Biochemistry
Thesis Advisor	Lerson Tanasugarn, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

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ฉันทลักษณ์ อาจหาญ: การทำให้บริสุทธิ์บางส่วนและลักษณะสมบัติของหน่วยย่อยของไกลซินินจากถั่วเหลืองไทย Glycine max พันธุ์ ส.จ. 5 (PARTIAL PURIFICATION AND CHARACTERIZATION OF GLYCININ SUBUNITS FROM THAI SOYBEAN Glycine max VARIETY SOJO5) อาจารย์ที่ปรึกษา: อ.ดร.เลอสรร ธนสุกาญจน์, 105 หน้า, ISBN 974-17-1135-2.

เมื่อแยกไกลซินิน หรือ 11S โกลบูลิน จากถั่วเหลืองพันธุ์ไทย [Glycine max (L.) Merr] ได้แก่ ส.จ.4 ส.จ. 5 เชียงใหม่ 60 และ เชียงใหม่ 2 โดยโครมาโตกราฟีคอลัมน์ดีอีเออีเซฟฟาเด็กซ์เอ-50 พบว่า พันธุ์ ส.จ.5 ให้ปริมาณไกลซินินมีผลผลิตสูงที่สุด 0.08 เปอร์เซ็นต์จากสี่พันธุ์ เมื่อนำไกลซินินทั้งสี่พันธุ์มาวิเคราะห์โดย SDS-PAGE และ isoelectric focusing PAGE พบว่าโปรตีนประกอบด้วยสองหน่วยย่อยที่มีขนาดและตำแหน่งแตกต่างกันที่จุด isoelectric โดยหน่วยย่อยที่เป็นกรดมีน้ำหนักโมเลกุลอยู่ในช่วง 36,000-40,000 และหน่วยย่อยที่เป็นเบสมีน้ำหนักโมเลกุลประมาณ 20,000 จากการทำให้หน่วยย่อยของไกลซินิน จากถั่วเหลืองพันธุ์ ส.จ.5 บริสุทธิ์บางส่วนโดยโครมา-โตกราฟีคอลัมน์ดีอีเออีเซฟฟาเด็กซ์เอ-50 ที่มียูเรีย และจากผลการวิเคราะห์ SDS-PAGE พบว่าหน่วยย่อยของไกลซินิน (peak 3) ที่ประกอบด้วยหนึ่งหน่วยย่อยที่เป็นกรดที่มีน้ำหนักโมเลกุลประมาณ 37,000-38,000 และหนึ่งหน่วยย่อยเป็นเบสที่มีน้ำหนักโมเลกุลประมาณ 20,000-21,000 เชื่อมกันด้วยพันธะไดซัลไฟด์ จากการไดอะไลซิสหน่วยย่อยของไกลซินินที่บริสุทธิ์บางส่วนใน โพแทสเซียมฟอสเฟตบับเฟอร์ 3.5 มิลลิโมลาร์ (pH 7.6) และเซนตริฟิวจ์ใน sucrose density gradient พบว่ามี 4 peak และเมื่อละเว้นขั้นตอนการไดอะไลซิส พบว่ามีสองหน่วยย่อยที่มีขนาดเท่ากับหน่วยย่อยที่เป็นกรดและหน่วยย่อยที่เป็นเบส และเมื่อศึกษาโครงสร้างสามมิติของไกลซินินและหน่วยย่อยของไกลซินินโดย Electron microscopy ยืนยันว่าไกลซินินมีโครงสร้างเป็นวงแหวนของหกหน่วยย่อยซ้อนกันสองวงและแต่ละคู่ของ intermediary subunits ประกอบด้วยหนึ่งหน่วยย่อยที่เป็นกรดและหนึ่งหน่วยย่อยเป็นเบสซึ่งเชื่อมกันด้วยพันธะไดซัลไฟด์ ในการศึกษานี้เน้นลักษณะสมบัติของหน่วยย่อยเช่นเดียวกับผลความสัมพันธ์ระหว่างความสามารถในการละลายของไกลซินิน หน่อยย่อยของไกลซินิน และ สมบัติทางโครงสร้างเมื่อความแรงของไอออนเปลี่ยนจาก 0.5 เป็น 0.2 หรือ 0.03 โดยพบว่าเมื่อเพิ่มเกลือในสารละลายก่อให้เกิดการเพิ่มความสามารถการละลายของทั้ง ไกลซินิน และหน่อยย่อยของไกลซินินที่บริสุทธิ์บางส่วน

ภาควิชา.....ชีวเคมี ..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2545..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4272244023 : MAJOR BIOCHEMISTRY

KEY WORD : GLYCININ / PARTIAL PURIFICATION / CHARACTERIZATION / ELECTRON MICROSCOPY

CHANTALAK ARJHAN : PARTIAL PURIFICATION AND CHARACTERIZATION OF GLYCININ SUBUNITS FROM THAI SOYBEAN Glycine max VARIETY SOJO 5

THESIS ADVISOR : LERSON TANASUGARN, Ph.D. 105 pp. ISBN 974-17-1135-2.

Glycinin (11S globulins) from Thai varieties of soybean [Glycine max (L.) Merr] Sojo4, Sojo5, Chiang Mai60, and Chiang Mai2 were separated by chromatography on DEAE-Sephadex A-50. It has been found that Sojo5 cultivar glycinin contained 0.80% yield, the highest yield among all four. Partially purified glycinin from all four cultivars were analyzed by sodium dodecyl sulfate as well as isoelectric focusing polyacrylamide gel electrophoreses. The results demonstrated that the proteins contain two sizes of subunits with different isoelectric points, the acidic subunits (MW ≈ 37,000-42,000) and the basic subunits (MW ≈20,000). The intermediary subunits of glycinin from Sojo5 cultivar were partially purified by chromatography on DEAE-Sephadex A-50 in the presence of urea and characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It was found that the partially purified intermediary subunits (peak **III** from ion exchange chromatography) consist of one acidic (MW≈37,000-38,000) and one basic subunit (MW≈20,000-21,000) held together by disulfide bonding. When the partially purified intermediary subunits were dialyzed against the 3.5mM potassium phosphate buffer (pH 7.6) and centrifuged in sucrose density gradient, four protein peaks were observed. When the dialysis step was omitted, two major peaks with sizes corresponding to the acidic and basic subunits were obtained. Electron microscopy experiments confirmed the reported 3-dimensional structure of glycinin and that of the intermediary subunits. The results are consistent with the model of glycinin with two annular-hexagonal structures packed one on top of another, each composed of 6 subunits. Each pair of the intermediary subunits consists of one acidic and one basic subunits linked by disulfide bonding. This study emphasizes the characterization of intermediary subunits as well as confirms published results on the quarternary structure and the solubility behavior of glycinin. When the ionic strength was lowered from 0.5 to 0.2 or 0.03, the addition of salt to the solutions led to increased solubility of both partially purified glycinin and partially purified intermediate subunits.

Department……Biochemistry…….. Student's signature……..

Field of study…..……Biochemistry…… Advisor's signature……..

Academic year……..2002…….. Co-advisor's signature……

ACKNOWLEDGEMENT

I would like to express my deepest appreciation and gratitude to my advisor, Dr. Lerson Tanasugarn, for his excellent instruction, guidance, encouragement, attention and support throughout this thesis. Without his kindness and understanding, this work could not be accomplished.

My gratitude is also extended to Associate Professor Piamsook Pongsawasdi, Assistant Professor Tipaporn Limpaseni and Associate Professor Aran Incharoensakdi for serving as thesis committee, for valuable comments and also for useful suggestions.

My appreciation is also expressed to Miss Prukswan Chetanachan of The National Institute of Health, Department of Medical Sciences, Ministry of Public Health for her help in electron microscopy determination and data analysis. Thank you very much to Miss Mukda Sawadwongchai for her help in microdensitometry determination and data analysis.

Sincere thanks are extended to all staff members and friends of the Biotechnology Program and Biochemistry Department, especially the members in room 604 for their assistance and friendship.

Finally, the greatest gratitude is expressed to my parents, Miss Jeerawadee klaipim, Miss Supaporn Budeewong, Mr. Rungson Mungkong for their support, infinite love and understanding.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
µg	microgram
µl	microlitre
A	Absorbance
BSA	bovine serum albumin
cm	centrimeter
DEAE	diethylaminoethyl
EDTA	Ethylenediamine tetraacetic acid
g	gram
hr	hour
HCl	hydrochloric acid
kDa	kilodaton
l	litre
M	molar
2-ME	mercaptoethanol
mA	miliampere
mg	miligram
min	minute

ml	mililiter
mM	milimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	Sodium chloride
NaN_3	Sodium azide
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethyl ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
V/V	volume by volume
W/V	weight by volume

CHAPTER I

INTRODUCTION

1.1 Soybean

Soybean, *Glycine max* (L.) Merrill belongs to the family *Leguminosae,* and the subfamily *Papilionoideae*. It is one of the most economical and valuable agricultural commodities because of its unique chemical composition. Among cereal and other legume species, it has the highest protein content of around 40 percents. This is in contrast to other legumes, with have protein contents between 20 and 30 percents, and cereals, with protein contents in the range of 8-15 percents. Soybean also contains about 20 percents oil, the second highest content among all food legumes (Salunkhe *et al*., 1983). Other valuable components found in soybean include phospholipids, vitamins, and minerals. Furthermore, soybean contains many minor substances, some of which, such as trypsin inhibitors, phytates, and oligosaccharides, are known to be biologically active.

Soybean proteins have good nutritional and physicochemical functions among plant proteins. Thus, soybeans have been utilized for many kinds of traditional foods in East Asia. More than 80% of the annual production of soybean seeds is used for oil extraction. The amount of protein in the residues after oil extraction is ~35 million tons/year. Most of the residues are used as feed for domestic animals and as fertilizer. It is known that soybean proteins have a physiological role in lowering cholesterol levels

in human serum (Kito *et al*., 1993). Therefore, the residual protein may be valuable in solving the current problems in the increase of heart disease and hypertension caused by high cholesterol levels and also in the future problem of food shortages. Further growth in the soy market is anticipated because of the 1999 US FDA ruling that approved use of the label claim that soybeans can lower cholesterol. Since physiological functional of the components in soybeans have very important shown in table 1.

Table 1 Physiological functionalities of soybean components.

components	Physiological functionalities
Protein	Control of cholesterol level in the blood, antimutagenic effect.
Peptides	Hypotensive effect, control of cholesterol level, detoxification of harmful substances, antivirus effect, promotion of bone and teeth growth.
Oligosaccarrides	Regulation or control of intestinal.
Dietary fiber	Function, (prevention of constipation, improvement of bacterial flora, prevention of colon cancers, immunoactivation), regulation of blood sugar content (inhibition of insulin and glycogen secretion, prevention of diabetes mellitus) control of regulation of cholesterol (prevention of gallstone formation, decrease in fats, prevention of obesity, hypotensive effect) bifidobacterium activation.
Unsaturated fatty acids	Decrease in cholesterol, hypotensive effect (decrease in viscosity of blood, increase of blood deformability) prevention of breast, colon and prostate gland cancers improvement of allergy diseases.
Tocopherol lecithin	Antioxidation, same as unsaturated fatty acids decrease in cholesterol, decrease in fats, improvement of fat liver.
Saponin isoflavonoids	Antioxidation, prevention of peroxidation of lipids, regulation of liver functions, immunoactivation.

Source: Proceedings of World Soybean Research Conference V, Chiang Mai, Thailand

Figure 1 Soybean tree and soybean seed.

(Source: www.doae.go.th/library/html/crop_all.html)

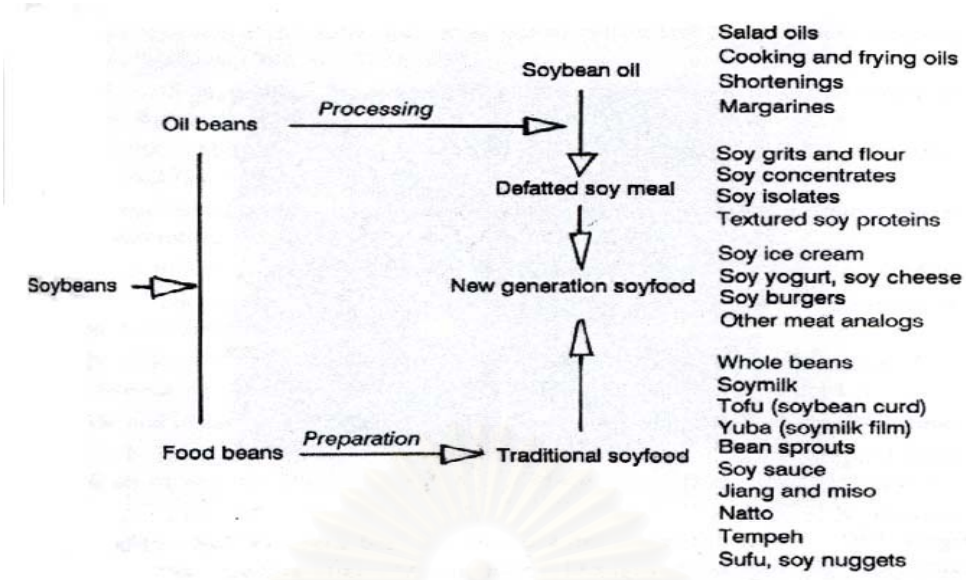


Figure 2 A general outline of soybean food based on classification of oil and food beans (Liu *et al.*,1995b).

Among the major economic crops, soybean has played an important role in the economic and social development plans in Thailand over the last two decades. Soybean is used as soy meal and soy oil. Soy meal is used mainly as feed for livestock and part of it is used as soy starch for local food mixtures. Soy oil is used in two ways. One is in seafood product canneries and the other is as the basis for cooking oil, light oil paint and other canneries. As well, there is some local soy milk produced (Hann, 1991).

As shown in Table 2 production of soybean in the country increased by 229 percent over the last ten years. The increase resulted from more intensive activity but not from land expansion (Dalodom, 1997). Production of grain soybean was 132,000 tons from a planted area of 800,000 rai with an average yield of 165 kg per rai in 1981/82. In 1991/92, the total production area of grain soybean increased to 2,715,475 rai, but the average yield remained at 201.9 kg per rai.

Table 2 Imports and exports of grain, meal and oil of soybean in Thailand

Unit: 1,000 tons

Crop Year	Import			Export		
	Grain	Meal	Oil	Grain	Meal	Oil
1991/92	79.2	506.7	-	0.7	-	-
1992/93	123.5	575.1	-	0.8	-	-
1993/94	97.9	788.6	-	0.2	-	0.01
1994/95	166.4	854.0	-	0.4	-	-
1995/96	425.7	819.6	1.5	0.3	-	0.3
1996/97	657.4	905.8	-	0.2	-	8.3
1997/98	574.2	825.8	-	0.4	-	12.3
1998/99	925.0	1,174.0	-	1.0	-	0.4
1999/00	1,078.9	1,191.7	-	0.5	-	39.6
2000/01	1,423.0	1,300.0	-	0.5	-	1

Source: Office of Agricultural Economics, Department of Agricultural Extension, Bangkok, Thailand.

Problems of the soybean are: Production and Marketing, as reviewed by Chavalvut Chaiuvati, Deputy director general, Department of agriculture extension, Ministry of Agriculture and cooperative of Thailand. (http://www.fao.org)

First, Seed availability is a big problem. The government cannot provide sufficient seed to meet farmers' demands, so the private sector has to provide the rest. But the quality of this seed is rather poor, as compared to the government seed (Chainuvati *et al*., 1997). Timing of seed supply is also not good. Most of the time the government seed supply is too late, so farmers have to seek seed from local traders where most of the time, the seed's quality is low and of mixed varieties. The problem of seed germination is also a burden. Another main problem is considered the non- or only partial, acceptance of new, high technology by the farmers that relate to lack of money and/or time and labor. Therefore, farmers are reluctant to adopt such technologies. Besides, disease and pest problems, as well as natural disasters are also contributing to low yields.

Second, The lack of bargaining power of the farmers to sell their products, and lack of competitiveness of soybean and soy products, to the other major soybean producing countries and other countries, all contribute to a low soybean price at the farm gate.

Soybean protein ingredients must possess appropriate functional properties for food applications and consumer acceptability. These are the intrinsic physicochemical characteristics which affect the behavior of protein in food systems during processing, manufacturing, storage and preparation, e.g., absorption, solubility, gelation, surfactancy, ligand-binding and film formation. These properties reflect the composition and conformation of the protein, their interactions with other food components, and they are affected by processing treatments and the environment. Because functional properties are influenced by the composition, structure and conformation of ingredient proteins, systematic elucidation of the physical properties of component protein is expedient for understanding the mechanism of particular function traits. The composition and properties of the major components of soy proteins are important for assessing and extending the application of soy proteins in foods and for developing new functional ingredients.

Soy proteins have four major water-extractable fractions (2S, 7S, 11S and 15S) that can be isolated on the basis of their sedimentation coefficients. Soybean proteins are composed of two major globulin components, β-conglycinin and glycinin, accounting for about 30% and 40 % of the total seed proteins, respectively (Utsumi,1992; Utsumi *et al.*,1997). These proteins may be recovered by ultracentrifugation as 11S and 7S fractions, respectively. A heavier (15S) and a lighter (2S) protein fractions also exist but do not need to be considered further, as the 15S proteins are mostly insoluble in the soy meal extract and the 2S (whey) proteins remain soluble throughout the fractionation process.

1.2 Glycinin

Glycinin, 11S globulin, is one of the major storage proteins in soybean. It is the more abundant of the two storage proteins. The amounts of these two storage proteins differ in soybean seeds depending on their genetics and where they are grown. Where the application of a protein product calls for more sulfur amino acids then a higher glycinin content is preferred. Thus, glycinin plays an important role in the properties of food made from soybean.

The glycinin extracted from seeds has a hexameric structure with a molecular mass of 300-380 kDa. Its quaternary structure is stabilized by electrostatic and hydrophobic interaction and by disulfide bridges between the acidic (34-44 kDa) and basic polypeptides (20 kDa) which are abbreviated A and B, respectively. (Peng *et al.,* 1984).

There are five genetic variants of glycinin, A1aB1b, A2B1a, A1bB2, A5A4B3, and A3B4 (Nielsen, 1985). Based on homology in subunit sequences, glycinin is classified into group I (A1aB1b, A2B1a, A1bB2) and group II, which is further divided into two subgroups called IIa (A5A4B3) and IIb (A3B4). These three groups (I, IIa, and IIb) differ especially in the carboxyl terminal end of the acidic subunit (Table 4). About 60 and 30 amino acid residues are deleted from the sequence of group IIa to form those of groups I and IIb, respectively (Nielsen *et al.,* 1989). The group I subunits have more uniform apparent MW and contain more methionine than members of group II. This last feature is important for breeders to increase the methionine content in seeds.

Five different subunits have been identified and sequenced. The subunits are classified into two groups based on the amino acid sequences. The sequence identity is about 80 percents within each group and about 45 percents between groups. The protein subunits are first synthesized as single protein precursors called preproglycinins (Adachi, 2001). A signal sequence is removed cotranslationally in the endoplasmic reticulum and the resulting proglycinins are assembled into trimers which are transported to the storage vacuoles via the golgi apparatus. There is thought to be some sort of sorting signal for the trimers to be transported to the vacuoles but this signal has not yet been identified (Adachi, 2001). After proteolysis the acidic and basic polypeptides are formed and assembled into a single subunit. The subunits then form the hexamers that make up the mature protein.(Adachi *et al.,* 2001; Katsube *et al*., 1999). The mechanism behind hexamer formation from the trimers is not fully understood. Only one of the proglycinins has been structurally determined. X-ray crystallography revealed that the A1aB1b proglycinin contains 25 strands and five helices. The protein is folded into two jelly-roll domains and two extended helical domains, which stack on adjacent subunits (Adachi *et al*., 2001).

Table 3 Five major soybean glycinin subunits and their paired acidic and basic Polypeptides (Nielsen, 1989).

Group	Gene	Subunit	Mol.wt.(KD)	No. of Methionine
I	G1	A1bB2	58	5-6
I	G2	A1aB1b	58	5-6
I	G3	A2B1a	58	7-8
IIa	G4	A3B4	62	3
IIb	G5	A5A4B3	69	3

Comparison of amino acid sequence of five major subunits (Figure 3) revealed that the acidic and basic polypeptides each exhibited considerable NH_2-terminal sequence homology (Moreira *et al*.,1979). There is also a high degree of homology in the interior portion of the acidic polypeptides. By using absorption spectroscopy, Wolf reported that natural glycinin contained 0.62 to 2.2 –SH groups/mol with a mean value of 1.4 ± 0.5. When reduced with 2-mercaptoethanol (ME) in the absence of denaturants, the –SH contents increased and leveled off at 21 –SH groups/mol in the range of ME concentration of 0.04-0.1 M (Wolf, 1993). Result of SH measurements are compared with the current model for glycinin, which have contents at 12-SH groups/mol (Badley *et al*., 1975). Apparently, there was a large discrepancy in the number of –SH groups measured vs. the number of predicted from the current model of glycinin structure.

By amino acid sequence modeling the secondary structure of glycinin was predicted to be 25% α-helices, 25% β-sheet, 42% turns, and 8% unordered (Argos *et al.,* 1985). Recently, Abbott *et al*. (1996) interpreted fourier transform infrared (FTIR) spectra of glycinin as containing 24% α-helices, 30% β-sheet, 31% turns, and 12% unordered. Their data also indicated that glycinin had the same secondary structure in solution and in hydrated solids. Nothing is known about the tertiary structure of glycinin. Nevertheless, glycinin has a complex quaternary structure consisting of two layers of trimers. Each trimer has three acidic and three basic polypeptides paired and held together by disulfide and hydrogen bonds, with alternating acidic and basic peptides (Badley *et al.,* 1975). These bonds can be disrupted by urea, strong acid, strong base, heat, or sodium dodecylsulfate in combination with a disulfide reducing agent. As a result, the quaternary structure is altered.

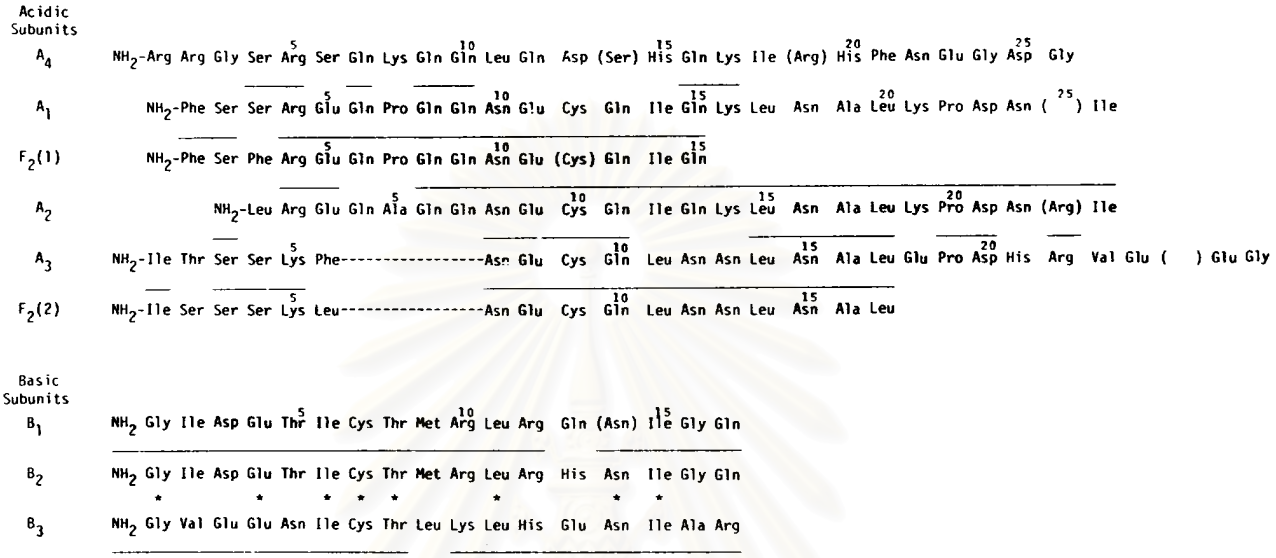


Figure 3 The NH_2-terminal sequences of the 9 glycinin subunits aligned for maximal homology.

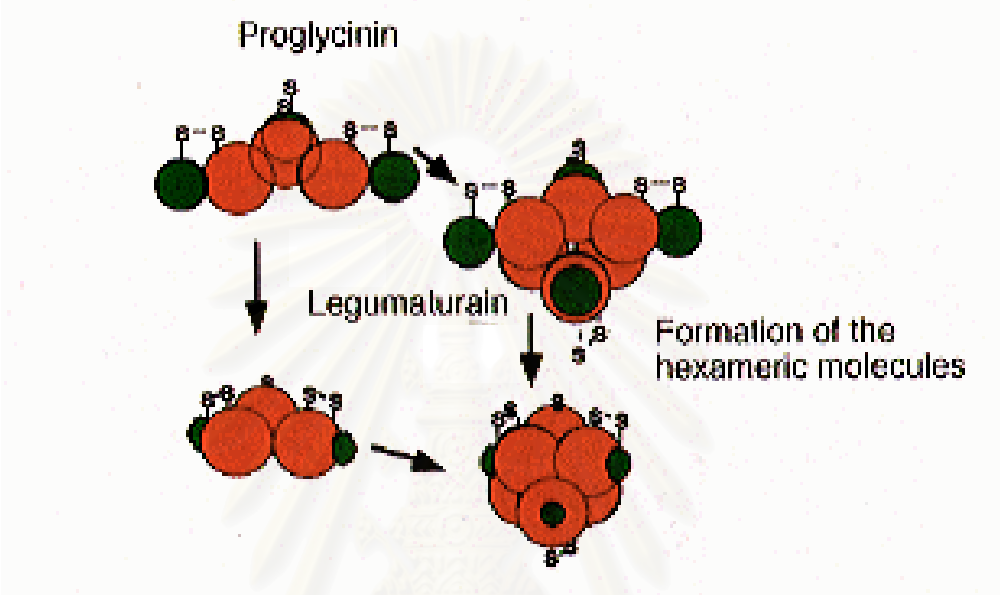
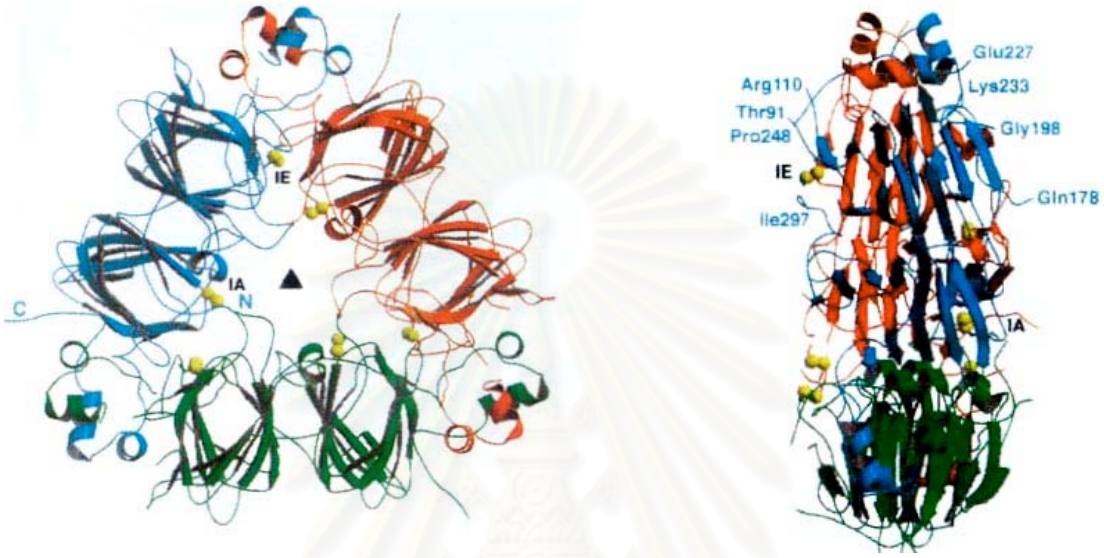


Figure 4 Schematic representation of the post-translational processing of a storage protein.

The Glycinin (11S globulins) from legumes have not yet been crystallized. The difficulty in the crystallization of these proteins may be due to molecular heterogeneity. Success in the high-level expression of glycinin in *Escherichia coli* (Kim *et al*., 1990) and *Saccharomyces cerevisiae* (Utsumi *et al*., 1991) should facilitate crystallization, because such an expression system enables the production of a single molecular species. In fact, Utsumi *et al.* succeeded in crystallizing proglycinin expressed by *E.coli* (the expressed proteins accumulated as proglycinins since *E.coli* does not have the enzyme responsible for the processing of proglycinins to the mature form (Utsumi *et al*., 1993) In addition to, normal recombinant proglycinin, Utsumi also crystallized its modified version having food functions different from those of the normal glycinin and proglycinin. The three-dimensional structure of the normal proglycinin was determined at 6 A° resolution (Utsumi *et al*., 1999). Adachi *et al.* have determined the crystal structure of the proglycinin A1aB1b homotrimer by X-ray crystallography at 2.8 A° resolution (Figure 5) and revealed the core structure consists of two jelly-roll barrels and two extended helix domains in analogy with 7S glybulins (Adachi *et al*., 2001).



A B

Figure 5 Three-dimension view of the ribbon diagram. Three protomers in the proglycinin trimer are shown in red, cyan and green, respectively. The disulfide bonds in the trimer molecule are represented by yellow spheres with a radius of 1.2 A°. A. View from a three-fold symmetry axis is shown by a filled triangle. B. Viewed after 180° rotation around the vertical axis (Adachi *et al*., 2001).

1.3 Separation methods for Glycinin

Wolf *et al.* first obtained 91-93% ultracentrifugally pure glycinin by cryoprecipitation followed by fractionation with ammonium sulfate. They reported a 25 % yield for the purified glycinin (Wolf *et al.*,1962). Factors influencing yield and purity of the glycinin fraction, such as extraction ratio, temperature, pH, salt and sugar content, and reducing reagent (2-mercaptoethanol, 2-ME), have been investigated (Wolf, 1967).

Koshiyama reported a procedure for glycinin and β-conglycinin (7S) fractionation (Koshiyama, 1965). The glycinin fraction was first cryoprecipitated, and then 0.025M $CaCl_2$ was added to remove the residual cold-insoluble protein. The 7S fraction, which contained mostly β-conglycinin, was precipitated by adjusting the pH of the supernatant to 4.5. After gel filtration a homogeneous β-conglycinin fraction was obtained as evaluated by ultracentrifugal analysis, which does not differentiate among different proteins of comparable mass.

Thanh *et al.* developed a straightforward process for glycinin and β-conglycinin separation based on differential solubilities of glycinin and β-conglycinin at pH 6.1-6.6. 0.03M Tris-HCl buffer (pH 8.0) containing 10mM 2-mercaptoethanol was used to extract soy proteins (Thanh *et al.*, 1975, 1976). Glycinin was separated by adjusting the pH to 6.4 and collecting the precipitate after centrifuging at 2-5 °C. β-conglycinin was precipitated at pH 4.8 and purified by redissolving the precipitate in the 0.03M Tris buffer and

adjusting the pH to 6.2. The β-conglycinin fraction was kept at 3-5 °C overnight and centrifuged to remove undissolved polymerized form. This particularly useful method for preparing the two soybean globulins has been used for many years, because glycinin and β-conglycinin components can be separated simultaneously. However, cross contamination of proteins in both globulin fractions is a continuing problem. The glycinin fraction contained 79% glycinin, 6% β-conglycinin, and 15% other components. The β-conglycinin fraction contained only 52% β-conglycinin, 3% glycinin, and 45% other. The method of Thanh *et al* requires procedures such as gel filtration and affinity chromatography for purification, which are costly and difficult to scale up.

Nagano *et al.* modified the method of Thanh *et al.* in three way: (i) soy proteins were extracted by using water at pH 7.5 instead of Tris buffer; (ii) sodium bisulfite was used as a reductant; and (iii) three protein fractions were precipitated at pH 6.4, 5.0 and 4.8 instead of two at pH 6.4 and 4.8 (Nagano *et al.*, 1992). They claimed the purities of crude glycinin and β-conglycinin were greater than 90% as measured by densiometer scans of gel from sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Marcone *et al*. reported a simple alternative technique for the purification of the 11S soybean globulin from crude globulin using a single Sephacryl S-300 gel filtration chromatographic technique (Marcone *et al*.,1994). This procedure yielded 11S globulin of purity comparable to those described above but was superior in its simplicity and was less time consuming. All supporting data demonstrated that the 11S globulin was purified to homogeneity with no contamination from the 7S soybean globulin.

Saito *et al.* showed a novel method for separating soybean β-conglycinin and glycinin from defatted soymilk by a phytase treatment (Saito *et al*., 2001). [Phytate (*myo*-inositol hexaphosphate) is the major storage form of phosphorus in soybean seeds. Phytate is also considered an antinutrient because it chelates minerals and forms insoluble complexes with proteins and interferes with essential mineral bioavailability.] Phytate may affect the solubility and related functions of soy proteins in commercial food applications. It may also interfere with the fractionation and characterization of the β-conglycinin and glycinin. Hence, it seems worthwhile to obtain reduced-phytate proteins to make high-quality foods. Phytate in defatted soymilk was hydrolyzed by phytase at pH 6.0, and the mixture incubated for 1 h at 40 °C. This procedure separated β-conglycinin and glycinin without seeding a reducing agent or cooling into the soluble and insoluble fractions, respectively. Simultaneously, most of the phytate in both proteins was removed.

Thiering *et al*. showed a novel protein fractionation technique using carbon dioxide as a volatile electrolyte to isoelectrically precipitate soybean proteins (Thiering *et al*., 2001). The pH of the protein solution was controlled by the carbon dioxide pressure, and the precipitate was recovered by centrifugation. Thiering *et al.* claimed the advantage of this methodology was the ability to ensure that the pH did not drop below the set point at any time within the fraction vessel. The purified glycinin and β-conglycinin were 95 and 80 percents, respectively. This volatile electrolyte technology was also

applied as a continuous process in order to eliminate the particle recovery concerns associated with batch precipitation and to demonstrate the potential for scale-up.

1.4 Purification methods for Glycinin

Numerous methods have been reported in the literature for purifying the glycinin fraction. Nearly all current methods involve separating an initial crude glycinin fraction from an alkaline soy protein extract by precipitation in the cold at pH 6.3-7.0 (Iyengar, 1981). The glycinin fraction may then be purified by chromatography on a hydroxyapatite column followed by Sepharose 6B chromatography (Badley *et al.,* 1975), or simply chromatographed on Sepharose 6B only (Moreira *et al.,*1979). Another method involves purification of the crude glycinin fraction by ion exchange chromatography (Utsumi *et al.*,1981). A more detailed procedure (Kitamura *et al.,*1976) involves the use of ammonium sulfate to fractionate the crude glycinin component followed by concanavalin A and Sepharose 6B chromatography. The glycinin peak was subjected to ion exchange chromatography to remove most of the final contaminating proteins (98-99%). The published information of Thanh and coworkers (Thanh *et al.,* 1976), However, confirmed the effectiveness of DEAE sephadex A-50 ion-exchange chromatography in separating glycinin from β-conglycinin.

A new purification technique using a single Sephacryl S-300 gel filtration chromatographic was developed (Marcone *et al.*, 1994). This procedure which yielded

11S globulin of purity comparable to other techniques was superior in its simplicity and was less time consuming. As reviewed earlier, carbon dioxide has been used as a volatile electrolyte to isoelectrically precipitate two major protein constituents of soybean. Carbon dioxide was shown to be effective in purifying glycinin and β-conglycinin in a three-step process, resulting in 95 and 80 percents concentrated fractions with yields of 28 and 21 percents, respectively (Thiering *et al*., 2001).

1.5 Objectives

The objectives of this biochemical study of glycinin in Thai varieties of soybean are:

1. To select the best cultivar of soybean which gives the highest glycinin contents among varieties of Thai soybean cultivars .
2. To extract glycinin subunits (intermediary subunits) from the cultivar with the highest glycinin content
3. To partially purify and characterize the intermediary subunits.

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CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Centrifuge, benchtop centrifuge: Model H-11n, Kokusan, Japan

Centrifuge, refrigerated centrifuge: Model J-21C,Beckman Instrument Inc, U.S.A.

Centrifuge, Hettich Zentrifugen: Model MIKRO 12-24, Swittzerland

Conductivity meter: CDM 83 Radiometer Copehapen, Denmark

Electric blender & mill: Model SMB-001, Srithai Marketing Co.,Ltd., Thailand

Electrofocusing unit: Model 111 MINI IEF cell, Bio-Rad, U.S.A.

Electron microscope: Model JEM-1010, JEOL, Japan

Electrophoresis unit: Model Mini PROTEIN® 3 System,Bio-Rad, U.S.A.

Freeze—dryer: Model Flexi-Dry™ μP Stone Ridge, New York, U.S.A.

Fraction collector: Model 2211 Pharmacia LKB, Sweden

Gradient makers: Hoefer SG30, Hoefer Pharmacia Biotech Inc, U.S.A.

Magnetic stirrer and heater: Model IKAMA® GRH, Janke & Kunkel Gmbh & Co. KG, Japan

Microdensitometer: Model PDM-7, Konica, Japan

Peristaltic pump: Model Pump p-1, Pharmacia LKB, Sweden

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark

Power Supply for electrophoresis: Model EC135-90 Bio-Rad, U.S.A.

Rocker platform: Model back-to-basics® Bellco, New York, U.S.A.

Spectrophotometer: Spetronic® genesys™8, Spectronic Instrument Inc, England

Ultracentrifuge: Model L8-80 Ultra-8™, Beckman Instrument Inc, U.S.A.

Vortex: Model K-550-GE, Scientific Industrial, U.S.A.

2.2 Chemicals

- Acetone (67-64-1) was obtained from BDH, England.

- Diethyl ether (3011-01) was obtained from Merck, Germany.

- Ampholyte (80-1124-80), Standard molecular weight marker proteins (17-0446-01), Standard pI marker proteins (17-0471-01) were obtained from Amersham Pharmacia Biotech, Sweden.

- Acrylamide (A 8887), Coomassie brilliant blue G-250 (B 5133), Coomassie brilliant blue R-250 (B 7920), DEAE-Sephadex A-50 (A-50-20), Glycine (G 7126), N,N′-methylene-bis-acrylamide (M 7256), 2-mercaptoethanol (M 6250) and Sodium azide (S 2002) were obtained from Sigma Chemical Co., U.S.A.

- Aguasorb was obtained from Bio-medical Laboratory, Thailand.

- Gas Nitrogen was obtained from Wattanachok Trading Co. Ltd., Thailand

- Glycerol (M 778-09) was obtained from J.T.Baker Chemical, U.S.A.

- Sucrose (84097), Trichloroacetic acid (911228) were obtained from Fluka, Switzerland.

Other common chemicals were obtained from Fluka or Sigma. All chemicals are of reagent grade unless otherwise specified.

2.3 Other materials

Dialysis tubing: (D-977) Sigma, U.S.A.

Filter paper No.1: Whatman International LTD., Englang.

Glass wool: (1686930) BDH, England.

2.4 Plant material

Glycinin was purified from soybean cultivars (*Glycine max* (L.) Merr.) Sojo4, Sojo5, Chiang Mai 60 and Chiang Mai 2, which were obtained from the soybean breeding program at Field Crops Research Institute, Department of Agricultural Extension, Ministry of Agriculture and Cooperatives of Thailand. These are high yield hybrids whose seeds contain on a dry weight basis about 40%, 42%, 36% and 37% protein, respectively. These cultivars are selections from the cross *Acadian X Tainung, Tainung X Sojo2, Chiang Mai 60 X IAC, Williams X Sojo4*, respectively. The seeds were harvested prior to the onset of dehydration. Individual seeds were frozen in liquid nitrogen and stored at -80°C. In order to stop germination of soybean seeds, they were kept at -20°C.

2.4.1 Preparation of soybean meal

Frozen seeds were ground using an electric blender & mill and defatted with hexane until the extracted solid was pale yellow to colorless. The meal was washed with

cold acetone (-20°C) once and in diethyl ether twice, dried on a piece of filter paper, and stored in a desiccator at -20°C.

2.4.2 Preparation of crude glycinin

Crude glycinin (11S globulin) fraction was prepared according to the method of Thanh and Shibasaki (1976). The isolated globulins were dissolved in 0.03M Tris-HCl buffer containing 0.01M 2-mercaptoethanol (ME) (pH 8.0) at room temperature for 1 hr. The protein solutions were centrifuged (15,344g, 20 min). The extract was adjusted to pH 6.4 with 2N HCl. The protein solutions (0.2%) were centrifuged (15,344g, 20 min, 4°C). The 11S globulin was collected by centrifugation. No significant precipitates were detected. All the protein solutions used in this investigation contained 0.01M ME and experiments were carried out at room temperature (20 to 22°C).

2.5 Purification of glycinin and subunit dimers

2.5.1 DEAE-Sephadex A-50 column chromatography

DEAE-Sephadex A-50 was swelled in 0.03M Tris-HCl buffer containing 0.01M ME, pH 8.0. Complete swelling took 1-2 days at room temperature. The swelled gel was degassed and packed into a glass column (2.4 x 90cm.). The DEAE-Sephadex A-50 column (2.4 x 80cm.) was equilibrated with a buffer for 5 column volumes at of 20ml/hr flow rate to allow stabilization of bed volume of the column.

2.5.2 Partial Purification of glycinin

Chromatographic fractionation of crude glycinin fraction was performed on a column of DEAE-Sephadex A-50 equilibrated with 0.035M potassium phosphate buffer (pH 7.6) containing 0.25M NaCl, 10mM 2-Mecaptoethanol and 0.025% NaN_3 at 5°C. Elution with NaCl in gradient concentration of 0.25M to 0.5M was carried out. Column effuents were collected in 3.2ml fractions and the absorbance at 280nm was measured. Protein concentrations were determined by the method of Bradford *et al*, (1976). The purity of glycinin was assessed by SDS-PAGE under reducing and non-reducing conditions using 10-15% gradient gels in a Mini PROTEIN® 3 System Gel Electrophoresis unit. The protein bands were stained with coomassie brilliant blue.

2.5.3 Preparation of intermediary subunits

The partially purified glycinin was dialyzed against 0.035M potassium phosphate buffer (pH 7.6) containing 0.4M NaCl. After exclusion of ME, the glycinin solution was resuspended with 0.09M sodium phosphate buffer (pH 6.6) containing 6 M urea and 0.001M EDTA, degassed with nitrogen, and then applied to the column of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was performed with the buffer containing a linear concentration gradient of 0 to 0.3 M NaCl. All operations were performed at 5 °C and all the buffers were degassed with nitrogen.

2.6 Protein determination

Protein concentration was determined by the Coomassie blue method according to Bradford (1976), using bovine serum albumin as standard.

One hundred microlitres of the sample was mixed with 5 ml of Bradford reagent (0.117mM) and incubated at room temperature for 5 minutes before its absorbance at 595nm was recorded.

2.7 Determination of glycinin and dimer glycinin

2.7.1 Polyacrylamide gel electrophoresis (PAGE)

Denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE) were employed for analyzing the partially purified glycinin and intermediary subunits.

2.7.1.1 Non-denaturing polyacrylamide gel electrophoresis

The system was modified from the method of Cameo and Blaquier (1976). A discontinuous PAGE was performed on a slab gel (10 x 8 x 0.075 cm) made of 8% (w/v) separating gel and 5.0% (w/v) stacking gel. Tris-glycine buffer pH 8.3 was used as electrode buffer. The preparations of polyacrylamide gels are described in Appendix D. Four parts of the protein from each step was mixed with one part of the 4 X sample buffer before being loaded onto the gel. Protein bands were electrophoresed from the cathode towards the anode at a constant current of 20mA per slab at room temperature in a Mini

PROTEIN® 3 System Gel Electrophoresis unit. Following electrophoresis, the gel was stained for protein as described in section 2.7.1.4

2.7.1.2 SDS-polyacrylamide gel electrophoresis

The denaturing gel was performed according to the modified method described by Laemmli (1970). The slab gel system consisted of a stacking gel (10 X 2 X 0.1 cm) made of 3% (w/v) acrylamide and a separating gel (10 X 8.6 X 0.1 cm) made of 12.5% (w/v) acrylamide. The gel preparation was described in Appendix D. Samples to be analyzed were treated with the sample buffer and boiled for 5 minutes prior to application on the gel. The electrophoresis was performed at a constant current of 20mA per slab, at room temperature in a Mini PROTEIN® 3 System Gel Electrophoresis unit. The standard molecular weight markers used were phosphorylase b (MW = 94 kDa), bovine serum albumin (MW = 67 kDa), ovalbumin (MW = 43 kDa), carbonic anhydrase (MW = 30 kDa), trypsin inhibitor (MW = 20.1 kDa) and α-lactalbumin (MW = 14.4 kDa). Following electrophoresis, the gel was stained for protein as described in section 2.7.1.4

2.7.1.3 Urea gel

The system was modified from the method of Davis (1964). Discontinuous PAGE was performed on slab gels (10 x 8 x 0.075 cm). Alkaline gel electrophoresis in 7M urea was performed in 7.5 % polyacrylamide gels with a slight modification in which

7M urea was included in all the gel solutions. The preparation of polyacrylamide gel is described in Appendix D.

2.7.1.4 Detection of protein

Gel from electrophoresis preparations were stained for 30 minutes with a staining solution consisting of (0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 7% (v/v) acetic acid, at room temperature with moderate shaking. Destaining was performed by immersing the gel for 30 minutes in destaining solution I (40% methanol and 7% acetic acid) followed by an overnight soak in destaining solution II (5% methanol and 7 % acetic acid).

2.7.1.5 Detection of disulfide linkage

Glycinin solutions were dialyzed against 0.035 M potassium phosphate buffer (pH 7.6) containing 0.25M NaCl and 0.025% NaN_3 at 5°C without 2-mercaptoethanol for 24 hours and run on SDS-PAGE in the absence of 2-mercaptoethanol.

2.7.2 Isoelectric focusing (IEF) polyacrylamide gel electrophoresis

The native isoelectric focusing polyacrylamide gel on mini gel system was used for determining the pI value of glycinin. The gel (10 X 9.4 X 0.075cm) was prepared as described in Appendix D. The IEF system was run at a pH range of 3.0 to 10.0. The partially purified glycinin obtained from DEAE-Sephadex A-50 column chromatography

was mixed with the sample buffer (60% glycerol, 4% ampholyte). The cathode solution was 25mM NaOH and the anode solution was 20mM acetic acid. Electrophoresis was performed at room temperature for 1.5 hour at 200V constant voltage. Then voltage was increased to 400V and kept constant for an additional 1.5 hour. After electrophoresis was completed, the gel was fixed by immersion in 10% trichloroacetic acid (TCA) for 10 minutes followed by 1% TCA for at least 2 hours to remove ampholyte. The fixed gel was stained for protein as described in 2.7.1.4. A standard pI calibration kit (pI 3.0-10.0) was used as standard pI markers.

2.8 Characterization of intermediary subunits

2.8.1 Determination of solubility

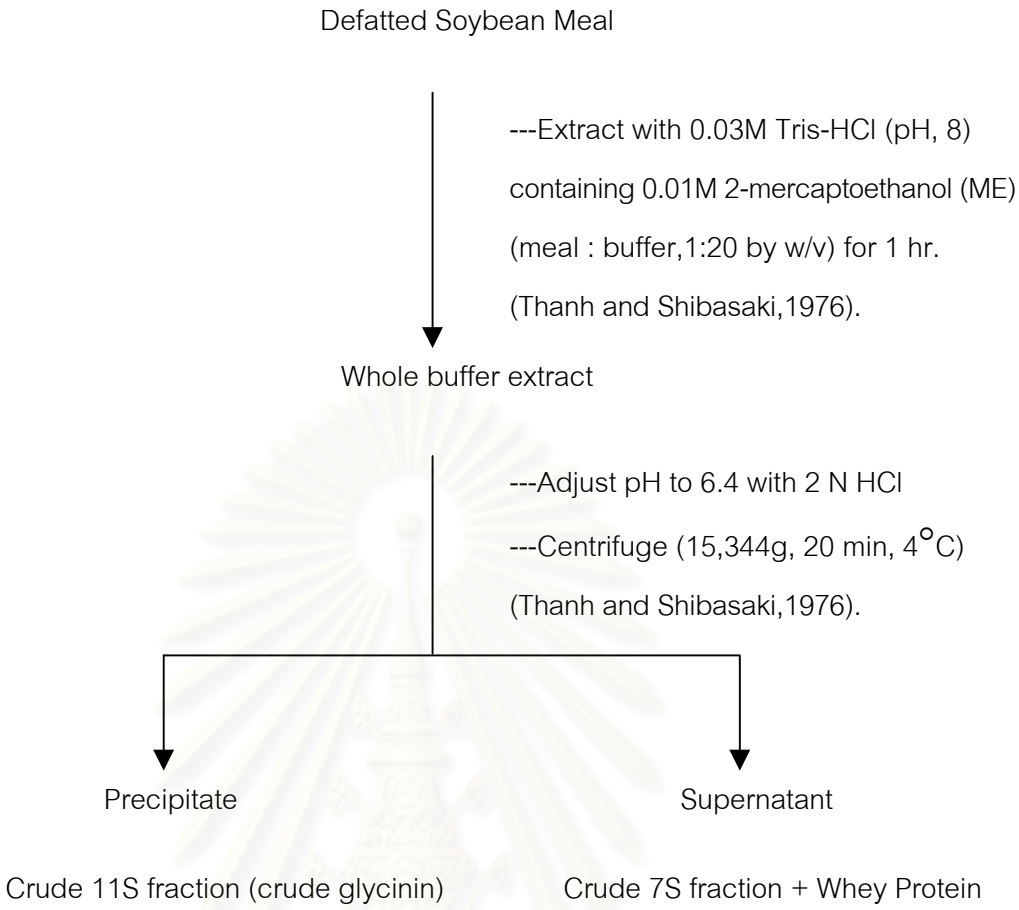
The I = 0.5 buffer consisted of 35mM potassium phosphate and 0.4M NaCl, the I = 0.2 buffer consisted of 35mM potassium phosphate and 0.1M NaCl, and the I = 0.03 buffer consisted of 10mM potassium phosphate. The pH of glycinin solutions (0.6 mg/mL) in pH 7.6 buffer at I = 0.5, 0.2, or 0.03 was lowered by adding different amounts of HCl stock solutions (0.05 to 5M) to obtain final pH values ranging from 7.6 to 2.5. After incubation of the glycinin samples for 16 h at 20°C, the samples were centrifuged for 5 min at 14,454g and 20°C. The precipitate consists of particles larger than approximately ~0.5 μm as was determined using Stokes equation (f=6$\pi\eta$r). The protein concentrations of the supernatants were determined in triplicate by the Bradford assay (Bradford, 1976) using BSA as standard.

2.8.2 Determination of dissociation-association

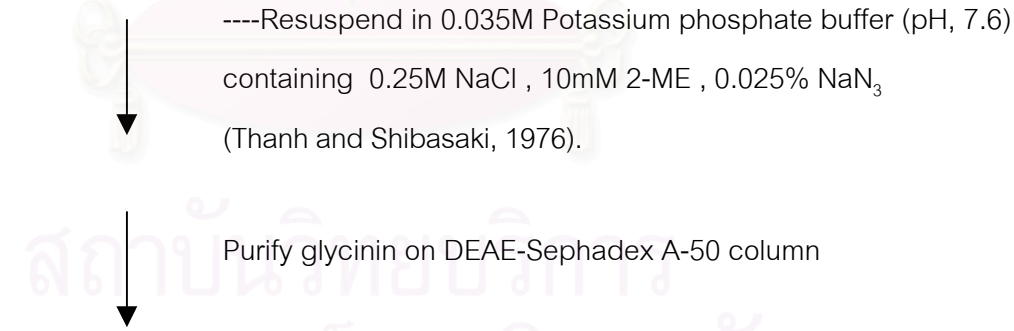
The intermediary subunits was optionally dialyzed against 0.035M potassium phosphate buffer (pH7.6). After dialysis, the sample was layered on 4.5 ml of 10~30% (w/v) linear sucrose density gradient and centrifuged at 120,516g (42,000 rpm in a 70.1 Ti rotor) for 6.5 hour. After centrifugation, the gradient was collected into 0.8ml fractions, Then the absorbance of each fraction at 280nm was measured.

2.9 Electron microscopy

A carbon coated copper grid was floated on top of a drop of a glycinin solution in the standard buffer (0.2-1 mg/ml) that was placed on a hydrophobic surface. The grid was then floated in a similar way on top of a drop of the staining solution and left there for several minutes. Either a 2% solution of potassium phosphotungstate pH 7.0 or a 4% solution of sodium silicotungstate pH 7.0 (from Taub Laboratories, Emmer Green, Reading, U.K.) was used as negative stain. Alternatively, the protein solution was dropped on to the grid and rinsed with 3-4 vol. of the negative stain. Excess liquid was drained off with a filter paper. Both staining procedures yielded similar results. No improvement was observed when the protein was fixed for 20 min in dilute (0.5%) glutaradehyde solution in the standard buffer prior to being negatively stained.



- Protein determination by Bradford's assay



- Protein determination by Bradford's assay
- Investigation of partially purified glycinin by SDS-PAGE and Non-denaturing-PAGE
- Electron microscopy

Figure 6a Flow chart for partial purification of glycinin.

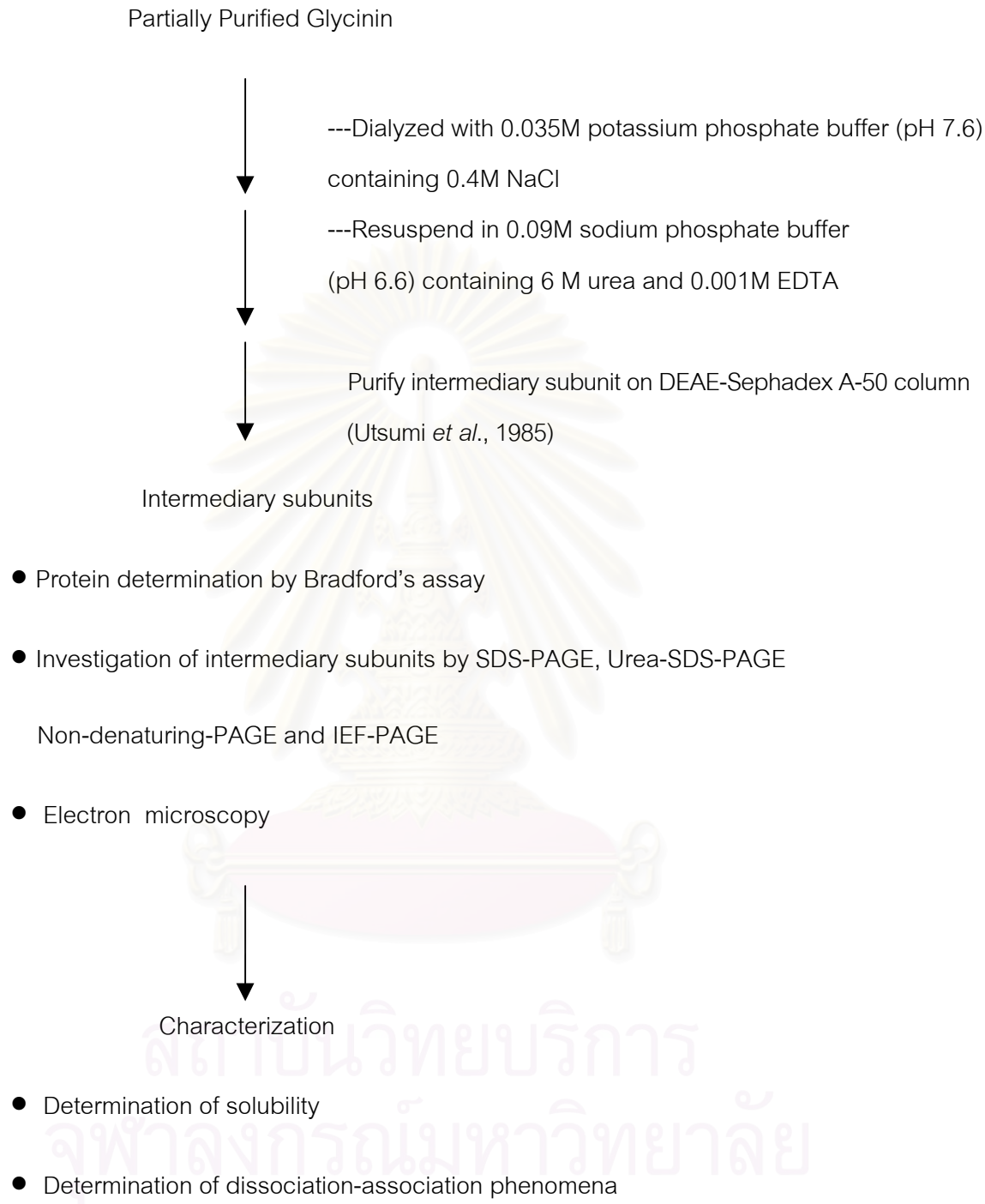


Figure 6b Flow chart for partial Isolation of intermediary subunits.

CHAPTER III

RESULTS

3.1 Preparation of defatted soybean meal

Soybean meal was prepared according to the methods outlined in section 2.5.1. (Figure 7). Before the fat was removed, milled soybean was yellow in color. The lipid was removed in the organic solvent fraction. After the fat was removed, the extracted solid turned into white and lightweight powder.

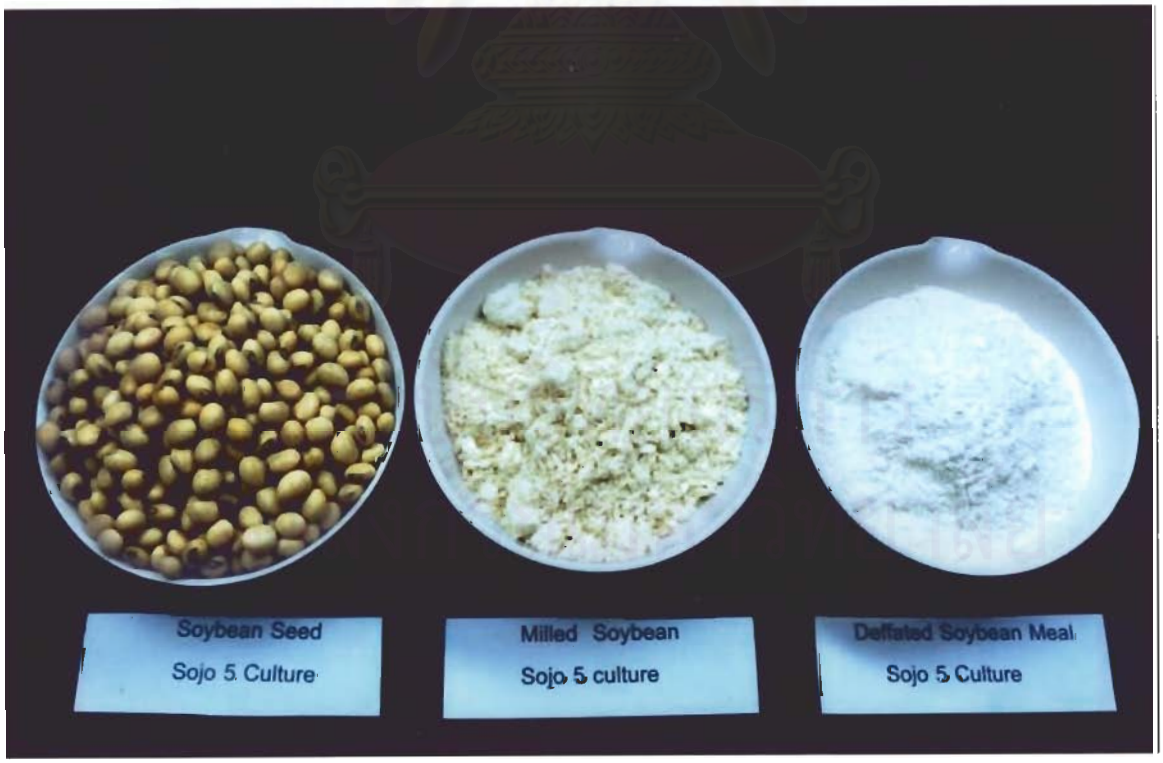


Figure 7 Preparation of defatted soybean meal from soybean seeds.

3.2 Partial purification of Glycinin from soybean meal

Crude glycinin from defatted soybean meal was purified according to the methods outlined in section 2.5.2. The two major soybean proteins were simultaneously isolated by a simple method based on their solubilities in dilute tris buffers at pH 8 (Figure 6a). The resuspended crude glycinin fraction was further resolved into at least three components by DEAE-Sephadex A-50 chromatography (Figure 8). The unbound proteins were eluted from the DEAE-Sephadex A-50 column with equilibrating buffer. Glycinin bound to DEAE-Sephadex A-50 column was eluted with linear gradient of 0.32 to 0.85 M sodium chloride. Glycinin was eluted as single peak that was comparatively sharper than other peaks. As monitored by A280, most proteins were eluted within fraction numbers 140-180, which were then pooled. The protein concentrations were determined by the method of Bradford (1976). Purified glycinin from 10 grams of soybean meal from each cultivars (Sojo4, Sojo5, Chiang Mai 60 and Chiang Mai 2) was obtained with 0.27, 0.80, 0.43, 0.14 % yield, respectively (Table 4). The resulting protein concentrations suggested that cultivar Sojo5 was the one that gave highest glycinin content among varieties of Thai soybean cultivars. Source of glycinin was reported to be the major protein in soybean meal and this was confirmed by non-denaturing PAGE (Figue11), so glycinin content was presented by total protein content.

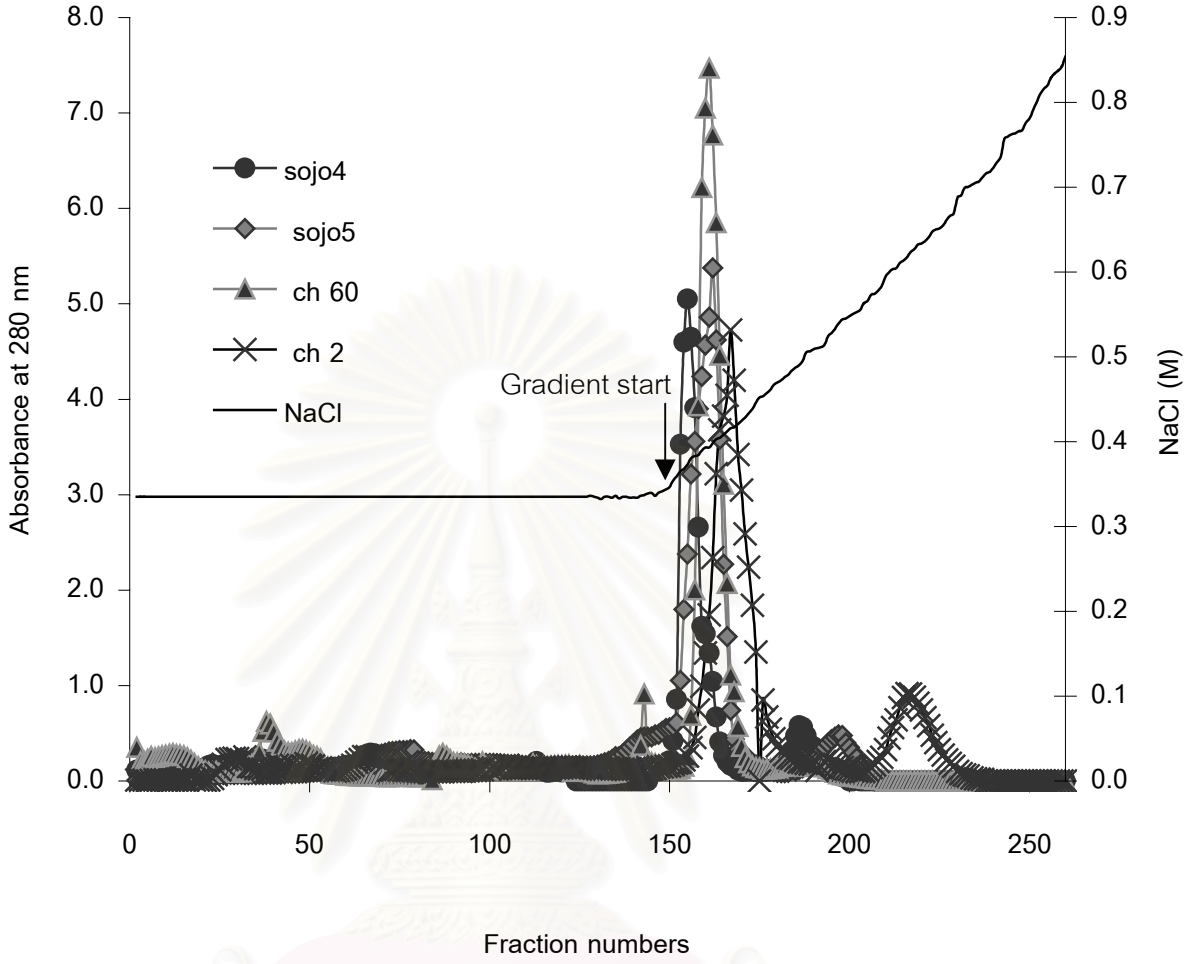


Figure 8 Chromatographic profile on DEAE-Sephadex A-50 column of glycinin from crude glycinin of different soybean cultivars.

Crude glycinin was resuspended with 35 mM potassium phosphate buffer (pH 7.6) containing 0.25 M NaCl, 10 mM 2-mercaptoethanol and 0.025% NaN_3 and applied on the column (1.5 X 90 cm) previously equlibrated with the same buffer at 5°C. Elution with a 0.32 M to 0.85 M NaCl gradient was carried out at 20 ml/hr flow rate and 3.2 ml fraction volume was collected.

Table 4 Purification of glycinin from crude glycinin

Cultivar	Yield		
	soybean meal	Crude glycinin %	Purified glycinin %
Sojo4	79.38	0.90	0.27
Sojo5	75.55	1.01	0.80
Chiang Mai 60	65.08	0.75	0.43
Chiang Mai 2	74.98	0.85	0.14

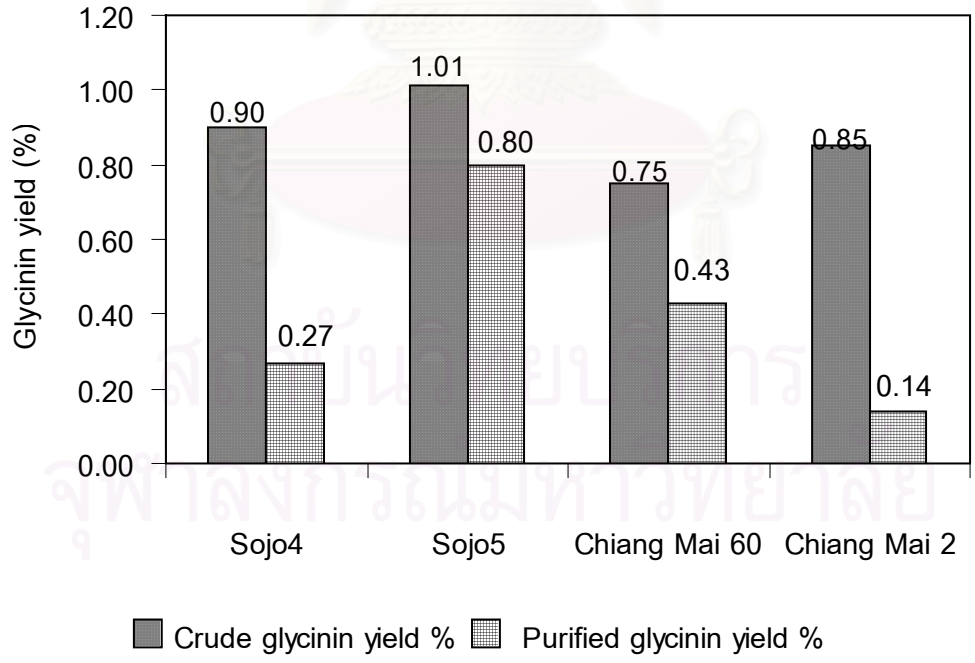


Figure 9 Summary of glycinin yields from varieties of Thai soybean cultivars.

3.3 Partial purification of Intermediary subunits from glycinin

The intermediary subunits of glycinin from the cultivar Sojo5 was derived from the partially purified glycinin in the preceding section according to the methods described in section 2.5.3. The intermediary subunits of glycinin were fractionated chromatographically on a DEAE-Sephadex A-50 column in the presence of 6 M urea and 0.2 M 2-mercaptoethanol (Figure 10). As shown in figure 10, the proteins were separated into four major peaks, **I, II, III, IV** in order of elution from the column. The unbound proteins (peaks **I** and **II** in Figure 10**)** were eluted from DEAE-Sephadex A-50 column with the equilibrating buffer. The intermediary subunits (peaks **III** and **IV**) bound to DEAE-Sephadex A-50 column were eluted with a linear gradient of 0.09 to 0.52 M sodium chloride. The fractions within each peak were pooled and the protein concentration was determined by the method of Bradford (1976). The intermediary subunits was obtained with 8.42% yield.

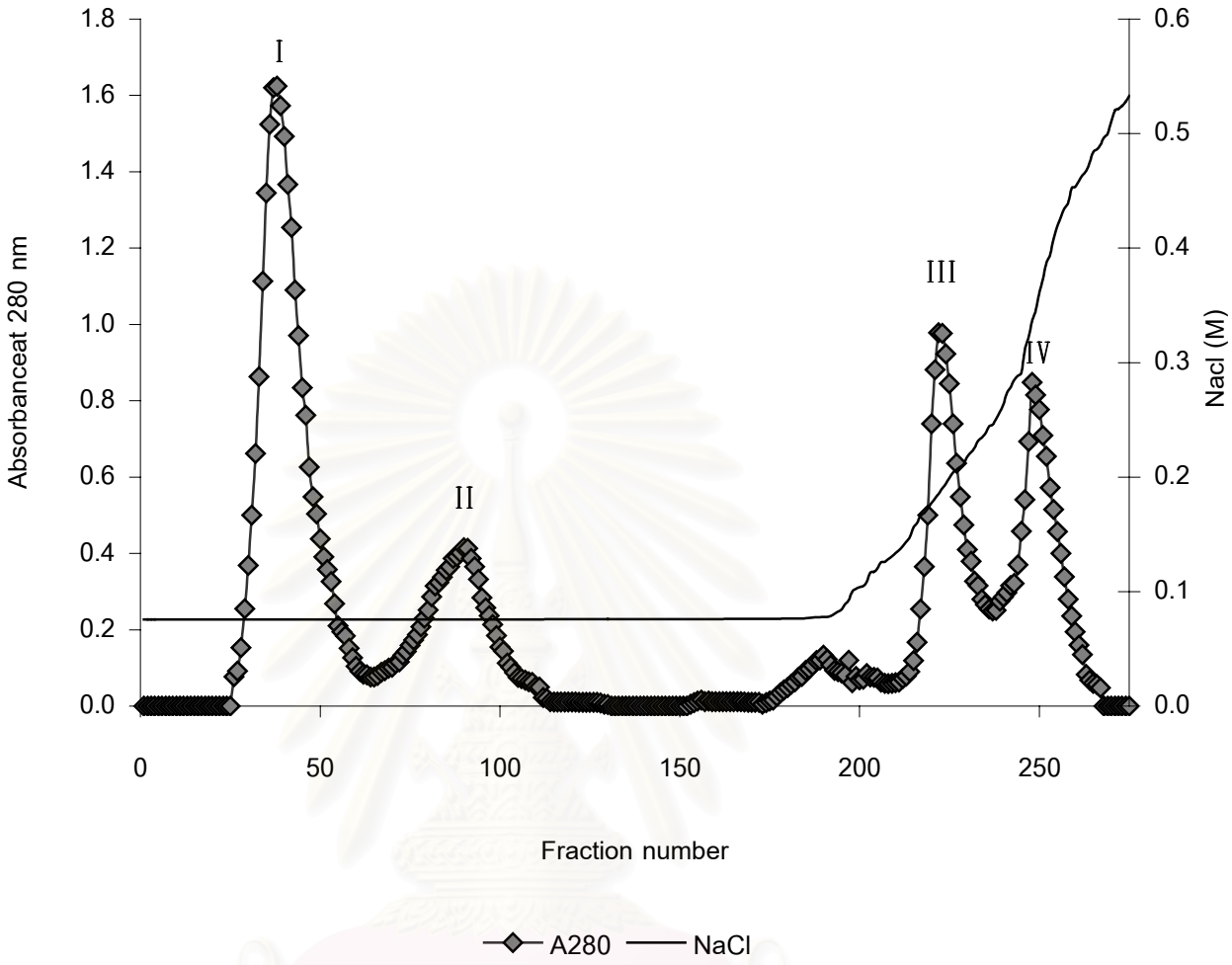


Figure 10 Chromatographic profile on DEAE-Sephadex A-50 column of glycinin subunit dimers from partially purified glycinin SOJO5 cultivar.

Dialyzed partially purified glycinin was resuspended with 0.09 M sodium phosphate buffer (pH 6.6) containing 6 M urea and 0.001 M EDTA and applied on the column (1.5 X 90 cm) which had been equlibrated with the same buffer at 5°C. Elution with a NaCl gradient of 0.09 M to 0.5 M was carried out at 20 ml/hr flow rate and 3.2 ml fraction volume was collected.

3.4 Determination of protein pattern on polyacrylamide gel electrophoresis

3.4.1 Non-denaturing-PAGE

The partially purified glycinin (4 cultivars) from section 3.2 and the partially purified intermediary subunits were analyzed by non-denaturing polyacrylamide gel electrophoresis and stained for protein according to the methods described in section 2.7.1.1. The results are shown in Figure 11. The protein pattern from DEAE-Sephadex A-50 column showed one major protein band (lane 1 and 2)

3.4.2 SDS-PAGE

The partially purified glycinin from section 3.2 were analyzed by denaturing polyacrylamide gel electrophoresis and stained for protein according to the methods in section 2.7.1.2. The results are shown in Figure 13. The samples from DEAE-Sephadex A-50 column showed four major proteins bands (lane 4-7). As judged by molecular weights of 36-40 kDa and 20 kDa, bands 1 and 2 correspond to the acidic and bands 3 and 4 to the basic polypeptides respectively. Electrophoresis of glycinin was performed on SDS-polyacrylamide gels (see figure 14) in the presence (lanes 2 and 4) and absence (lanes 3 and 5) of 2-mercaptoethanol. There are 2 major bands in lanes 2 and 4 (MW 37 kDa and 20 kDa) and 3 major bands in lanes 3 and 5 (MW 61 kDa, 38 kDa and 20 kDa).

The partially purified intermediary subunits were analyzed by denaturing polyacrylamide gel electrophoresis and stained for protein according to the methods in section 2.7.1.2. The results are shown in Figure 15. Crude glycinin which in buffer pH 7.6 in the presence of 0.01M 2-ME shows primarily six protein bands with the molecular weights in denaturing state of 19, 22, 32, 37, 53 and 60 kDa. Crude glycinin which in buffer pH 6.6 in the presence of 6M urea shows primarily twelve protein bands with the molecular weights in denaturing state of 15, 16.5, 19, 21, 25, 31, 32, 33, 37, 53, 75 and 90 kDa. Peak I (as defined in Figure 10) principally contains a basic subunit with a molecular weight about 19 kDa. Peak II principally contains the intermediary subunit IS I (MW 54,000), which consists of the acidic subunit (MW 35 kDa) and the basic subunit (MW 19 kDa). Peak III principally contains the intermediary subunit IS II (MW 58,000), which was composed of acidic subunit (MW 38 kDa) and basic subunit (MW 20 kDa). Peak IV shows primarily four protein bands with the molecular weights in denaturing state of 19, 23, 35 and 57 kDa.

3.4.3 Urea-SDS-PAGE

The partially purified intermediary subunits were subjected to denaturing polyacrylamide gel electrophoresis and stained for protein according to the methods in section 2.7.1.3. The results are shown in Figure 16. Peak I (as defined in Figure 10)contains a basic subunits with molecular weights of about 16 to 19 kDa. Peak II and peak III contained intermediary subunits with molecular weights of about 43 kDa and 62 kDa, respectively. Peak IV shows one protein band with molecular weight in denaturing state of 23 kDa.

Figure 11 Non-denaturing PAGE of partially purified glycinin from different cultivars in 7.5 % polyacrylamide gel with coomassie blue staining (in Section 2.7.1.4)

Lane 1 : Crude glycinin cultivar SoJo5 (20 μg)

Lane 2 : Purified glycinin cultivar SoJo 5 (20 μg)

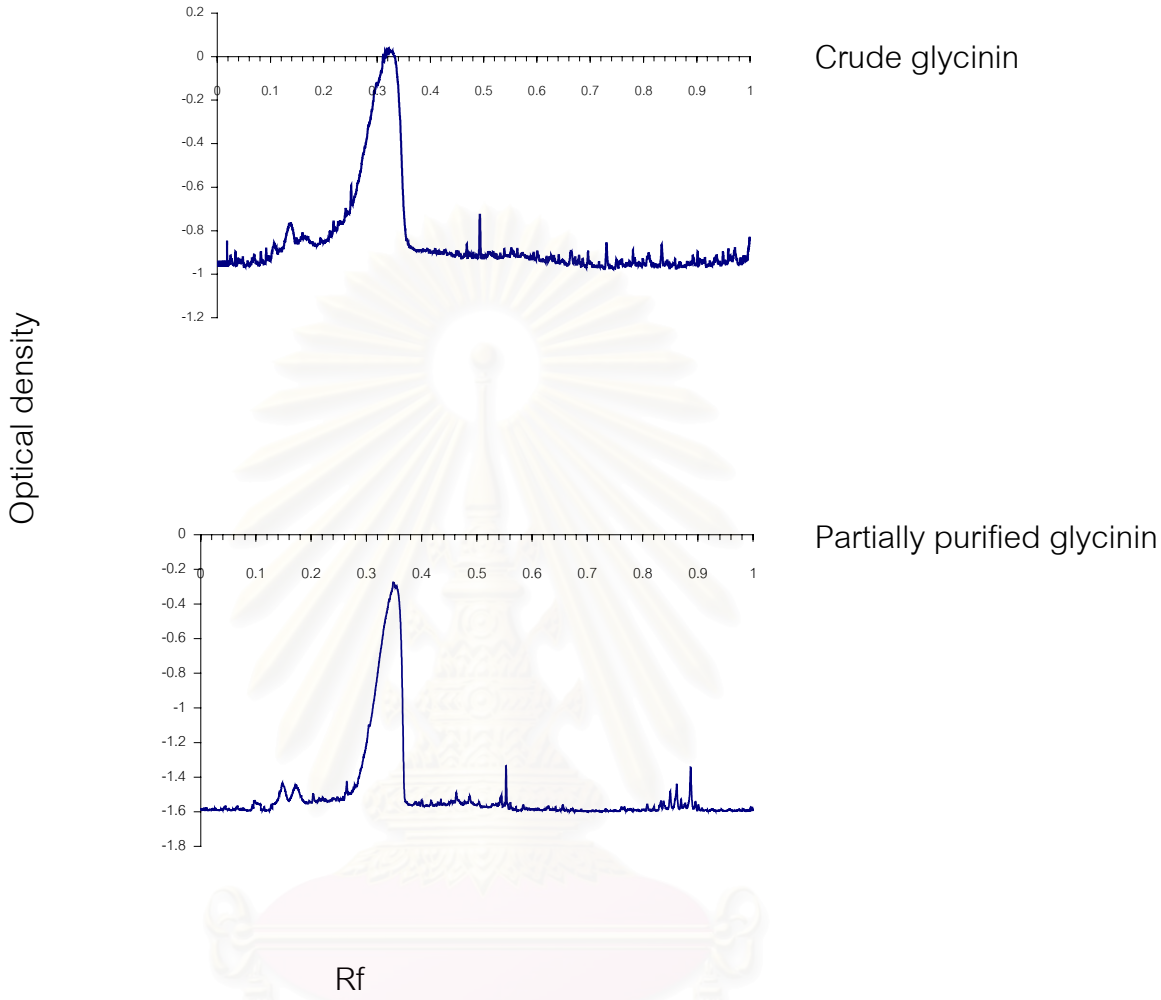


Figure 12 comparison of migration pattern of crude glycinin and partially purified glycinin as scanned by microdensitometer.

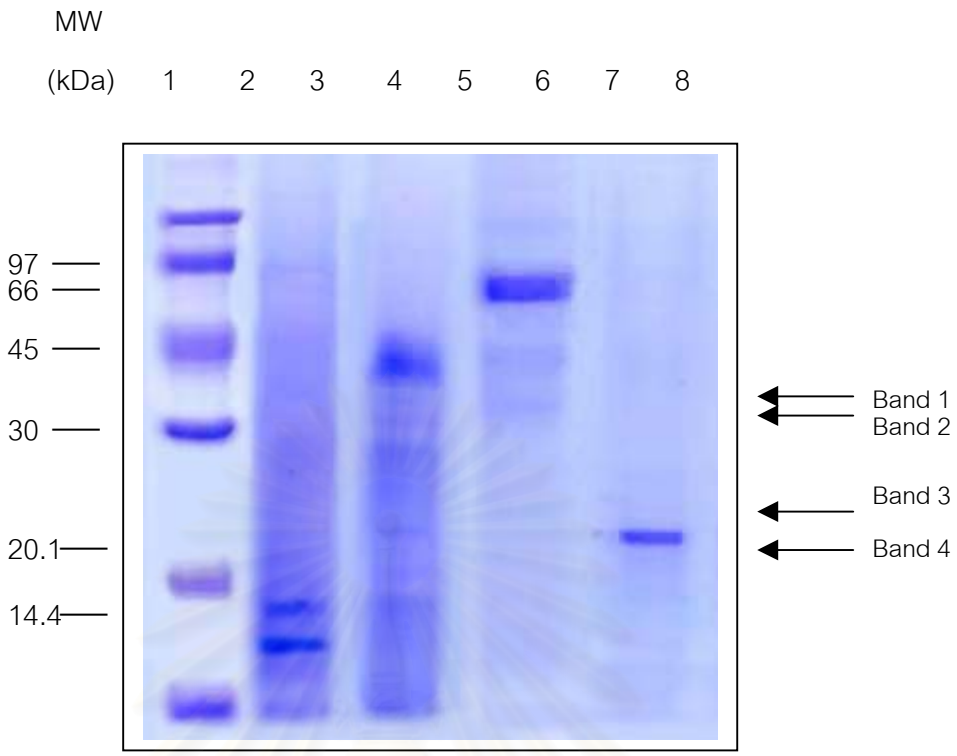


Figure 13 SDS-PAGE of partially purified glycinin from different soybean cultivars

Lane 1, 8 : Molecular weight marker proteins. Phosphorylase b (94 kDa), Bovine serum albumin (67 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Lane 2 : Crude con-glycinin (30 μg)

Lane 3 : Crude glycinin (20 μg)

Lane 4 : Partially purified glycinin cultivar SoJo 5 (20 μg)

Lane 5 : Partially purified glycinin cultivar Chiang Mai 60 (20 μg)

Lane 6 : Partially purified glycinin cultivar SoJo 4 (20 μg)

Lane 7 : Partially purified glycinin cultivar Chiang Mai 2 (20μg)

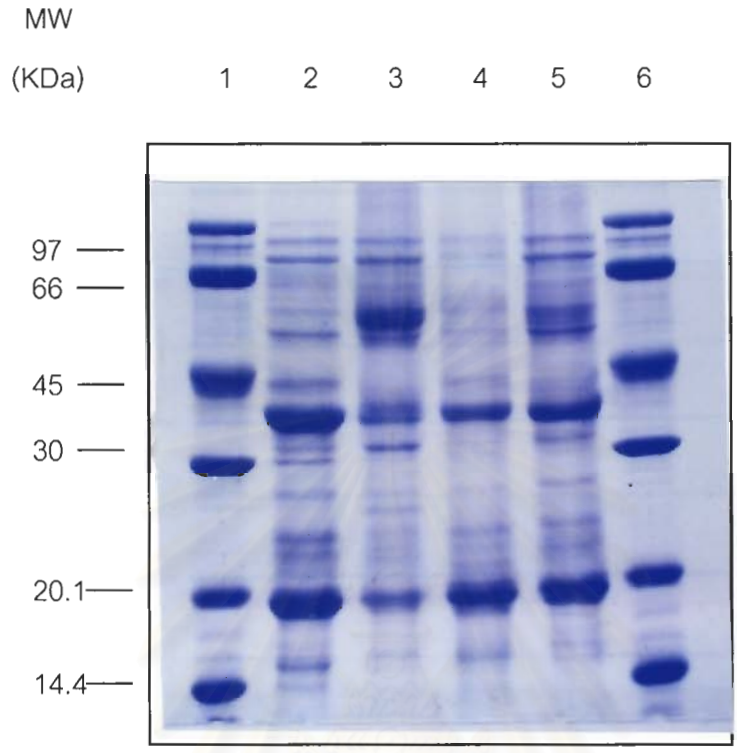


Figure 14 SDS-PAGE of partially purified glycinin cultivars SoJo5 in a 12.5% SDS-PAGE.

Lane 1, 6 : Molecular weight marker proteins.

Lane 2 : Glycinin (20 μg) heated in the presence of 10mM 2-ME.

Lane 3 : Glycinin (20 μg) not heated in the absence of 2-ME.

Lane 4 : Glycinin (20 μg) in the presence of 10mM 2-ME.

Lane 5 : Glycinin (20 μg) heated in the absence of 2-ME.

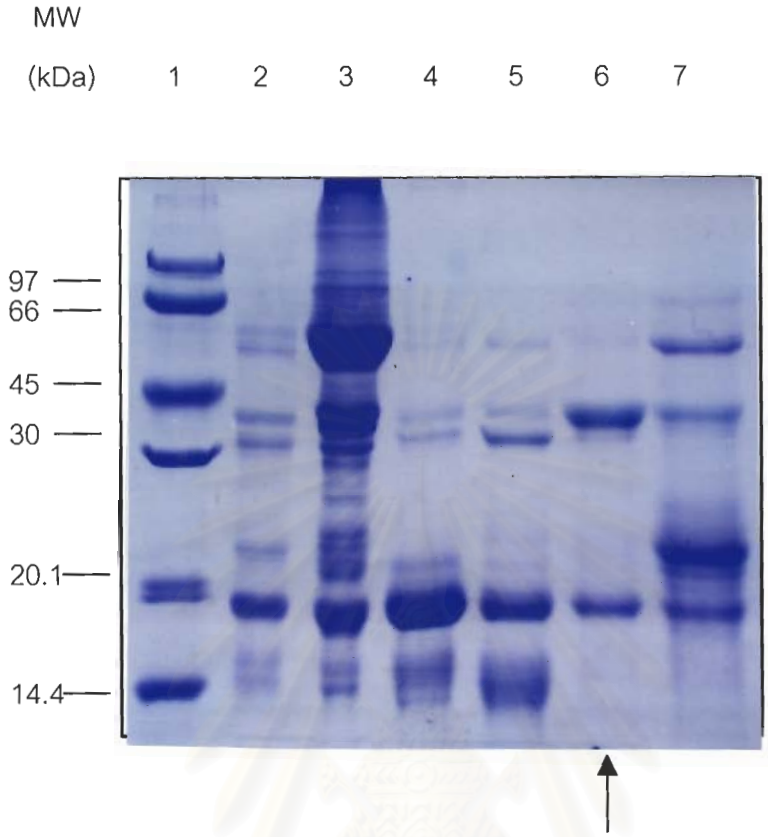


Figure 15 SDS-PAGE (12.5% gel) of each peak fractionated by DEAE-Sephadex column chromatography (as shown in figure 10).

Lane 1 : Molecular weight marker proteins.

Lane 2 : Crude glycinin in Buffer pH 7.6 in the presence of 10mM 2-ME (10 μg)

Lane 3 : Crude glycinin in Buffer pH 6.6 in the presence of 6M Urea (20 μg)

Lane 4 : Peak I (20 μg) in the presence of 10mM 2-ME

Lane 5 : Peak II (20 μg) in the presence of 10mM 2-ME

Lane 6 : Peak III, the intermediary subunits (20 μg) in the presence of 10mM 2- ME

Lane 7 : Peak IV (20μg) in the presence of 10mM 2-ME

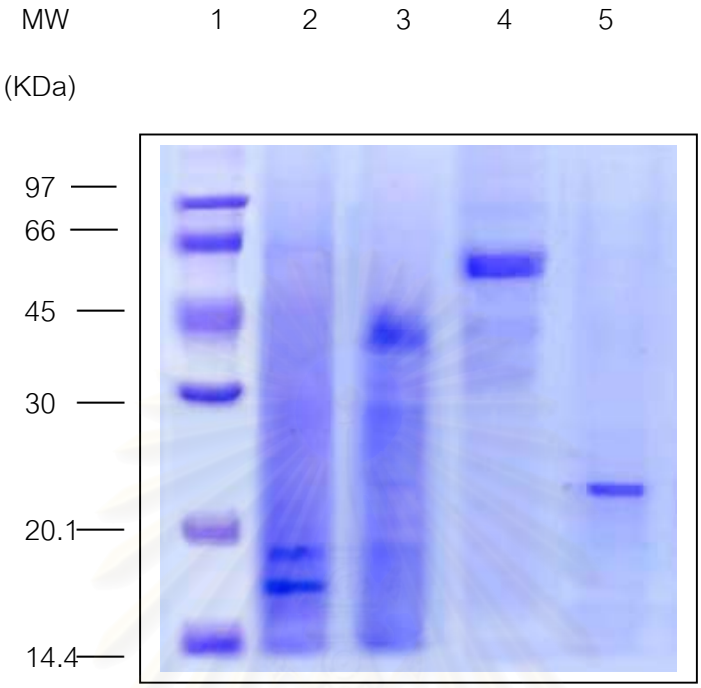


Figure 16 SDS-urea PAGE (12.5% gel) of intermediary subunits.

(Refer to Figure 10 for the definition of peaks I, II, III and IV)

Lane 1 : Molecular weight marker proteins.

Lane 2 : peak I (20 μg) in the absence of 2-ME.

Lane 3 : peak II (20 μg) in the absence of 2-ME.

Lane 4 : peak III (20 μg) in the absence of 2-ME.

Lane 5 : peak IV (20 μg) in the absence of 2-ME.

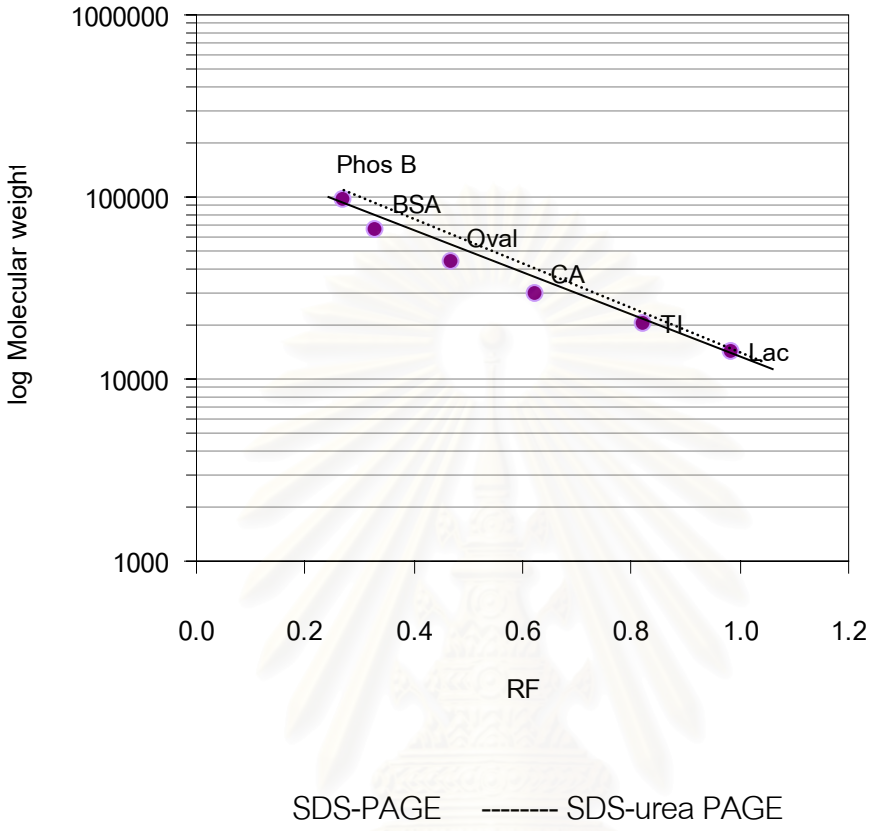


Figure 17 Calibration curve for molecular weight on the 12.5% SDS-PAGE and SDS-urea PAGE .

Phos B : Phosphorylase B MW = 97 kDa

BSA : Bovine serum albumin MW = 66 kDa

Oval : Ovalbumin MW = 45 kDa

CA : Carbonic anhydrase MW = 30 kDa

TI : Trypsin inhibitor MW = 20.1 kDa

Lac : α-Lactabumin MW = 14.4 kDa

3.5 Native IEF polyacrylamide gel electrophoresis

- **Native IEF polyacrylamide gel electrophoresis**

The isoelectric points of the partially purified glycinin and intermediary subunits were determined by IEF gel electrophoresis (pH 3-10) in comparison with standard p*I* markers (Appendix E). The partially purified glycinin showed one broad band with p*I* between 6.55 and 6.85. The intermediary subunits (IS II) from DEAE-Sephadex A-50 Column showed several bands with p*I* of 4.5, 5.2, 5.9, 6.2, 6.5 and 6.7 superimposing on a diffuse background (Figure 18).

Microdensitometry scan of standard p*I* marker proteins shows eleven major peaks (Figure 19). The partially purified glycinin shows one broad peak and the intermediary subunit shows eight major peaks.

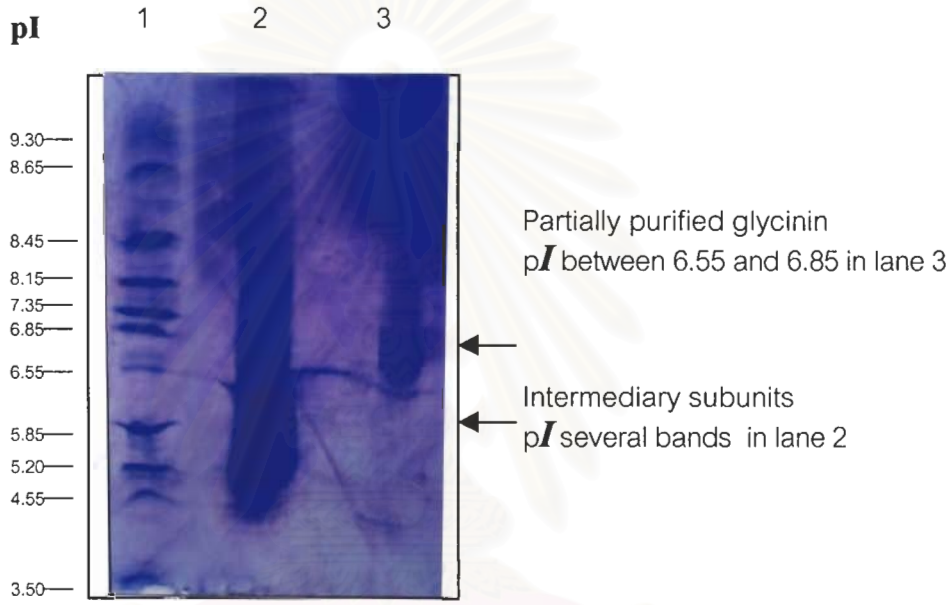


Figure 18 Isoelectrofocusing gel of partially purified glycinin and subunits using ampholyte pH 3-10.

Lane 1 : Standard p*I* marker proteins.

Lane 2 : Intermediary subunits (IS II) (peak III from figure 14) (7 μg)

Lane 3 : Partially purified glycinin (5 μg)

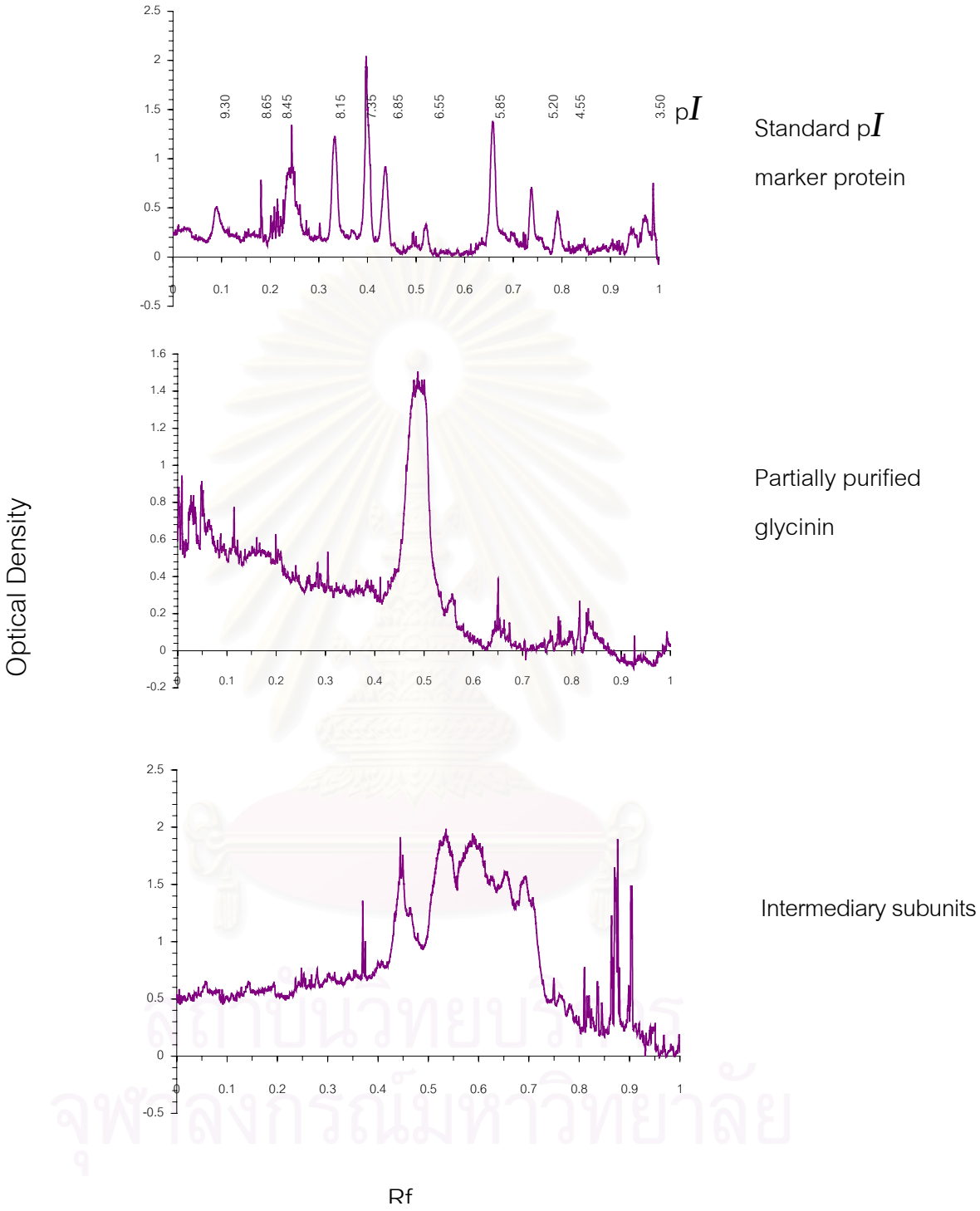


Figure 19 comparison of migration pattern of standard pI marker protein, partially purified glycinin and intermediary subunits as scanned by microdensitometer.

- **SDS-PAGE of partially purified glycinin and intermediary subunits**

Electrophoresis of partially purified glycinin and intermediary subunits were performed on SDS-polyacrylamide gels (see figure 20) heated in the presence (lanes 2 and 3) and absence (lanes 4 and 5) of 2-mercaptoethanol. There were six major bands in lane 2 (MW 14.6, 19, 20, 42, 58, and 65 kDa), two major bands in lane 3 (MW 19 and 42 kDa), four major bands in lane 4 (MW 35, 43, 57 and 63 kDa) and two major bands 5 (MW 42 and 63 kDa)

As shown in figure 21, microdensitometic scan of the molecular weight marker proteins showed six major peaks. The partially purified glycinin heated in the presence of 10mM 2-ME showed six major peaks. The partially purified glycinin showed four major peaks and the intermediary subunits heated in the presence and absence of 10mM 2-ME showed two major peaks.

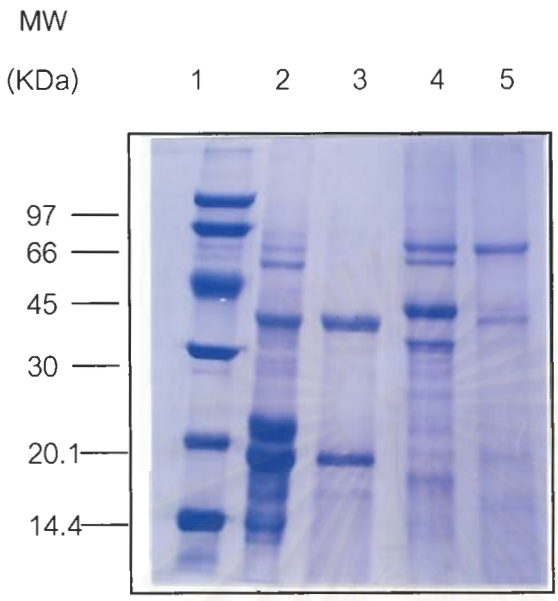


Figure 20 SDS-PAGE of partially purified glycinin and intermediary subunits (peak III) in a 12.5% SDS-PAGE.

Lane 1 : Molecular weight marker proteins.

Lane 2 : Purified glycinin (20 μg) heated in the presence of 10mM 2-ME.

Lane 3 : Intermediary subunits (20 μg) heated in the presence of 10mM 2-ME.

Lane 4 : Purified glycinin (20 μg) in the absence of 10mM 2-ME.

Lane 5 : Intermediary subunits (20 μg) in the absence of 10mM 2-ME.

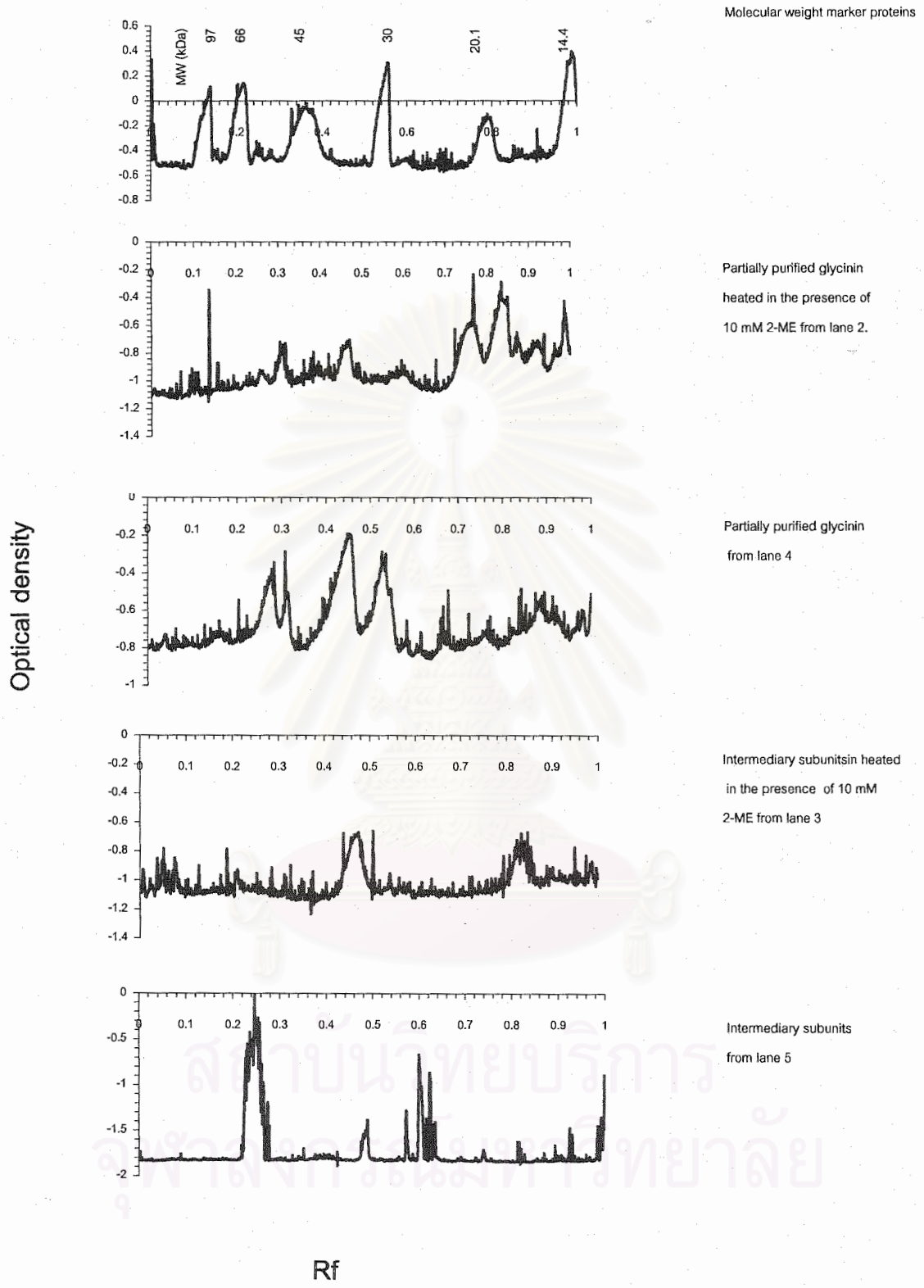


Figure 21 Comparison of purified glycinin and intermediary subunits at different conditions.

3.6 Characterization of intermediary subunits

3.6.1 Determination of solubility

These investigations were aimed at measuring changes in the solubility of glycinin and intermediary subunits at different ionic strengths and pH's. Figure 22 shows that the solubility of glycinin at 20°C depends strongly on ionic strength and pH. At I = 0.5 and pH 6.6 all glycinin was soluble, but the solubility gradually decreased when the pH was lowered from 5.6 to 2.6. The precipitation of glycinin below pH 3.6 was not instantaneous but requires several hours of incubation. For I = 0.2 and 0.03 the solubility profiles showed one minimum; at I = 0.2, the protein precipitated in the pH range from 4.6 to 5.6 at the minimum value around 0.10 A_{280} unit, whereas at I = 0.03 complete precipitation occurred in a range between pH 4.0 and 6.0 at the minimum value around 0 A_{280} unit. In the latter case the precipitation occurred almost immediately after adjustment of the pH. Glycinin was soluble below pH 3.8 at both I = 0.2 and 0.03.

Figure 23 features similar trends of glycinin solubility as figure 21. At I = 0.5 and pH 6.0 all intermediary subunits were soluble, and the solubility gradually decreased when the pH was lowered from 5.6 to 2.6. At I = 0.2 and 0.03 the solubility profiles showed one minimum; at I = 0.2, the protein precipitates at the minimum value around 0.15 in the pH range from 3.6 to 6.0, whereas at I = 0.03 complete precipitation at the minimum value around 0.10 occurred in range of pH 3.6 and 5.6.

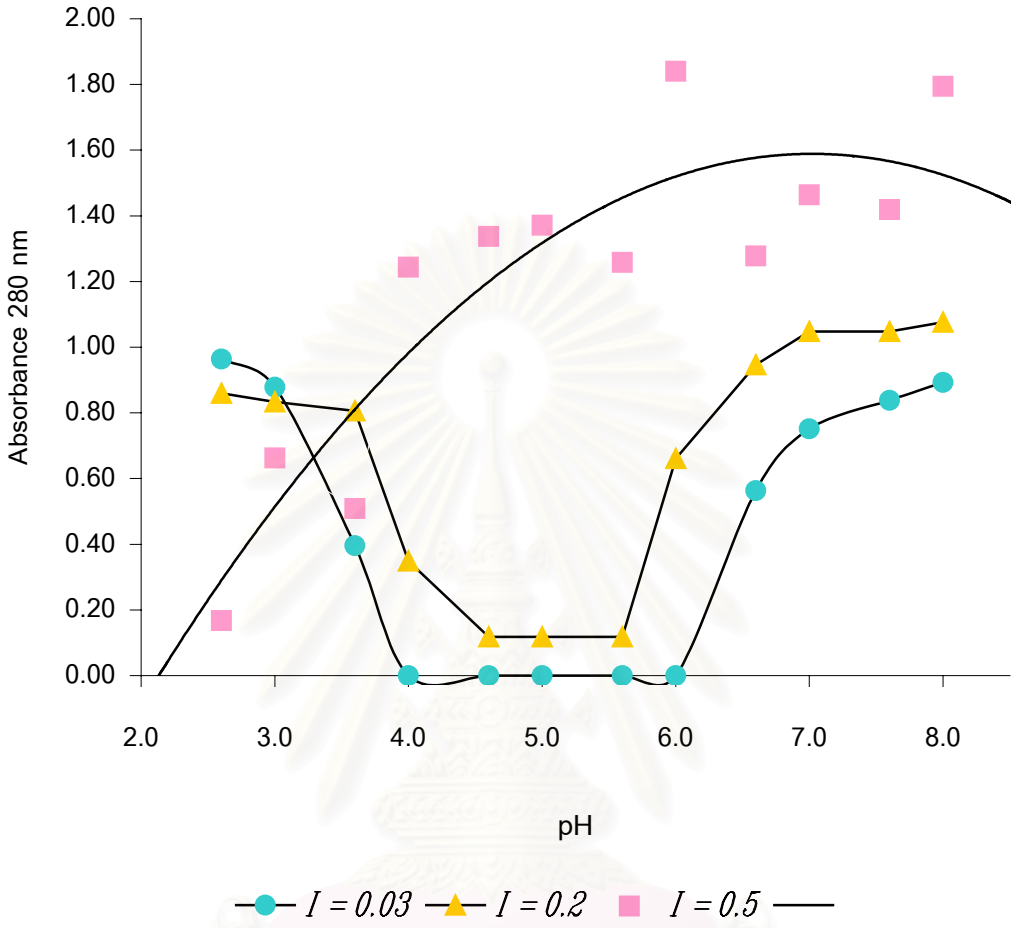


Figure 22 pH-dependent solubility profile of glycinin.

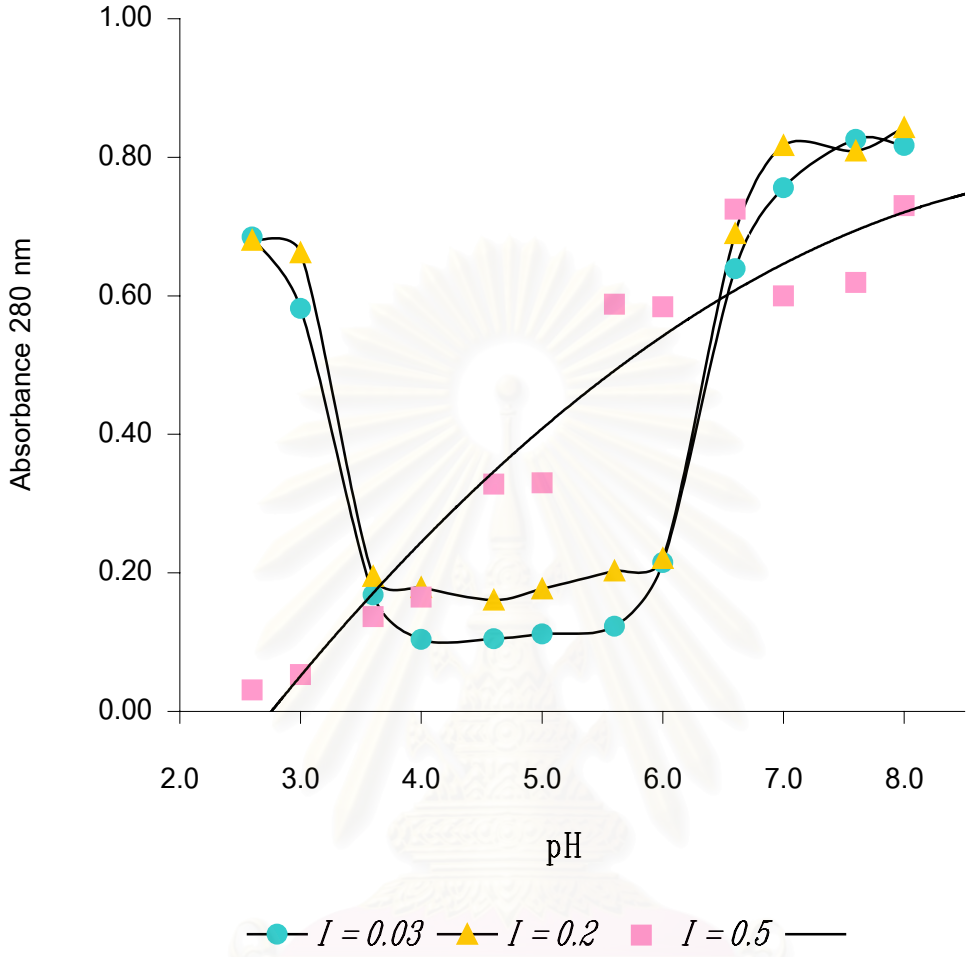


Figure 23 pH-dependent solubility profile of intermediary subunits.

3.6.2 Determination of dissociation

The purified intermediary subunits in the standard buffer was dialyzed against 0.035 M potassium phosphate buffer (pH7.6) for 24 hours at 4°C. The solution became slightly turbid after the dialysis. The dialyzed sample was then fractionated by sucrose density gradient and centrifugation (10 to 30 percent sucrose), as described in section 2.8.2. As shown in figure 24, The gradient profile of intermediary subunits displays four major peaks, the electrophoretic mobility profile of which are shown Figure 25.

When another batch of intermediary subunits were taken from the ion-exchange column without dialysis to get rid of NaCl, the sucrose density gradient profile is show in Figure 26. Here there are to central A_{280} peaks, a tiny rise in A_{280} at the bottom of the tube and a marked increase in absorbance at the top of the centrifuge tube. In the Figure 26, Peak A was pool fractions number 2 to 8 and peak B was pool fractions number 9 to 13. The pooled fractions were then analyzed by SDS-polyacrylamide gel electrophoresis, in order to estimate the molecular weight as shown in lane 2 and 3 in Figure 27. Peak A (lane 2) consists primarily of a higher molecular weight peptide (37 kDa) and peak B (lane 3) consists primarily of a lower molecular weight peptide (19 kDa).

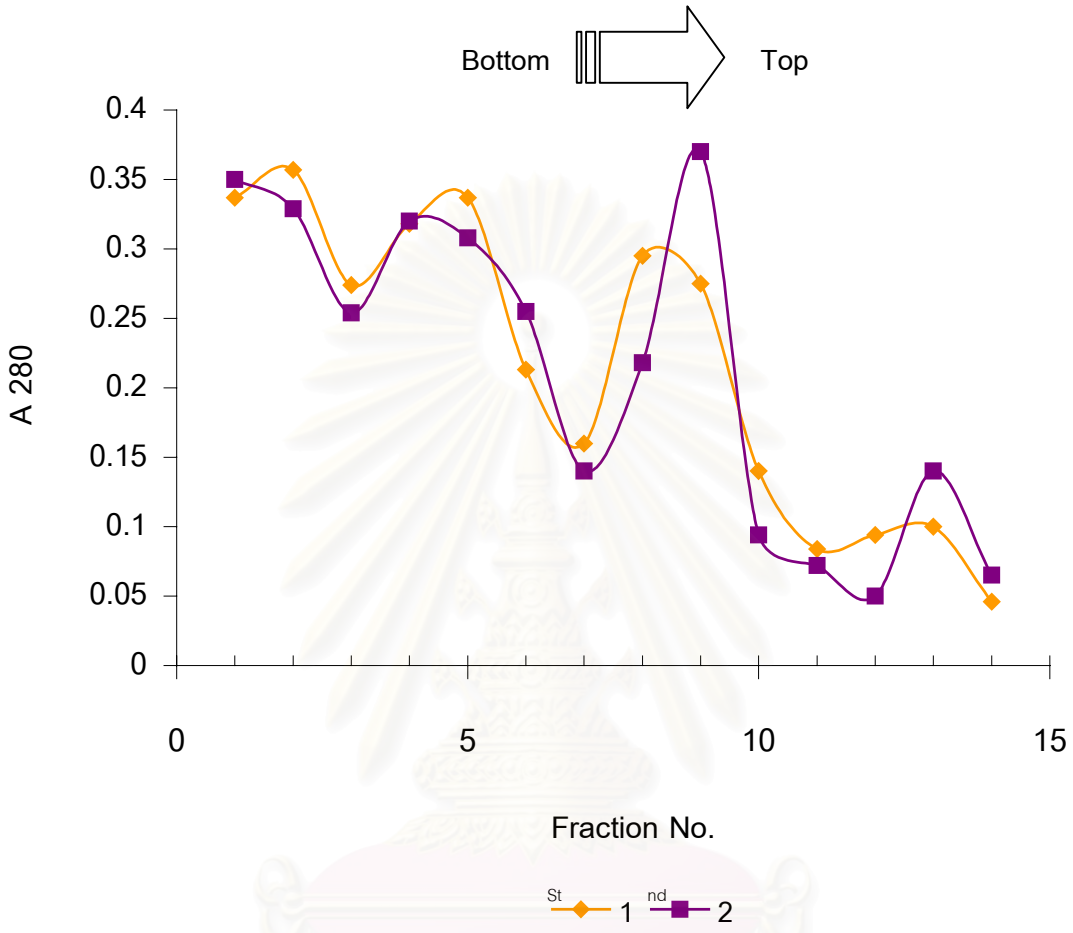


Figure 24 Sucrose density gradient centrifugation of the intermediary subunits.

Before being applied to the gradient, the purified intemediary subuntis eluted from the ion exchange column were dialyzed against 35mM phosphate buffer pH 7.6 for 24 h at 4°C. The SDS-PAGE of the subunits before and after dialysis are shown in lane 4 through 7 of Figure 27.

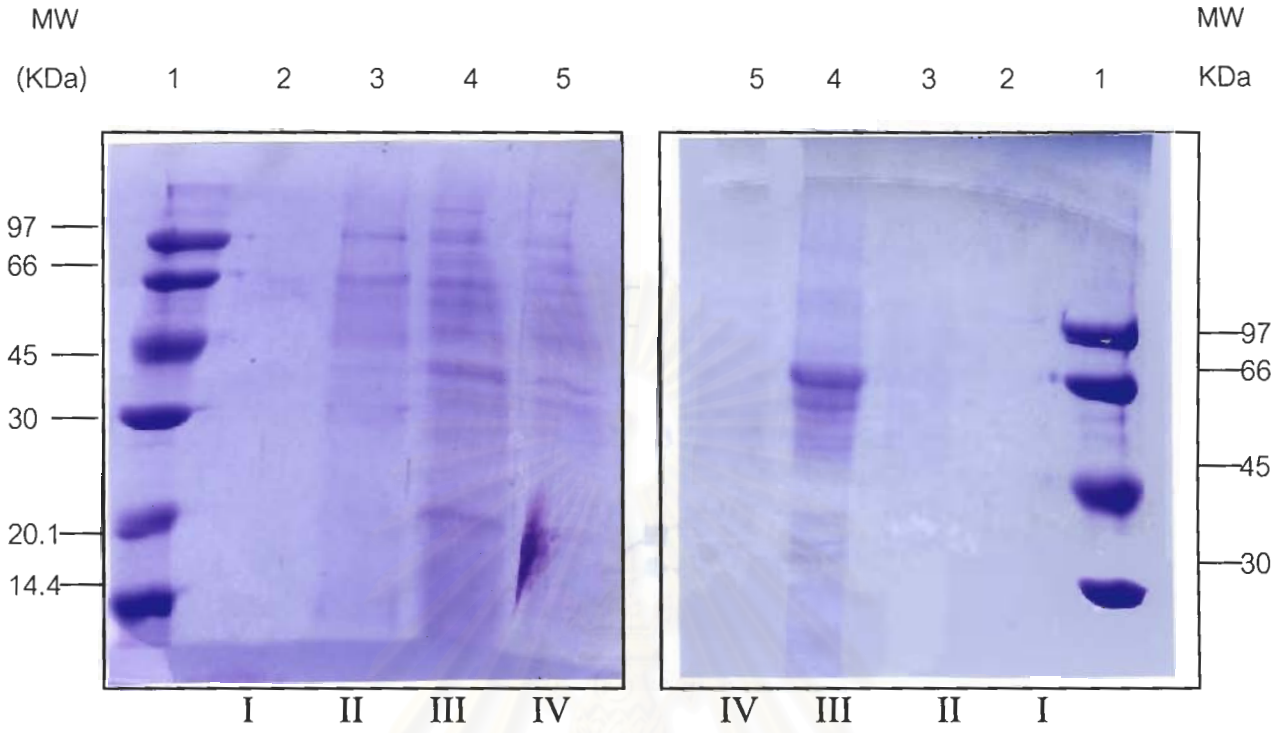


Figure 25 SDS-PAGE 12.5% of intermediary subunits subjected to sucrose density gradient centifugation.

Left gel : with 2-ME Right gel : without 2-ME

Lane 1 : Molecular weight marker proteins.

Lane 2 : Peak I

Lane 3 : Peak II

Lane 4 : Peak III

Lane 5 : Peak IV

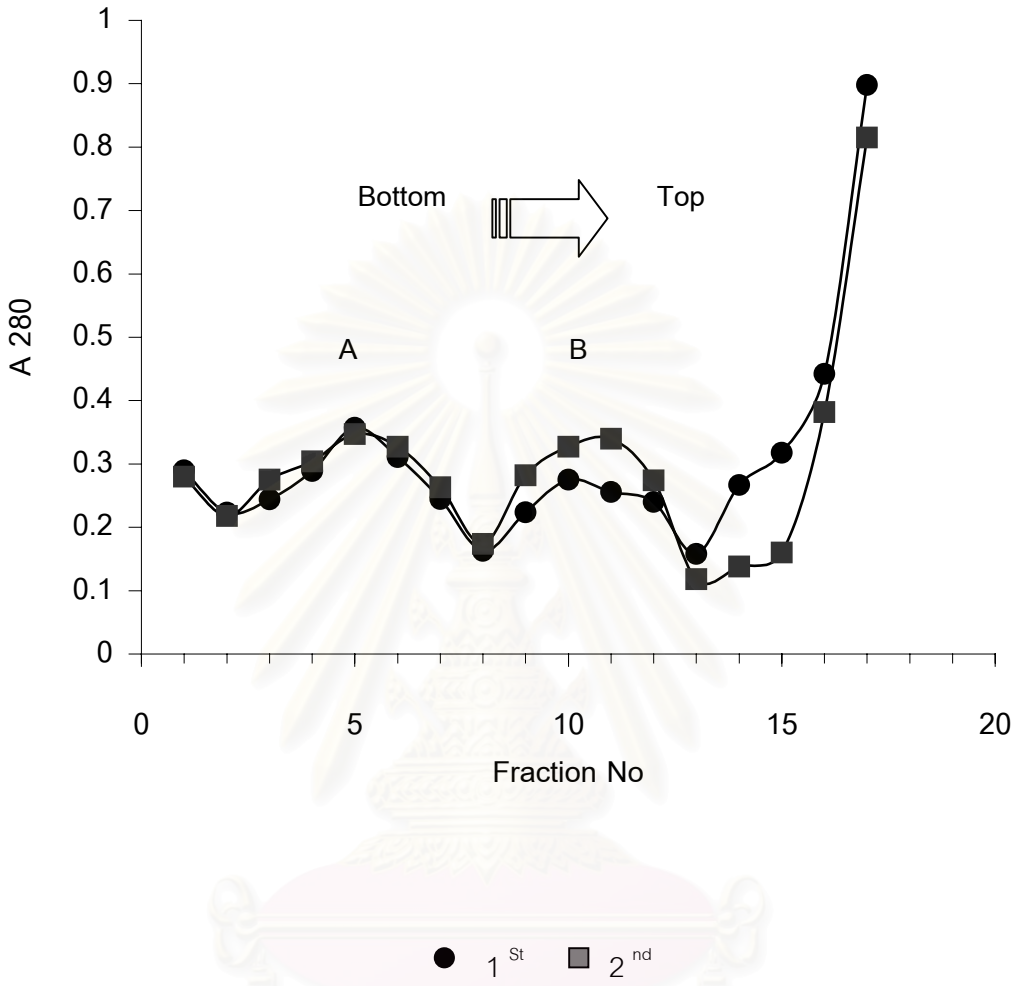


Figure 26 Sucrose density gradient centrifugation of the intermediary subunits.

The intermediary subuntis were not dialyzed prior to the sucrose density gradient fractionation. The subunits were in 0.2M NaCl. Proteins in peaks A and B wee identified by SDS-PAGE, as shown in Figure 27.

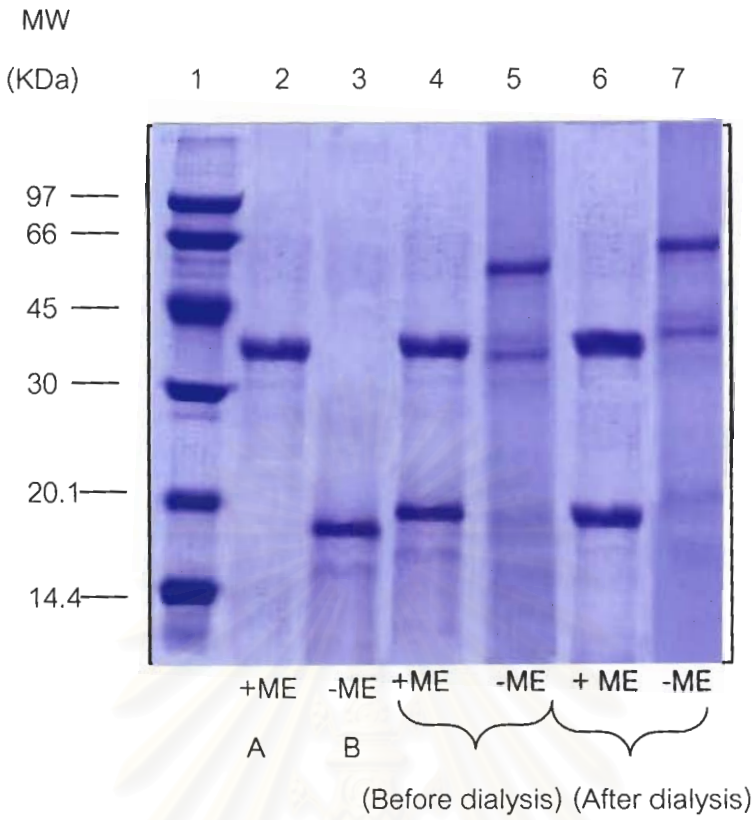


Figure 27 SDS-PAGE (12.5% gel) of intermediary subunits [dimer glycinin].

Lane 1 : Molecular weight marker proteins.

Lane 2 : Peak A (10 μg)[1] after sucrose density gradient

Lane 3 : Peak B (10 μg)[1] after sucrose density gradient

Lane 4 : Intermediary subunits (15 μg) heated in the presence of 10mM 2-ME.

Lane 5 : Intermediary subunits (15 μg) in the absence of 10mM 2-ME.

Lane 6 : Intermediary subunits (15 μg) heated in the presence of 10mM 2-ME.

Lane 7 : Intermediary subunits (15 μg) in the absence of 10mM 2-ME.

Note [1] : In lanes 2 and 3, the intermediary subunits were not dialyzed to remove 0.2M NaCl prior to the fractionation step, as shown in lanes 4 and 5. The samples in both lanes 2 and 3 were heated in 2-ME.

Note [2] : Dialysis prior to sucrose density gradient centrifugation was performed in order to remove 0.2M NaCl from the ion exchange chromatography

3.7 Electron microscopy

Figure 28 shows a representative electron micrograph of a negatively stained preparation of glycinin. Many polygonal structures, particularly pentagonal and hexagonal are visible. The protein molecules trapped in the stain layer are present in all possible orientations. When the magnification was raised approximately 10 times from that of figure 28, the electron micrograph in figure 29 was produced. The top most outlined structure shown in figure 29b represents one typical orientation. It is identified as a glycinin molecule resting on its flat face and viewed from the top. The subunits appear to be packed in an approximately hexagonal arrangement with a suggestion of a stain filled hole in the center. The middle-outlined structure in figure 29b represents a slant view of the hexameric structure. The lower outlined structure in the same figure represents a view of the stacked configuration of two hexameric rings.

An electron micrograph of a negatively stained preparation of intermediary subunits taken at 53,333 magnification is shown in figure 30. It shows patches of aggregated intermediary subunits molecules, which probably result from the negative staining procedure. When the magnification was increased to a magnification of 600,858 an electron micrograp in figure 31 was produced. The outlined structures shown in figure 31 represents pairs of intermediary subunits resting on its flat face and viewed from the top. The subunits appear to be two domain dispersed in the negative stain.

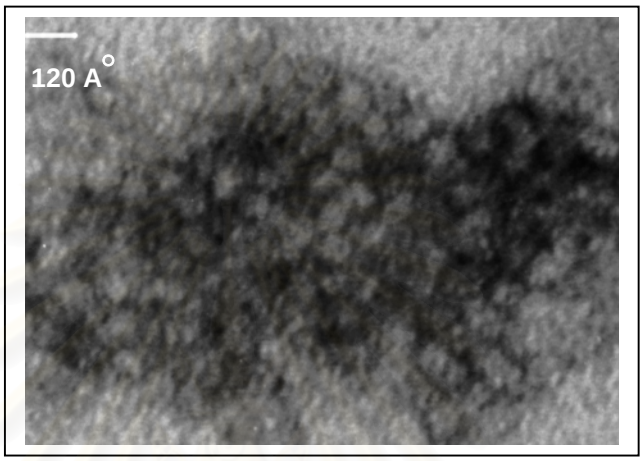


Figure 28 Typical view of negatively stained glycinin.

The sample of partially purified glycinin was stained by 4% uranyl acetate for 5 min

magnification : 58,333 X

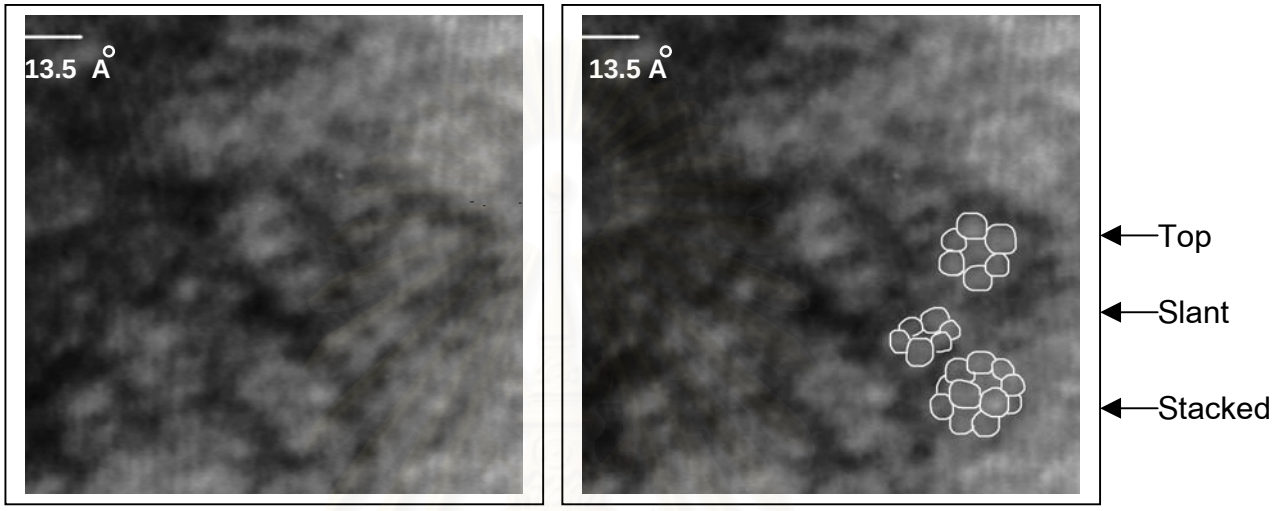


Figure 29 Top view. Image obtained views from figure 28.

The sample partially purified glycinin was stained by 4% uranyl acetate for 5 min

magnification : 592,592 X

a : Original scanned image.

b : The same image as in a superimposed by outlined of representative structure:

Top = Flat face view showing the hexameric structure

Slant = Slant view of the structure

Stacked = Slant view of the stacked hexameric structure

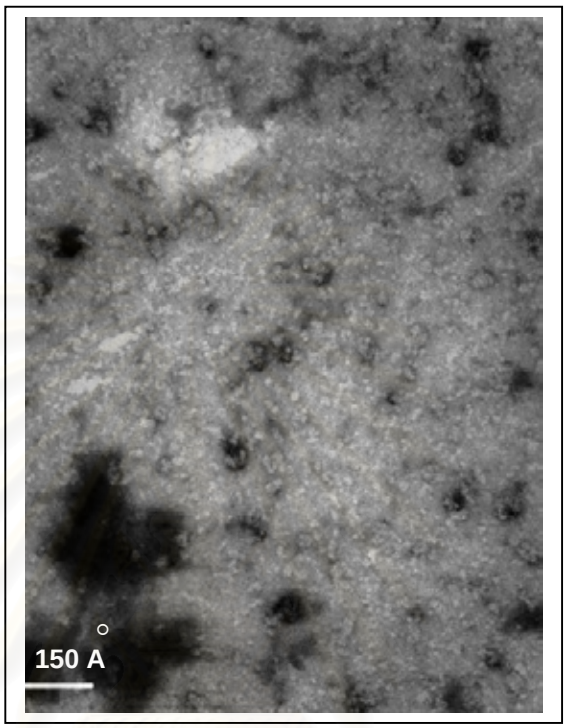
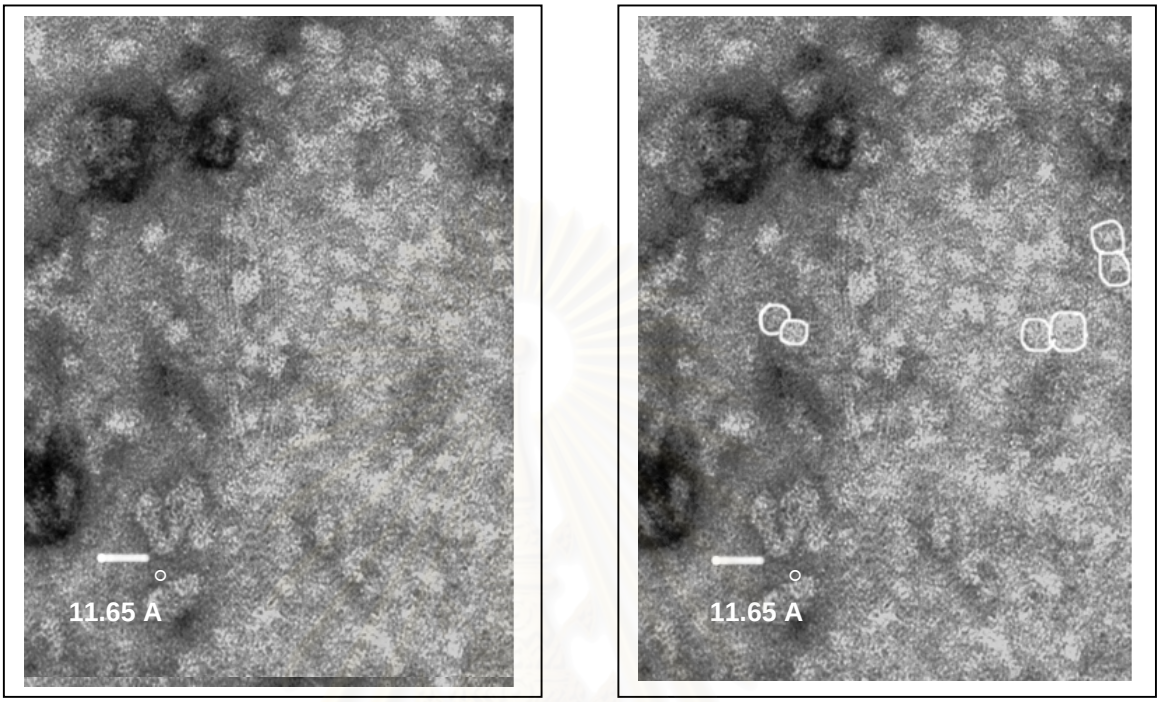


Figure 30 Electron micrograph of intermediary subunits

The sample partially purified glycinin was stained by 4% uranyl acetate for 5 min

magnification : 60,000X



a b

Figure 31 Top view. Image obtained from figure 30.

The sample partially purified glycinin was stained by 4% uranyl acetate for 5 min

magnification : 600,858 X

a : Original scanned image.

b : The same image as in a superimposed by outlined of representative structure:

Top = Flat face view showing the hexameric structure.

Slant = Slant view of the structure.

Stacked = Slant view of the stacked hexameric structure.

CHAPTER IV

Discussion

It is well known that soy proteins tend to behave differently in food systems and variety is an important determinant to be considered in assessing the functional properties of soy proteins, especially the two major constituents, glycinin (11S globulin) and conglycinin (7S globulin) (Badley *et al*., 1975; Kitamura *et al.,* 1976). The aim of this research was Thai soybean that gives the highest glycinin yield. Additionally, glycinin and intermediary subunits from the selected Thai cultivar were to be characterized and compared with published results based on foreign varieties.

4.1 Preparation of glycinin and intermediary subunits

Soybean glycinin and intermediary subunits need to be partially purified before being characterized. The preparation procedure used in our experiments was based on previous purification methods of the glycinin and the intermediary subunits from other sources. (Badley *et al*., 1975; Kitamura *et al.,* 1976; Moreira *et al.,* 1979; Iyengar, 1981; Utsumi *et al*., 1981)

Thanh and Shibasaki's method was the most suitable for preparing glycinin because of its simplicity and rapidity. The procedure for glycinin separation from other proteins was based on differential solubilities of glycinin at pH 6.1-6.6 (Thanh *et al*., 1976). Tris-HCl buffer (0.03M, pH 8.0) containing 10mM 2-mercaptoethanol (ME) was chosen to extract soy proteins. Glycinin was separated by adjusting the pH to 6.4 and

collecting the precipitate after centrifugation at 4°C. One advantage of Thanh and Shibasaki's method is that it can afford large-scale isolation of the major proteins.

The above method for glycinin preparation was based on the different solubilities of glycinin and conglycinin in dilute Tris buffers (0.03-0.06M) in the pH region of 6.1-6.6. It is known that conglycinin precipitates at pH values between 4.0 to 5.6, whereas glycinin precipitates between 4.4 to 6.8, that is, in the pH 6.1-6.6 region, conglycinin dissolves while most of the glycinin precipitates (Thanh and Shibasaki, 1976). The resulting glycinin precipitate reported here fits the proteins expected property at pH 6.4, its isoelectric point (Thanh and Shibasaki, 1976). Such pH is also within the optimum pH range for glycinin-conglycinin separation.

The intermediary subunits were prepared according to Utsumi *et al* (1981) owing to the simplicity of the method and the apparatus. The procedure for preparing the intermediary subunits was different from that described by Kitamura *et al.* (1976), because of the probability of disulfide bonds formation in our preparation of glycinin in the absence of 2-ME. In the presence of 0.01M 2-ME at neutral pH and an ionic strength of over 0.38, the glycinin monomer has been shown to be stable (Wolf and Briggs, 1958).

4.2 Purity of the partially purified glycinin and intermediary subunits

After, the glycinin and intermediary subunits were prepared to partially purified procedure. Nevertheless, cross contamination of proteins in fraction was a continuing problem. To remove cross contamination, DEAE Sephadex A-50 ion exchange

chromatography was used in the next step. From Figure 13 lanes 2 and 3 the use of ion exchange chromatography separate glycinin from conglycinin, as Utsumi *et al.* (1981) rationalized the effectiveness of their ion exchange purification of glycinin by stating that contaminating conglycinin was not bound to the column at 0.25M NaCl and above.

In this study, DEAE Sephadex A-50 chromatography was chosen to purify the crude glycinin fraction (Figure 8). Slow flow rates were essential, and we generally used 20 ml/hr for the entire analysis. The unbound proteins eluted with same buffer could be derived from the 7S component, which contaminated the glycinin fraction. Since the pH 6.4, 0.32 ionic strength, precipitate contained little conglycinin, the chromatography step could have been repeated in order to obtain better purification.

From the result of glycinin purification, different yields were found among the cultivars (Figure 9). For instance, the concentrations of glycinin in Sojo4 and Chiang Mai2 were lower than those of the other two cultivars. Furthermore, Chiang Mai2 contained the lowest concentration of glycinin compared with other cultivars (Table 4). In this study, it was found that Sojo5 cultivar contained the highest partially purified glycinin contents, which agrees with information obtained from Field Crops Research Institute, Department of Agricultural Extension, Ministry of Agriculture and Cooperatives of Thailand (Chainuvati *et al*., 1997). This means that there may be a mechanism in Sojo5 seeds that regulates the amount of storage protein that are synthesized and stored in protein bodies. The Sojo5 cultivar, which gave highest yield of glycinin, was subsequently used is all other experiments reported here.

The isolated glycinins were tested for purity using SDS-polyacrylamide gel electrophoresis. Figure 13 shows that the two most prominent glycinin bands are the acidic and basic subunits. The acidic and basic subunits have molecular weight ranges of 36–40 kDa and 18 to 20 kDa, respectively, which are in agreement with results reported by Kella *et al.* (1986) and others.

The intermediary subunits of glycinin, the acidic and basic subunits were partially purified by DEAE-Sephadex column chromatography in the presence of 6M urea. Trace concentrations (approximately 8μM) of 2-ME may be present after a dialysis in the presence of oxygen (no attempt was made to exclude oxygen during the normal preparation of glycinin) which may serve as a catalyst to promote reformation of SS bonds as noted in regeneration of reduced lysozyme (Saxena and Wetlaufe, 1970). As shown in Figure 10, glycinin was separated into four major peaks. The basic subunits were not absorbed by the column and eluted as one peak, while the intermediary subunits were fractionated into two peaks.

Each peak was then analyzed by electrophoresis on SDS-polyacrylamide gel (Figure 15). The basic subunit group consisted of two components with molecular weights of 19 kDa and 14 kDa. Peak II showed several components with major bands at 14, 19 and 35 kDa, peak III showed several components with major bands at 20 and 38 kDa. In peak IV shows primarily four protein bands with the molecular weights of 19, 23, 35 and 57 kDa. It is interesting to note that, on SDS-PAGE, peaks I and III showed very similar bands to the corresponding peaks obtained from ion exchange chromatography

repeated by Utsumi *et al.* (1981). Peak II and peak IV, however, showed different band pattern from the corresponding peaks reported by Utsumi *et al.* (1981). As shown in figure 15, the 14 kDa band in peak II and the 20-25 kDa bands in peak IV were not present in Utsumi's preparation. The 14 kDa band could represent proteolytic products of other protein bands while the 20-25 kDa bands could be different forms of the basic subunit (Nielsen, 1989).

4.3 Solubility of the glycinin and the intermediary subunits

Solubility is one of the most basic physical properties of proteins and a prime requirement for any functional application. Solubility of a protein under specified conditions is governed by factors that influence the equilibrium between protein-protein and protein-water interactions (Damodaran, 1994). Intrinsic factors including hydrophobic, hydrophilic, size, charge, steric properties, and extrinsic factors such pH, types and ionic strength of various salts, and interaction with other components affect the solubility of proteins.

Many studies on soy protein have been performed at pH 7.6 and I = 0.5. However, this investigation was aimed at measuring changes in the solubility of glycinin when the ionic strength and pH were lowered to conditions more representative for food products so that results may be compared to repeated values (Lakemond *et al*., 2000).

From result of glycinin, at I = 0.2 precipitation occurs between pH 5.6 and 4.6, which was in about the same range of the pI values, of the acidic polypeptides (pH 4.8-

5.4) (Catsimpoolas, 1969). At I = 0.03 this region of precipitation occurs at slightly higher pH (6.0-4.0) (Figure 22), suggesting that it shifts toward the isoelectric points of the basic polypeptides, which vary from pH 8 to 8.5 (Catsimpoolas, 1969). On the other hand, the solubility of intermediary subunits displays similar trends of glycinin. From Figure 23, at I = 0.2 precipitate occurs between pH 6.0 and 3.6. At I = 0.03 complete precipitate at the minimum value around 0.15 occurs in rang of pH 5.6 and 3.6. The decreased solubility of glycinin below pH 4.0 (Figure 22) can be attributed to pH denaturation of the protein caused by protonation of the carboxyl groups. It is only observed at I = 0.5, where apparently the screening of positive charges of the salt is as efficient to overcome electrostatic repulsions. No minimum in solubility could be observed between pH 7.6 and 3.8 (Figure 22) at 20°C, probably due to the *salting in* effect. At 20°C at I = 0.5 and pH 7.6 the solvent exposure of the acidic polypeptides is maximal. The solubility behavior of glycinin at low pH is related to the relative arrangement of acidic and basic polypeptides at pH 7.6. The solubility profile of glycinin and intermediary subunits were in this results agree with the results of Thanh and Shibasaki (1976). But the solubility base line of the intermediary subunits was slightly difference might be cause the intermediary subunits was incomplete precipitation in range 3.6-6.0 and effect of the average hydrophobicity of amino acid residues and charge frequency.

Because the aggregation state of soybean proteins depends on the environmental conditions, the pH was varied from 2.6 to 8.0, and the influence of such

changes on the apparent solubility was analyzed by spectrophotometry. Results so obtained are shown in Figure 22 and 23. As the pH approached the isoelectric point, the aggregation of the soybean proteins resulted in the precipitation due to interaction between protein molecules. The electrostatic change can be used to predict soybean protein dispersion and association. The pH is the major parameter that affects the peptide charge. As a consequence, proteins precipitate at their isoelectric point. Far from the isoelecric point, the protein molecules disperse in a solution because of electrostatic repulsion. The results show that the glycinin is precipitated in a much narrower interval of pH values than the intermediary subunits, because of the greater van der waals and hydrophobic forces among glycinin molecules. The greater the attractive interaction forces are, the smaller is the solubility. In addition, the addition of salt to the solutions leads to increased solubility of the proteins as indicted in Figure 22 and 23, unlike the common *salting out* effect generally expected for the proteins in solution. Similar shapes of the solubility profiles of curd and soy protein isolate were seen by Shan (1976), but of different magnitude used for determination of soluble proteins by Biuret analyses. This *salting in* effect is due to thermodynamic linkage between the free energy of salt binding and solubility of the soybean proteins; however, it is usually found at NaCl concentrations that are at least an order of magnitude lower than the ones used in this study.

4.4 Quarternary structure of glycinin and intermediary subunits

So far there has never been a 3-dimensional model of glycinin based on x-ray crystallography due to the size and complexity of the molecule. Nevertheless, a 3-dimension model, based on electron microscopy was proposed for glycinin (Badley *et al*., 1975). Up to now electron microscopy is the most refined technique used to study the structure of glycinin and intermediary subunits. Figure 29 supports the model of glycinin consisting of two annular-hexagonal structures packed one on top of another and each composed of 6 subunits (Badley *et al*., 1975). It would suggest that alternation of acidic and basic subunits within the structure contributes ionic interactions to the stability of the molecule. In Figure 31, the intermediary subunits appear to be pairs of domains dispersed in the negative stain. Figure 31a also shows single domains of such structure, some of which probably represented an end-on view of the dimers (the intermediary subunits). This interpretation is confirmed in Lane 5 of Figure 20, which shows a mixture of the 42 kDa single subunits and 63 kDa dimers consisting of the two bands in Lane 3 that were separated by 2-ME. These results appear to be consistent with those reported by Badley *et al*, (1975).

In the study of the quaternary structure of proteins containing disulfide bonds, isolation and study of constituent intermediary subunits is obviously of importance because it could provide at least a partial answer to the question of the protein structure. According to Kitamura *et al*. (1976), the intermediary subunits of glycinin are formed from one basic and one acid subunit being linked together by disulfide bonds. It has

been reported that 11S component of broad bean also has disulfide bonds between subunits, (Wright and Boulter, 1974) whereas the specificity of the interactions has not been elucidated. As shown by SDS-PAGE in Lane 2 of Figure 14, partially purified glycinin dissociated into the sub-components of molecular weights 20 and 37 kDa at, in the presence of 10mM 2-ME. The bands for the acidic and basic subunits were prominently detected without the ~60 kDa band for the presumed intermediary subunits, as shown in lane 3 of the same Figure. As shown in Figure 15 (lane 6), in the presence of 10mM 2-ME, the bands for the presumed intermediary subunits (61 kDa in lane 3 of Figure 14) disappeared and only the bands for the acidic and basic subunits were observed at 38 and 20 kDa, respectively. Thus, the disappearance of the bands with molecular weights of 61 kDa was correlated with the appearance of acidic and basic subunits. As shown in Figure 20, the 61 kDa band in lane 5 disappeared completely with the emergence of the 42 and 19 kDa, corresponding respectively to the acidic and basic subunits. The results indicate that glycinin of Sojo5 cultivar contains such intermediary subunits that have very similar structure and behavior as those reported for Raiden cultivars (Kitamura *et al*., 1976).

The subunit structure of the intermediary subunit was studied by SDS-PAGE (with or without 6M urea) in the absence and in the presence of 2-ME. Data are shown in Figures 15, 16 and 20. Without 2-ME treatment of the sample, the intermediary subunits showed a major band (approximately 86.5% of area under the microdensitometric scan) at approximately 62 kDa with fainter bands at approximately 40, 42, and 39 kDa in SDS-

PAGE (lane 5 of Figure 20) and two faintly smeared bands around 35 and 40 kDa in SDS-PAGE with 6M urea (lane 4 of Figure 16). Additionally, very faint bands at approximately 20 and 15 kDa are also visible in SDS-PAE (lane 5 of Figure 20) but are not apparent in SDS-Urea PAGE (lane 4 of Figure 16) gaven that 2-ME reductively cleaves disulfide bonds to release the bound subunits from one another, our data confirm published results on the disulfide linkage between the two types of glycinin intermediary subunits. With the assumption that Coomassie brilliant blue binds quantitatively and with the same specificity to all the glycinin subunits, the ratio of one acidic to one basic subunit (Wolf, 1993) is supported.

A photomicrograph of an IEF gel of the partially purified glycinin and intermediary subunits is shown in Figure 18. The partially purified glycinin shows one broad band at pI around 6.5-6.7 and the intermediary subunits shows several bands with pI between 5.2 and 6.7. The IEF results reported here show much broader bands compared with those reported by Nielsen *et al.* (1981), where the intermediate subunits from cultivar CX635-1-1-1 were subject to sucrose density gradient centrifugation before isoelectric focusing.

When the intermediary subunits in the standard buffer were dialyzed against 3.5mM potassium phosphate buffer (pH 7.6) and then fractionated by sucrose density gradient centrifugation, four A_{280} peaks were observed. There was not enough protein to verify the native molecular weights of the proteins in peaks 1, 2 and 4 (lanes 2, 3 and 5 in Figure 25 right and side) but peak III (mobility approximately 0.384) shows a couple of stronger bands around 66 and 60 kDa and a faint band around 38 kDa with no band

visible at 19 kDa (data not shown). In the presence of 2-ME roughly faint bands were visible at approximately 19 kDa, 38 kDa and 60 kDa, as well as at higher molecular weights. Peak III, therefore, appears to be the intermediary subunits. Peak II, however, appears consist of intermediary subunits that did not dissociate into the 19 and 38 kDa bands when heated in 2-ME.

When the intermediary subunits that were eluted from the ion-exchange column were allowed the remain in high salt (0.2M NaCl), sucrose density gradient centrifugation fractionated the intermediary subunits into two central peaks (Figure 26), which were identified as the acidic and the basic subunits by their mobilities on an SDS-PAGE (Figure 27).

It has been shown (Figure 27 lanes 4 and 5 and Figure 20 lanes 3 and 5) that the intermediary subunits in high salt (before dialysis) are mostly approximate 60 kDa structure with a slight mix of 38 kDa fractions. Breaking down the intermediary subunits into the acidic and the basic subunits requires the application of boiling in 2-ME (ie. Lane 4 of Figure 27). Nevertheless, sucrose density gradient centrifugation in the absence of ME yielded one peak consisting of almost electrophoretical pure acidic subunits and another peak consisting of almost clean electrophoretical pure basic subunits. Evidently, the acidic and the basic subunits had to somehow come apart during sucrose density gradient centrifugation, without the help of additional 2-ME.

One explanation of the above finding could be a quarternary structure framework where a disulfide linkage operates in series with ionic interaction. Any breaking of either

one of these would result the separation of the subunits. Superficially, this explanation does not fit the peptide map of glycinin (Staswick *et al.,* 1984) where the two subunits are directly linked to one-another via a disulfide bond. Nevertheless, other types of glycinin has been shown to lack disulfide bonds (Nielsen *et al.,* 1989) and therefore have to rely on other forms of binding. Glycinin in SoJo 5 variety has not been studied in this regard.

Another possible explanation has to do with the exposure of glycinin to low level (10mM) of 2-ME during extraction and before the first ion-exchange chromatography. If this exposure reduced a majority of disulfide bonds and the high ionic strength medium weakened any ionic interaction, it is conceivable that centrifugation could separate the two subunits apart. Ultracentrifugal studies indicate that the intermediary subunits of soybeans is capable of undergoing conformation changes probably involving dissociation into two subunits.

The results reported here are consistent with the model that glycinin (11S component) consists of two annular-hexagonal structures packed one on top of another and each composed of 6 subunits (Badley *et al*, 1975). In this model, the alternation of acidic and basic subunits within the structure contributes by ionic interactions to the stability of the molecule.

Top view Side view

Figure 32 Model for glycinin, the major soybean 11S globulin (Badley *et al*, 1975).

A: acidic subunits ; B : basic subunits

From work done by Wolf and Briggs (1958);

The glycinin of soybean proteins is stable in aqueous solution at neutral pH and at ionic strengths of 0.35 and above. Under a variety of other conditions of its solution (viz., ionic strength of 0.01, High and low pH, 6M urea at neutral pH and 0.5 ionic strength), the 11S molecules undergo stepwise and at least partially reversible conformation changes to forms which exhibit sedimentation constants of the order of 7S and 3 S. These forms of the protein could, conceivably, result from shape or volume (hydration) changes in the 350,000 molecular unit. However, the magnitude of the change in the sedimentation constants from 11S$\rightarrow$ $\overline{7S}\rightarrow$3S, the fact that these changes are stepwise and reasonably reversible, and the evidence that the same stepwise changes occur under the action of a variety of reagents and conditions; lend favor to the idea that conformation changes are in fact the result of dissociation of the 11S molecule to units of half its molecule weight ($\overline{7S}$) and further to units of one-eighth its molecular weight (3S). Wright and Boulter (1974) published a subunit structure for legumin of *Vicia faba*, in which the acidic and basic subunits formed “intermediary” subunits via disulphide bridges. These “intermediary” subunits could correspond to the 3S component depicted in the dissociation. The dissociation reaction may tentatively be written as follows:

$$11S \leftrightarrow \overline{7S} + \overline{7S}$$

$$\overline{7S} \leftrightarrow 3S + 3S + 3S + 3S \text{ (acidic and basic subunits)}$$

The effects of pH, ionic strength, and detergent indicate that such dissociation is caused by electrostatic repulsion between the subunits of the 11S molecule. The 11S molecule is vary stable entity at 0.5 ionic strength at pH 7.6, because at this high ionic strength the effectiveness of intramolecular repulsion forces arising from the electrostatic charges is reduced to the point where they are no longer able to counteract attraction forces acting between subunits. On lowering the ionic strength to 0.01 or less, or by dialysis at pH 7.6, the intramolecularr electrostatic repulsion becomes sufficiently great to

cause partial dissociation to the subunits. Increasing the number of negative charges on the molecule by raising the pH or by adding an anionic detergent, which is bound by the protein, results in greater dissociation. Complete dissociation can be effected at high concentration of detergent. The studies at low pH indicate that dissociation can also occur when, the 11S protein is positively charged (Wolf and Briggs, 1958).

According to the number and the position of the disulfide bond between the acidic and the basic polypeptides were determined by Staswick *et al*. (1984).

Only one disulfide bond was found to be involved in linking the acidic and the basic polypeptides of each subunit, and they were in analogous positions. All of the 11S globulin determined so far has cysteine residues in homologous positions (Utsumi, 1992). These facts strongly suggest that the disulfide bond between the acidic and the basic polypeptide of 11S globulin subunits is located in a homologous position regardless of the origin of the 11S globulins.

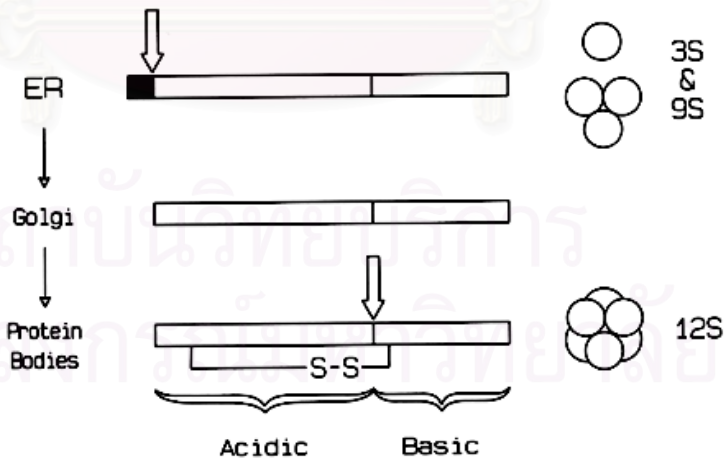


Figure 33 Schematic representation of events in the synthesis and assembly of soybeanglycinin(http://bldg6.arsusda.gov/sarl/byaklich/byaklich.html).

The effect of urea provides a possible clue to the nature of these secondary forces. Urea is generally recognized as an agent capable of breaking hydrogen bonds hence it is probable that hydrogen bonds constitute the forces of attraction responsible for the association of the 3S subunits in the 11S state. Since the dissociation of the 11S molecule proceeds through a well-defined intermediate state, i.e., the $\overline{7S}$ molecule, it appears that two groups of secondary forces are involved.

- One group is responsible for the combination of the 3S subunits to form the $\overline{7S}$ unit.
- The other acts in the association of two $\overline{7S}$ units to form the 11 S molecule.

The results of this study suggest that isolation and partially purification of soybean glycinin by precipitation followed by ion exchange chromatography does not yield electrophoretically pure glycinin. In order to produce pure glycinin, additional steps are needed, such as gel filtration and affinity chromatography. Further characterization of glycinin will be attempted in the future, including protein sequence analysis, x-ray crystallography and the genetic relationships of the subunits from varieties of Thai soybean cultivars. This type of knowledge should help determine the structure-function relationship of glycinin.

CHAPTER V

CONCLUSION

1. Glycinin was partially purified from Thai varieties of soybean (Sojo4, Sojo5, Chiang Mai 60 and Chiang Mai2) based on differential solubilities, at different pH's followed by chromatography on a DEAE-Sephadex A-50 column. It was found that Sojo5 cultivar yielded the highest glycinin contents of 0.80 percent yield.
2. The intermediary subunits from Sojo5 cultivar were fractionated chromatographically on a DEAE-sephadex A-50 column in the presence of 6 M urea and 0.01 M EDTA. The proteins were separated into four major peaks. The intermediary subunits were obtained with 8.42 percent yield.
3. Using SDS polyacrylamide gel electrophoresis, the two most prominent glycinin bands are the acidic and basic subunits, which have molecular weight ranges of 36 to 40 kDa and 18 to 20 kDa, respectively.
4. The partially purified Intermediary subunits were analyzed by electrophoresis on SDS-polyacrylamide gel. It was found that the acidic and basic subunits had molecular weight ranges of 37 to 38 kDa and 20 to 21 kDa, respectively.
5. The role of disulfide linkages, in linking the acidic and the basic polypeptides of each intermediary subunits is confirmed.

6. The isoelectric point of the purified glycinin was between 6.55 and 6.85. Intermediary subunits fraction from ion exchange chromatography shows pIs of 4.5, 5.2, 5.9, 6.2, 6.5 and 6.7, respectively.

7. Electron microscopy confirmed reported findings that glycinin consist of two annular-hexagonal structures packed one on top of another and each composed of 6 subunits. Electron micrography studies supported the model of the intermediary subunit consisting of one acidic subunit and one basic subunit linked by disulfide bonding.

8. The solubility behavior of glycinin and the intermediary subunits at low pH is related to the relative arrangement of acidic and basic polypeptides at pH 7.6. The addition of salt (I = 0.5) to the solutions leads to increased solubility of the proteins. These results agree with previously published findings.

9. Ultracentrifugal studies indicate that the intermediary subunits of soybeans is capable of dissociating into subunits which appear to be roughly one-half. When analyzed by polyacrylamide gel electrophoresis, the two subunits formed bands with molecular weights of around 19 and 38 kDa, respectively. This values correspond well to the molecular weight of the acidic and basic subunits.

10. The results are in agreement with the model that glycinin (11S dodecamer) can dissociate into two half units (7S hexamer) further into intermediary subunits (dimer) and finally into 3S monomer.

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APPENDICES

APPENDIX A

Approximate Composition of Soybeans and Their Structural Parts

	Percentage in Whole Seeds	Chemical Composition (%dry matter)			
		Protein	Lipid	Carbohydrate	Ash
Hull	8	9	1	86	4.3
Hypocotyl axis	2	41	11	43	4.4
Cotyledons	90	43	23	29	5.0
Whole seeds	100	40	20	35	5.0

Source : Data adapted from Wolf and Cowan (1975)

APPENDIX B

Quantity of lipid and protein in soybean culitvars developed by Department of Agricultural Extension, Ministry of Agriculture and Cooperatives of Thailand

Cultivar	Planted area	Yield (Kg/rai)	Quality (% dry weight)	
			%Lipid	%Protein
Sojo 4	Upper Northern, North East	280	19	40
Sojo 5	North, Central	275	19	42
Chiang Mai 60	North, Central	290	21	37
Chiang Mai 2	Lower Northern, North and Central	235	20	36

Source : Department of Agricultural Extension, Ministry of Agriculture and Cooperatives of Thailand (1999)

APPENDIX C

Preparation for buffer solution

- **0.03M Tris-HCl pH 6.4, 8.0**

  Tris (hydroxymethyl)-aminomethane 3.63 g

  Adjust to pH 6.4 or 6.8 by 1 M HCl and adjust volume to 1 litre with distilled water

- **0.035M Potassium phosphate buffer pH 7.6 (for extract glycinin)**

  KH_2PO_4 1.36 g

  K_2HPO_4 4.36 g

  NaCl 14.61 g

  NaN_3 0.2 g

  Adjust to pH 7.6 by 1 M HCl and adjust volume to 1 litre with distilled water

- **0.09M Sodium phosphate buffer pH 6.6 (for extract intermediary subunits)**

  $NaH_2PO_4.H_2O$ 9.94 g

  Na_2HPO_4 2.56 g

  EDTA 0.37 g

  Urea 360 g

  Adjust to pH 6.6 by 1 M HCl and adjust volume to 1 litre with distilled water

- **0.035M Potassium phosphate buffer pH 7.6 (for solubility study)**

I	NaCl (g)	KH_2PO_4 (g)	K_2HPO_4 (g)
0.5	23.37	1.36	4.36
0.2	5.84	1.36	4.36
0.03	-	0.39	1.25

Adjust to pH 7.6 by 1 M HCl and adjust volume to 1 litre with distilled water

- **0.035M Potassium phosphate buffer pH 7.6 (for study dissociation phenomena)**

KH_2PO_4	1.36 g
K_2HPO_4	4.36 g
NaCl	23.37 g
Glycerol	400 ml
NaN_3	0.2 g

Adjust to pH 7.6 by 1 M HCl and adjust volume to 1 litre with distilled water

APPENDIX D

Preparation for polyacrylamide gel electrophoresis

1. **Stock reagents**

30% Acrylamide, 0.8% bis-acrylamide,100 ml

acrylamide	29.2 g
N,N'-methylene-bis-acrylamide	0.8 g

Adjust volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.20 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.10 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

Solution B (SDS PAGE)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (SDS PAGE)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

Solution B (Urea Gel)

1.5 M Tris-HCl pH 8.8	75 ml
0.4% SDS	4 ml
Distilled water	21 ml

Solution C (Urea Gel)

0.5 M Tris-HCl pH 6.8	50 ml
0.4% SDS	4 ml
Distilled water	46 ml

2. **Non-denaturing PAGE**

7.5% separating gel

30% acrylamide solution	2.5 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
Distilled water	5.0 ml

10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

5.0% stacking gel

30% acrylamide solution	1.67 ml
1.5 M Tris-HCl pH 8.8	2.50 ml
Distilled water	5.0 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µl

Sample buffer

For analysis gel

1 M Tris-HCl pH 6.8	3.1 ml
Glycerol	5.0 ml
1% bromophenol blue	0.5 ml
Distilled water	1.4 ml

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g

Dissolved in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3)

3. SDS-PAGE

12.5% separating gel

30% acrylamide solution	4.17 ml
Solution B	2.5 ml
Distilled water	3.33 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

5.0% stacking gel

30% acrylamide solution	0.67 ml
Solution C	1.0 ml
Distilled water	2.3 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µl

Sample buffer

1 M Tris-HCl Ph 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffer was added to four part of sample. The mixture was

heated 5 minutes in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-aminomethane	3.03 g
Glycine	14.40 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water (pH should be approximately 8.3)

4. **Urea Gel**

7.5% separating gel

30% acrylamide solution	2.5 ml
Solution B	2.5 ml
Urea	4.8 g
Distilled water	1.4 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	5 µl

5.0% stacking gel

30% acrylamide solution	0.67 ml
Solution C	1.0 ml
Urea	1.9 g
Distilled water	0.93 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µl

Electrophoresis conditions and buffer solutions as well as staining and destaining solutions are the same as described for SDS-PAGE. 5x sample buffer should be made to contain 8 M Urea.

APPENDIX E

Preparation for isoelectric focusing gel electrophoresis

Monomer-ampholyte solution

30% acrylamide solution	2 ml
Ampholyte Ph 3-10	240 µl
Distilled water	9.7 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	5 µl

Fixative solution, 100 ml

Trichloroacetic acid	10 g

Immerse gels in this solution for 30 minutes.

Staining solution, 100 ml

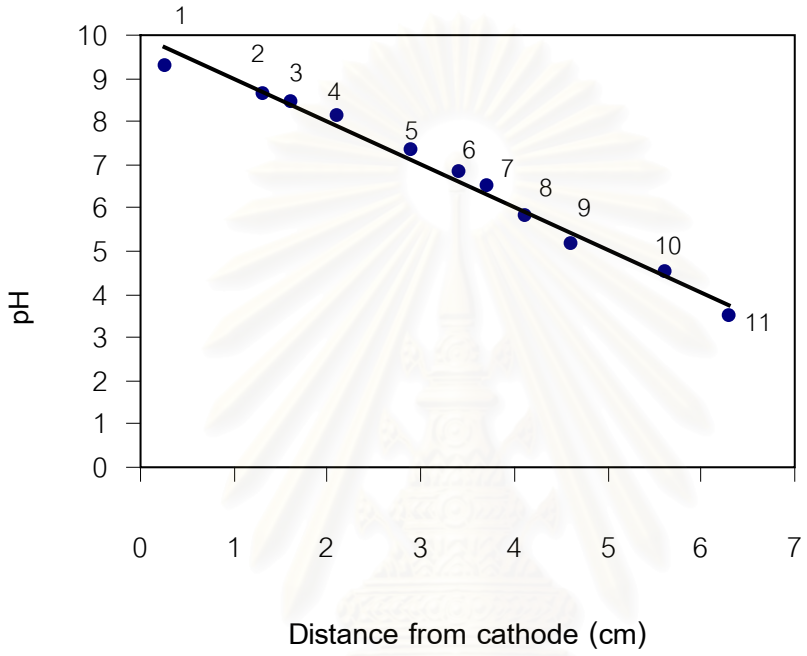
Ethanol	27 ml
Acetic acid	10 ml
Coomassie brilliant blue R-250	0.04 g
$CuSO_4$	0.5 g
Distilled water	63 ml

Dissolve the $CuSO_4$ in water before adding the alcohol. Either dissolve the dye in alcohol or add it to the solution at the end.

Immerse the gel in the stain for approximately 0.5-1 hours. After that Immerse the gel in destain solution (Appendix C) to remove the last traces of stain and $CuSO_4$.

APPENDIX F

Standard pI calibration curve (pI 3-10)



1- trysinogen (9.30)

2- lentil lectin-basic band (8.65)

3- lentil lectin-middle band (8.45)

4- lentil lectin-acidic band (8.15)

5- myoglobin basic band (7.35)

6- myoglobin acidic band (6.85)

7- human carbonic anhydrase B (6.55)

8- bovine carbonic anhydrase B (5.85)

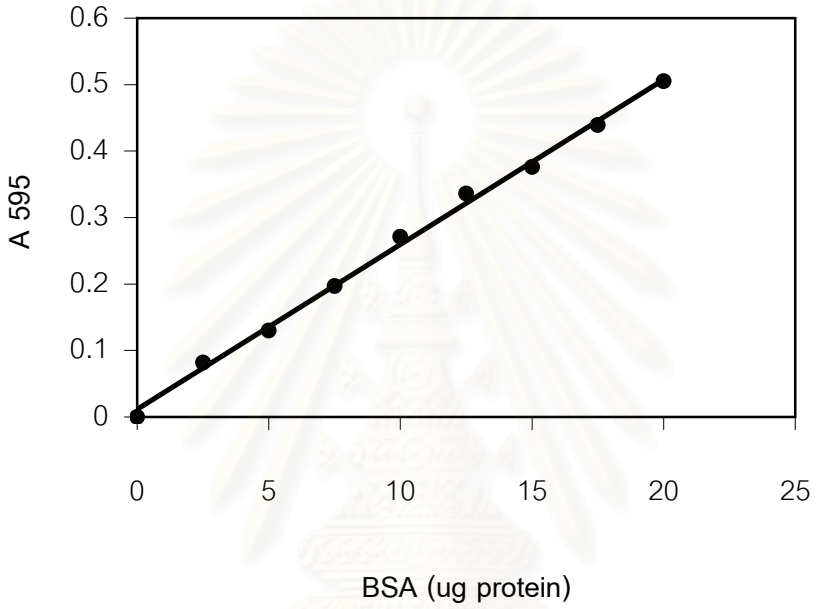
9-β-lactoglobulin A (5.20)

10-soybean trypsin inhibitor (4.55)

11- amyloglycosidase (3.50)

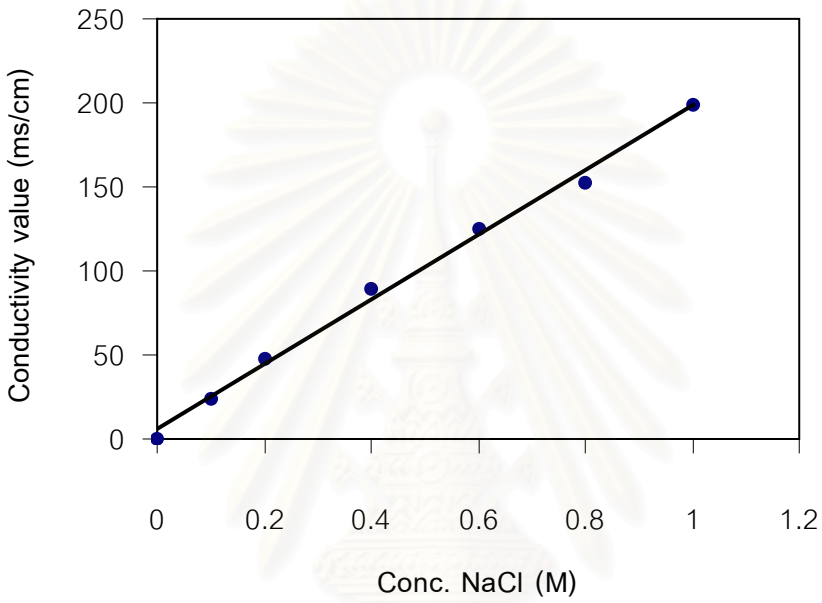
APPENDIX G

Standard curve for protein determination by Bradford's method



APPENDIX H

Standard curve of NaCl



BIOGRAPHY

Miss Chantalak Arjhan was born on November 4, 1977. She finished High school at Prachinrasadornumrung School, Prachinburi and enrolled in the Faculty of Science, Burapha University. She graduated with the B.Sc. in Chemistry in 1999 and continued studying for M.Sc. in Biochemistry Program, Faculty of Science, Chulalongkorn University in that year.