องค์ประกอบโปรตีนและฤทธิ์ทางชีวภาพจากเนื้อผลของมะระขี้นก

Momordica charantia Linn.

นางสาว สุภาพร นามมูลน้อย

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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PROTEIN COMPONENTS AND BIOLOGICAL ACTIVITIES FROM FRIUT PULP OF *Momordica charantia* Linn.

Miss Supaporn Nammoonnoy

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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สุภาพร นามมูลน้อย : องค์ประกอบโปรตีนและฤทธิ์ทางชีวภาพจากเนื้อผลของมะระขี้นก *Momordica charantia* Linn. (PROTEIN COMPONENTS AND BIOLOGICAL ACTIVITIES FROM FRIUT PULP OF *Momordica charantia* Linn.) อ. ที่ปรึกษา :รศ.ดร. พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาร่วม : รศ.สพ.ญ.ดร.ศิรินทร หยิบโชคอนันต์, 84 หน้า.

มะระขี้นก (*Momordica charantia* Linn.) ใช้เป็นอาหารและยา ในตำรายาพื้นบ้านของไทยใช้มะระขี้นกเป็นยาขมเจริญอาหาร แก้ไข้ ขับน้ำดี แก้ปวดข้อ ขับพยาธิ และเป็นยาระบาย วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาองค์ประกอบโปรตีนและหาฤทธิ์ทางชีวภาพจากเนื้อผลของมะระขี้นก โดยทำการสกัดโปรตีนด้วยฟอตเฟตบัฟเฟอร์และตกตะกอนโปรตีนด้วยแอมโมเนียมซัลเฟต แยกโปรตีนด้วยแอนไอออนเอ็กเชนจ์คอลัมน์โครมาโทกราฟี แล้วนำโปรตีนที่ได้ไปทดสอบฤทธิ์ทางชีวภาพคือฤทธิ์ hemagglutination และ ฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดส พบโปรตีนที่มีฤทธิ์ hemagglutination กับเลือดกระต่ายได้แก่โปรตีน 40U, 40B5, 40B10, 40B20, 40B30, 40B50, 40B100, 80U, 100U และ100B100 โปรตีนที่มีฤทธิ์ hemagglutination สูงสุดคือโปรตีน 40B5 มีค่า specific hemagglutination เท่ากับ 400 U/mg protein นำโปรตีน 40B5 หาลำดับกรดอะมิโนโดยเทคนิค peptide mass mapping พบว่ามีความคล้ายคลึงกับโปรตีน OSJNBa0084N21.4 ซึ่งแยกได้จากข้าว *Oryza sativa* ส่วนโปรตีนที่มีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสได้แก่โปรตีน 20U, 40B5, 40B50, 60U, 80U, 80B10, 80B20, 80B50, 100U และ 100B100 โปรตีนที่มีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสสูงสุดคือโปรตีน 100B100 มีค่า IC_{50} เท่ากับ 0.12 mg/ml เมื่อนำโปรตีน 100B100 ไปหาลำดับกรดอะมิโนโดยเทคนิค peptide mass mapping พบว่ามีความคล้ายคลึงกับโปรตีน hypothetical protein ซึ่งแยกได้จากองุ่น *Vitis vinifera*

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สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....สุภาพร นามมูลน้อย.....

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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SUPAPORN NAMMOONNOY : PROTEIN COMPONENTS AND BIOLOGICAL ACTIVITIES FROM FRUIT PULP OF *Momordica charantia* Linn.THESIS ADVISOR : ASSOC. PROF. POLKIT SANGVANICH, Ph.D. , THESIS COADVISOR :ASSOC.PROF. SIRINTORN YIBCHOK-ANUN, D.V.M., Ph.D , 84 pp.

Thai bitter gourd (*Momordica charantia* Linn.) has been used as vegetable as well as medicine. It has been used in traditional Thai medicine as bitter tonic, antipyretic, cholagogue, antirheumatic, antheminthic and laxative. The present study aimed to characterize components and bioactivities (hemagglutination and α-glucosidase inhibition) of proteins from Thai bitter gourd fruit pulp. Crude protein was extracted by phosphate buffer and fractional precipitation with ammonium sulfate in early step in purification step and separated by anion-exchange column chromatography. Afterwards, the protein fractions were tested for bioactivity, hemagglutination and α-glucosidase inhibition. Crude protein (40U, 40B5, 40B10, 40B20, 40B30, 40B50, 40B100, 80U, 100U and 100B100) showed hemagglutinating activity with rabbit red blood cell. Highest specific hemagglutinating activity found at 40B5 (400 U/mg protein). From peptides mass mapping by MALDI-TOF MS and amino acid sequence database searching, 40B5 was similar to partial amino acid sequence of OSJNBa0084N21.4 protein from *Oryza sativa* Linn. α-Glucosidase inhibitions were found in 20U, 40B5, 40B50, 60U, 80U, 80B10, 80B20, 80B50, 100U and 100B100. 100B100 showed highest α-glucosidase inhibition with IC_{50} of 0.12 mg/ml. The amino acid sequence from peptide mass mapping database searching from 100B100 were similar to partial amino acid sequence of hypothetical protein from *Vitis vinifera* Linn.

Field of study…Biotechnology…Student's signature…S. Nammoonnoy…
Academic year …..2007……..Advisor's signature…Polkit Sangvanich……
Co-advisor's signature…S. Yibchokanun…

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BSA	Bovine serum albumin
CCA	α-Cyano-4-hydroxycinnamic acid
°C	degree Celsius
cm	centimeters
CM	Carboxymethyl
CID	collision-induced dissociation
DTT	Dithiothreitol
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DC	Direct current
DEAE	Diethylaminoethyl
ESI	Electrospray ionization
EtOH	Etanol
EDTA	Ethylenediaminetetraacetic acid
g	gram
h	hour
HIV	human immunodeficiency virus
HPLC	High performance liquid chromatography
HU	Hemagglutinating unit
IAA	iodoacetamide
kDa	kilo Dalton
kg	kilogram
µl	microliter
MW	Molecular weight
MOWSE	molecular weight search
MS	Mass spectrometry
MS-MS	Tandem Mass spectrometry
MALDI	Matrix Assisted Laser Desorption Ionization
mM	millimolar
mA	milliampere
ml	milliliter

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mm	millimeter
mg	milligram
min	minute
m/z	mass per charge ratio
nm	nanometer
NCBI	National Center of Biotechnology Information
Native PAGE	Non-denaturing polyacrylamide gel electrophoresis
OD	Optical density
pI	Isoelectric point
ppm	part per million
PNPG	*p*-nitrophenyl -α-D-glucopyranoside
PMF	Peptide mass fingerprint
RF	Radio frequency
RPC	Reverse phase chromatography
RP-HPLC	Reverse phase High performance liquid chromatography
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethylenediamine
TOF	Time of flight
TCA	Trichloro acetic acid
Tris	Tris(hydroxymethy)-aminoethane
TFA	Trifluoro acetic acid
U	Unit

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CHAPTER I

INTRODUCTION

Momordica charantia Linn. Commonly known as bitter gourd, Kerela, Balsam pear, bitter cucumber and bitter melon, is cultivated in tropical areas, including part of Asia, Amazon, east Africa, and the Caribbean. It is used as vegetable as well as medicine [1]. In Thailand, there are two types of *Momordica charantia* fruit, Chinese bitter gourd (มะระจีน) and Thai bitter gourd (มะระขี้นก). Both types of immature green fruits used as vegetable. Thai bitter gourd has been used in traditional Thai medicine as bitter tonic, antipyretic, cholagogue, antirheumatic, antheminthic and laxative.

The last few decades many studies that have been studied on medicinal properties of *Momordica charantia* that include antibacterial [2],antiviral [3,4,5,6], antihyperglycemic [7,8], anti-ulcerogenic [2,9], antitumor [10], antispermatogenic [11], hypotriglyceridermic and hypocholesterolemic activity [12], and anti-human immunodeficiency virus (HIV) [4,6]. Most of the studies have been conducted using crude preparation of *Momordica charantia* and the chemical profile was not mentioned. In 1998, Kusamran *et al.* demonstrated chemo preventive potential of Thai bitter gourd but not by the Chinese variety. Moreover, Jiratchariyakul *et al.* found *Momordica* protein with a molecular weight of 28 kDa (MRK29) [6], while the Chinese one were reported to have a 30 kDa protein (MAP30) [4]. Both of proteins have the anti-HIV activities. In view of these findings, different variety may contain different biologically active chemicals. In this study, an interest in proteins from Thai bitter gourd fruit pulp, which the protein identification of Thai bitter gourd fruit pulp proteins can give information and knowledge, which is an important advantage for medicinal application and other utility.

The objective of this research is to characterize proteins from Thai bitter gourd fruit pulp. It will be extracted by fractional precipitation with ammonium sulfate in early step in purification step and separated by chromatography. Afterwards, the protein will be identified by using mass spectrometer and then determined protein profile by database searching.

CHAPTER II

LITERATURE REVIEWS AND THEORETICAL

2.1 *Momordica charantia* Linn.

2.1.1 General Background

2.1.1.1 Classification

Family: Cucurbitaceae

Genus: *Momordica*

Species: *Charantia*

Common names: Bitter melon, bitter gourd, balsam pear, balsam apple, bitter cucumber, karela

2.1.1.2 Description

Momordica charantia Linn. grows in tropical areas, including parts of Amazon, east Africa, Asia, and the Caribbean, and is cultivated throughout Thailand as food and medicine. It is a slender, climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf axils. The fruit appears as a warty gourd, usually oblong and resembling a small cucumber. The young fruit is emerald green, turning to orange-yellow when rip. At maturity the fruit splits into three irregular valves that curl backwards and release numerous brown or white seeds encased in scarlet arils. All parts of the plant, including fruit, taste very bitter. The fruit is most often eaten green [13].

Momordica charantia has a very large morphological variation with respect to fruit shape, size and colour (Figure 2.1) [14].

Figure 2.1 Variability in shape, size and colour of *Momordica charantia* fruits.

In Thailand, there are two types of *Momordica charantia* fruits [15].

1. Chinese bitter gourd (มะระจีน), phenotype is the smooth light green fruit with a round shape and normal length of 15-25 cm (Figure 2.2 A).

2. Thai bitter gourd, Ma-ra-kee-nok(มะระขี้นก), phenotype is the rough dark green fruit with tapered ends and normal length 5-8 cm (Figure 2.2 B).

A **B**

Figure 2.2 A : Chinese bitter gourd (มะระจีน), B : Thai bitter gourd (มะระขี้นก)

2.1.2 Chemical Constituents

There was determined the nutritional value of Thai bitter gourd fruits. Its fruits contain 100g, 83.20g moisture, 17.00 kilocalories, 1.00g fat, 12.00g fiber, 9.80g carbohydrate, 2.90g protein, 3.00 mg Ca, 140.00 mg P, 9.40 mg Fe, 2924 IU retinol, 0.09 mg thiamine, 0.05 mg riboflavin, 0.40 mg ascorbic acid, and 190.00 mg niacin [16, 17].

Momordica charantia contains biologically active phytochemicals including glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids. Several phytochemicals such as momorcharins, momordenol, momordicilin, momordicins, momordicinin, momordin, momordolol, charantin, charine, cycloartenols, diosgenin, elaeostearic acid, erythrodiol, galacturonic acid, gentistic acid, goyaglycosides, goyasaponins, multiflorenol have been isolated [13,18,19,20,21,22]

2.1.3 Used and Application [13]

MC has been used as food and traditional medicine. The fruit is edible when harvested green and cooked. All parts of the plant, including the fruit, taste very bitter. The leaves and fruits have both been used occasionally to make teas and beer, or to season soups in the Western world. The fruit is highly nutritious due to the iron and ascorbic acid content. MC has been used traditionally as medicine in many countries such as India, China, Brazil, Mexico, New Zealand, Philippine, Cuba, Ghana and Thailand. Thai people use immature green fruits as vegetable. Thai bitter gourd has been used in traditional Thai medicine as bitter tonic, antipyretic, cholagogue, antirheumatic, anthelminthic, and laxative. In India, *Momordica charantia* is used antidiabetic, abortifacient, anthelmintic, contraceptive, antimalarial and laxative and is used for treatment of dysmenorrhea, eczema, emmenagogue, galactagogue, gout, jaundice, kidney (stone), leprosy, leucorrhea, piles, pneumonia, psoriasis, rheumatism and scabies. In Turkish folk medicine, mature fruits of *Momordica charantia* are used externally for rapid healing of wounds and internally for treatment of peptic ulcers. In the Amazon, the plant has a long history of use by the indigenous peoples of the Amazon. A leaf tea is employed for diabetes, as a carminative for colic, topically for sores, wounds, and infections, internally and externally for worms and parasites, as an emmenagogue and as an antiviral for measles, hepatitis, and feverish conditions. In Brazilian herbal medicine, *Momordica charantia* is used for tumors, wounds, rheumatism, malaria, leucorrhea, inflammation, menstrual problems, diabetes, colic, fevers, worms, to induce abortions, and as an aphrodisiac. It is also employed topically for skin problems, vaginitis, hemorrhoids, scabies, itchy rashes, eczema, and leprosy.

2.2 Literature Reviews

The uses of natural drugs, such as plants and herbal remedies to treat diseases are very common in Asia and developing countries. The chemical diversity and unique biological activities of biologically active compounds have propelled further discoveries in both the chemical and biological sciences and provided therapeutic agents for many diseases. Some plant contains biologically active chemicals such as glycosides, saponins, alkaloids, triterpenes, steroids, and proteins [23]. Plant proteins

are exploited very extensively for food. A few plant proteins are used in medical, technical or analytical application such as lectins and α-glucosidase inhibition [24].

Lectins are proteins that recognize and bind to specific carbohydrate structural epitopes. They may be regarded as protein interpreters of the sugar code and represent convenient biochemical tools to probe protein-carbohydrate interactions [25]. They have the ability to induce cell agglutination phenomena. The promotion of erythrocyte agglutination by plant extracts was first identified by Stillmark in 1988, by searching for toxicity factor in *Ricinus communis* [26]. Lectins initiate several peculiar activities on the cell include blood-group-specific hemagglutinating activity, tumor-cell-specific agglutinating activity and mitogenic activity against lymphocytes [27].

One interesting activity is α-glucosidase inhibition activity. N-Linked oligosaccharides play many roles in the functions of glycoproteins. One function is to assist in the folding of proteins by mediating interactions of the lectin-like chaperone proteins calnexin and calretculin with nascent glycoproteins. The interactions can be prevented by inhibitors of the α-glucosidase and this causes some proteins to be misfolded and retained within the endoplasmic reticulum. It has been demonstrated in an animal model of chronic HBV that glucosidase inhibitors can alter glycosylation and have anti-viral activity [28].

Momordica charantia is naturally rich in beneficial phytochemical. Studies on phytochemical and bioactivity characteristic of *Momordica charantia* have been carried out since 1950 [15]. It is most widely studied with its antidiabetic effect. Most of the studies have been conducted using crude preparation of *Momordica charantia* for demonstrate hypoglycemic properties such as observations that the aqueous juice of MC fruit [29, 30, 31] or fractions extracted with water, alcohol or acetone from this fruits [32,33,34,35,36,37] exhibits a potent hypoglycemic activity in normoglycemic and streptozotocin-induced diabetic rats as well as in human subjects with diabetes mellitus type II. However, few studies have demonstrated hypoglycemic activity of *Momordica charantia* compound. At least three different groups have been reported to have hypoglycemic activity or other actions of potential benefit against diabetes mellitus. These include a mixture of steroidal saponins known as charantin, polypeptide-p, and triterpenoid. Charantin was isolated from the unripe fruits of

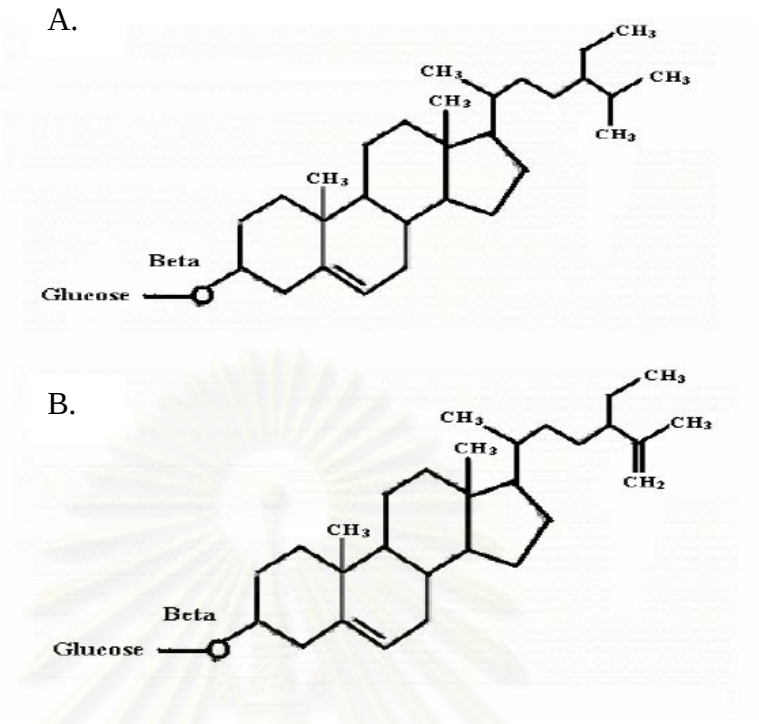

Figure 2.3 Chemical structure of A : sitosteryl glucoside ($C_{35}H_{60}O_6$), B: stigmasteryl glucoside ($C_{35}H_{58}O_6$)

Momordica charantia. It is composed of a mixture of sitosteryl glucoside ($C_{35}H_{60}O_6$) and stigmasteryl glucoside ($C_{35}H_{58}O_6$) [38, 39]. In 1981, Khana *et al.* reported isolation polypeptide-p from fruits, seeds, and tissue culture of seedlings of *Momordica charantia* by acid-ethanol extraction. It consisted of a methionine-containing protein with a minimum size of 11 kDa. It is a very effective hypoglycemic agent when administered subcutaneously to gerbils, langurs, and diabetic patients [32]. In 2006, Harinantenaina *et al.* evaluated the contribution of the cucurbitane triterpenoids of the ether fraction of *Momordica charantia* methanol extract to in vivo anti-diabetic effects, the major compounds are 5*β*, 19-epoxy-3*β*, 25-dihydroxycucurbita-6, 23(*E*)-diene, and 3*β*, 7*β*, 25-trihydroxycucurbita-5,23(*E*) dien-19-al have shown blood hypoglycaemic effects in the diabetes -induced male ddY mice strain at 400 mg/kg [36]. In the same year, Yibchok-anun *et al.* reported the first investigated the direct effect of the protein extract from Thai bitter gourd (*Momordica charantia*) fruit pulp on insulin and glucagons secretions using the pancreatic perfusion technique. The *Momordica charantia* protein extract, a slow acting chemical, was able to decrease plasma glucose levels, and increase plasma insulin secretion in both normal and diabetic rats. The molecular weights of two major

proteins were estimated to be 10 and 20 kDa [37]. In 2002, Matsuura *et al.* reported α-glucosidase inhibitory activities in aqueous methanol extracts of the seed of *Momordica charantia.* The structure of the compound was identified as D-(+)-trehalose which its inhibitory activity was compared with 1-deoxynojirimycin. Trehalose showed 45% inhibitory activity at the concentration of 2×10^{-3} M [40].

All parts of the plant, including the fruits, taste very bitter. Taste bitter and it has long been known that it contains a bitter principal. Various bitter principles have been isolated and their structures have been elucidated. The structure of momordicosides K and L, bitter principles in immature fruits of *Momordica charantia* were elucidated as 7-O-*β*-D-glucopyranosides of 3*β*,7*β*-dihydroxy-25-methoxy-cucurbita-5-23-dien-19-al and 3*β*,7*β*,25-trihydroxy-cucurbita-5-23-diren-19-al, respectively [41]. In 1984, Yasuda *et al.* isolated momordicines I, II and III from the leaves and vines of *Momordica charantia*. Struture of momordicines I, II and III were eusidated as 3*β*,7*β*,23ε-trihydroxy-cucurbita-5-24-diren-19-al, 23-0-*β*-glucopyranoside and 23-0-*β*-glucopyranoside of 3*β*-7*β*-23ε-trihydroxy-24-oxo-cucurbita-5, 25-dien-19-al, respectively [42].

Eight cucurbitane-type triterpene glycosides called goyaglycoside-a, -b, -c, -d, -e, -f, -g, and –h and three oleanane-type triterpene saponins termed goyasaponin I, II, and III was isolated from the fresh fruit of Japanese *Momordica charantia* [19]. Three cucurbitane-type triterpene and five cucurbitane-type triterpene glycosides were isolated from the dried fruit of *Momordica charantia* in Sri Lanka. They was called karavilagenins A, B, and C and karavilosides I, II, III, IV, and V respectively [43]. The structure of the compound were elucidated by spectroscopic methods. Triterpenes (momordicin, momordicinin, and momordicilin), a sterol (momordenol) and a monocyclic alcohol (momordol) were isolated from the flesh fruits of *Momordica charantia* [21].

The abortifacient proteins have been isolated and characterized from *Momordica charantia* seeds are called α- and β-momorcharins and they are glycoproteins with molecular weights around 30 kDa. They are all basic proteins devoid of half-cystine residues and possessing aspartic acid as the NH_2-terminal amino acid, although they are immunologically distinct. They terminate early pregnancy in mice by interfering with implantation onto the endometrium and

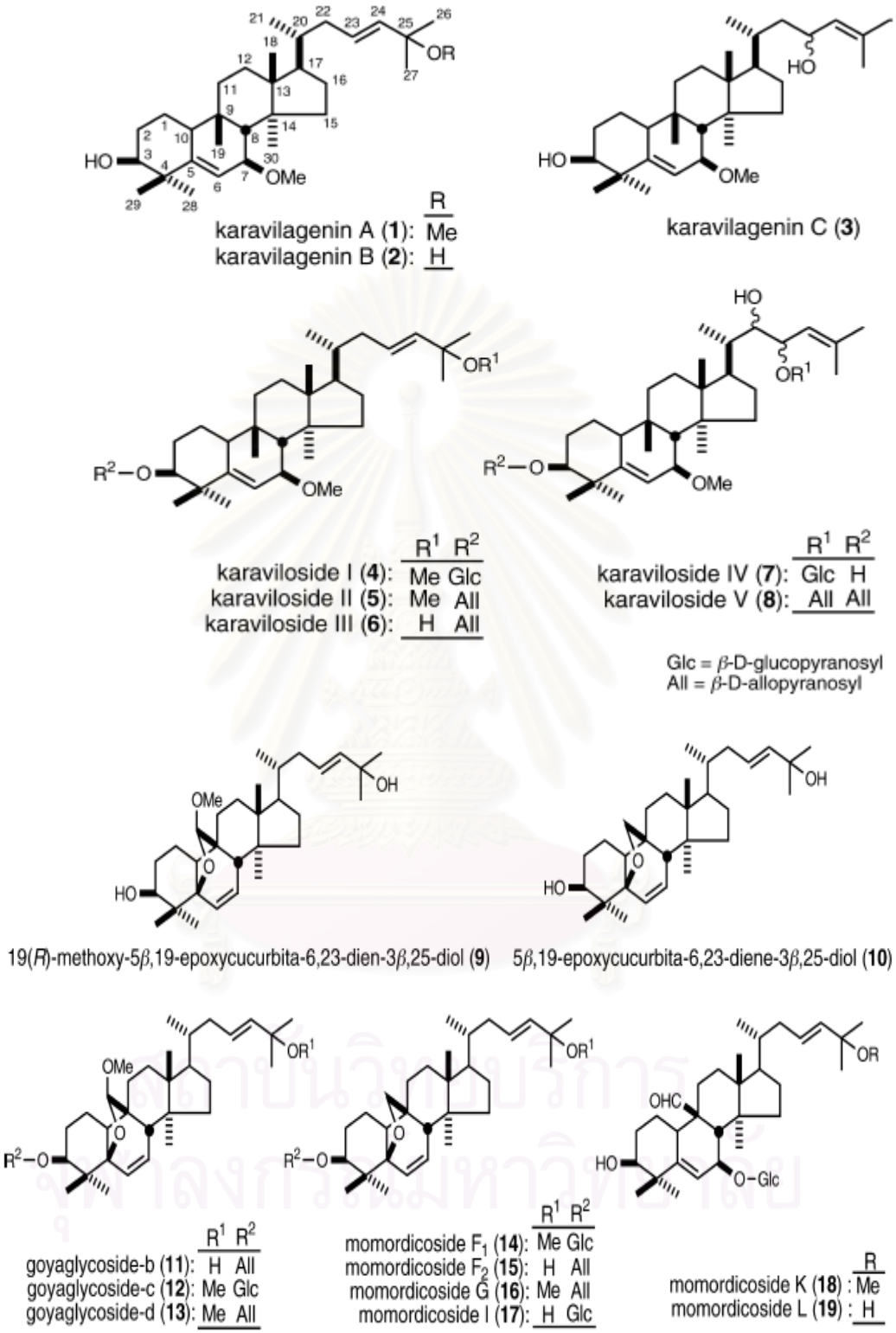

Figure 2.4 Chemical structure of cucurbitane-type triterpene and cucurbitane-type triterpene glycosides from the dried fruit of *Momordica charintia* Linn.

midterm abortion in rats by inducing necrosis of the trophoblast [44,45,46,47]. In 1999, Paul *et al.* isolated ribosome-inactivating protein (RIP) (δ-momorcharin) from the seeds and a candidate RIP (ε-momorcharin) from fruits of *Momordica charantia*. δ- and ε-momorcharin possessed a molecular weight of 30 and 24 kDa respectively and inhibited cell-free translation in rabbit reticulocyte lysate with an IC_{50} of 0.15 and 170 nM [48]. In 1996, Pu *et al.* characterized a small ribosome-inactivating protein, γ-momorcharin, which purified from the seeds of *Momordica charantia.* Its molecular weight is 11,500 Da. It can inhibit the protein synthesis in the rabbit reticulocyte cell-free system with ID_{50} of 55 nM [49]. Anti HIV activity of MAP30 (*Momordica* Anti-HIV Protein), a ribosomal inactivating protein, was isolated from the seeds and ripe fruits of *Momordica charantia.* It was found to be single chain polypeptide which inhibited the HIV-1 reverse transcription, integration and syncytium formation between the infected and the new white blood cells. Anti HIV activity of MAP30, recombinant MAP30, and proteolytic fragments of MAP30 were exhibited in several in vivo and in vitro studies. Moreover, MAP30 is non-toxic to normal non-infected cells, as it does not penetrate healthy cells [4,10,50]. In 2001, Jiratchariyakul *et al.* isolated Thai bitter gourd protein (MRK29) from seeds and ripe fruits of *Momordica charantia.* The purification was performed by ammonium sulfate fractionation. It was concentrated in the 30-60% salt precipitated fraction. Its molecular weight is 28.6 kDa. MRK29 inhibited HIV-1 reverse transcriptase. The salt-precipitated fraction of MRK29 caused 82% reduction of viral core protein p24 expression in HIV-infected cells and an increased in TNF activity [15].

Trypsin inhibitors are universally found in many plants. They contain high cysine content, the disulfide bridges playing an important role in their inhibitory activities. In 1988, Zeng *et al.* isolated and characterized three trypsin inhibitors, MCI-1, MCI-2 and MCI-3, from the seeds of *Momordica charantia*. MCI-1, 9 kDa, is composed of 77 amino acid residues and 7 pairs of disulfide bridges. MCI-2 is composed of 70 amino acid residues and 2 pairs of disulfide bridges. MCI-3, 7443 Da, is composed of 62 amino acid residues and contained no cysteine [51].

In 1983, Spreafico *et al*. studied in vitro on the immunological activity of *Momordica charantia* inhibitor (MCI). MCI, 30 kDa plant proteins, inhibited lymphoid cell responsiveness to PHA and ConA, but not to LPS and markedly

enhanced macrophage-dependent cytotoxicity [52]. In 1991, an inhibitor (BGIA) against an acid amino acid specific endopeptidase of *Streptomyces griseus* (Glu *S.griseus* protease) was isolated from seeds of *Momordica charantia* by Ogata *et al.* The molecular weight was calculated to be 7419 [53]. In 2002, Parkash *et al.* isolated a peptide designated charantin, with a molecular weight 9.7 kDa, from *Momordica charantia* seeds. Charantin inhibited cell-free translation in a rabbit reticulocyte lysate system with an IC_{50} of 400 nM [54].

Momordica charantia is widely studied. However, there are few reports about proteomic field in fruits pulp of Thai bitter gourd. Moreover, some biologically active chemicals depend on variety of *Momordica charantia* such as the studied of Kusamran et al. demonstrated chemopreventive potential of Thai bitter gourd fruit, while the Chinese variety uncertain [55] and one of report studied about Thai bitter gourd protein fraction which has revealed the presence of an anti-HIV protein with the molecular weight of 28.6 kDa and different amino acid sequence from MAP30 that has been isolated from seed and fruits of MC from China [15]. α-Glucosidase inhibitors have received considerable attention as they are potential therapeutic agents for the treatment of diabetes. Among the plant, *Momordica charantia*, some are traditionally recommended for diabetic treatment and several studies have been conducted to determine the antihyperglycemic effect, whereas no attention has been directed to α-glucosidase inhibition by protein from fruit pulp of *Momordica charantia*. From all these reasons, it led to idea of this research which concern with proteins of Thai bitter gourd fruit pulp which expect to found bioactive proteins from this plant which is an important advantage for medicinal application and other utility.

2.3 An Introduction to Proteins

Proteins are the major components of living organisms which regulate metabolic activity, catalyze biochemical reactions and maintain structural integrity of cell and organisms. Proteins are biopolymer of α - amino acid. The physical and chemical properties of a protein are determined by its constituent amino acids. The structure of an amino acid contains a central carbon atom with a primary amine group, a carboxylic acid group, and an R group which is varies and defines the structure, function, chemical properties, and physical properties of the different amino acids.

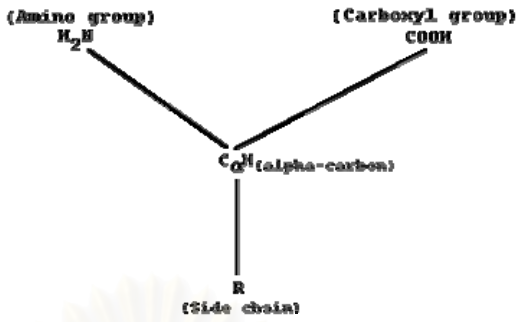

Figure 2.5 The structure of an amino acid

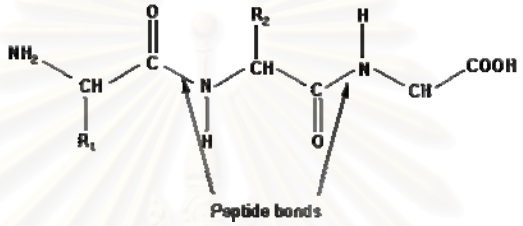

Figure 2.6 The structure of peptide bond

There are four levels of protein structure (Figure 2.7). The primary protein structure is the linear sequence in which amino acids are covalently connected to form a polypeptide chain. The individual amino acid subunits are linked by amide linkages called peptide bonds. The free amino acid group and free carboxyl group at opposite ends of peptide bonds with the free amino group ($-NH_3^+$) is called N-terminus and the end with the free carboxyl group ($-COO^-$) is called the C-terminus. Secondary protein structure occurs when the sequence of amino acid are linked by hydrogen bonds. The major secondary structures are pleated sheet and α helix. Tertiary protein structure is the folding of the secondary structure. Tertiary structures are stabilized by interactions of side chains of non-neighboring amino acid residues. Quaternary protein structure is the interaction of two or more polypeptide chains into multisubunit, or oligomeric, protein with a specific function [56].

2.4 Measuring the Concentration of Protein [57]

Quantification of protein concentration is required, for example, to estimate recovery at different stages during purification of proteins to measure specific activity Table 2.1 shows four methods that are commonly used to quantify proteins. Each of these methods has advantages and limitation.

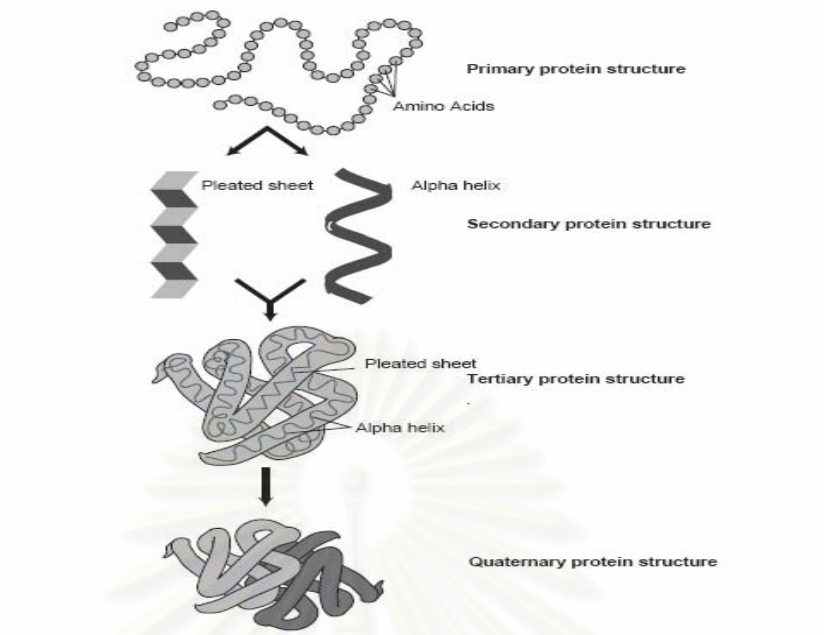

Figure 2.7 The structure of protein

2.4.1 Ultraviolet Absorption at 280nm

Proteins contain tyrosine, tryptophan and phenylalanine side chains that are fairy strong absorbers of light in the 275 – 280 nm (ultraviolet) regions. Proteins that contain these amino acids can be detected by their ability to absorb ultraviolet light. After suitable dilution to produce on scale absorbance readings, total protein can be estimated from UV absorbance spectra using quartz or fused silica cuvettes.

2.4.2 The Bicinchonic Acid (BCA) Assay

The BCA assay is a copper-dependent method based on the reduction by protein of Cu^{2+} to Cu^{+} under alkaline conditions. The cuprous (Cu^{+}) ion is detected by reaction with BCA, a water-soluble Cu^{+}-chelating agent. The intense purple-colored reaction product (measured at 562 nm) is formed by the interaction of two molecules of BCA with one cuprous (Cu^{+}) ion. The side chains of cysteins, cystine, tryptophan, and tyrosine residues are capable of reducing Cu^{2+} to Cu^{+} and contribute significantly to color formation in the BCA assay.

Table 2.1 Protein assays

Assay	Working Range	Applications	Major Interfering Substances
UV absorbance	A_{280} :20-300 μg	can be performed directly on the sample	Nucleic acids and many buffer salts and detergents
BCA method	Standard method: 5-200 μg/ml Micro method: 0.5-30 μg/ml	compatible with detergents	reducing agent, chelators, and lipids
Bradford assay	Standard method: 10-100 μg/ml Micro method: 1-10 μg/ml	compatible with reducing agent	detergents
Dot-blotting assay	0.5-5 μg	rapid screening of large number of samples	-

2.4.3 The Bradford Assay

The Bradford assay originally described by Bradford to provide a measure of total protein. This method relies on the binding of protein to Coomassie Brilliant Blue G-250 which causes an absorbance shift from 465 nm to 595 nm in an acidic solution (Figure 2.8). The absorption at 595 nm is directly related to proteins concentration. The Bradford assay is extremely fast (color development is complete after 2 minutes) and very sensitive (as little as 1 μg of protein can be detected) [58].

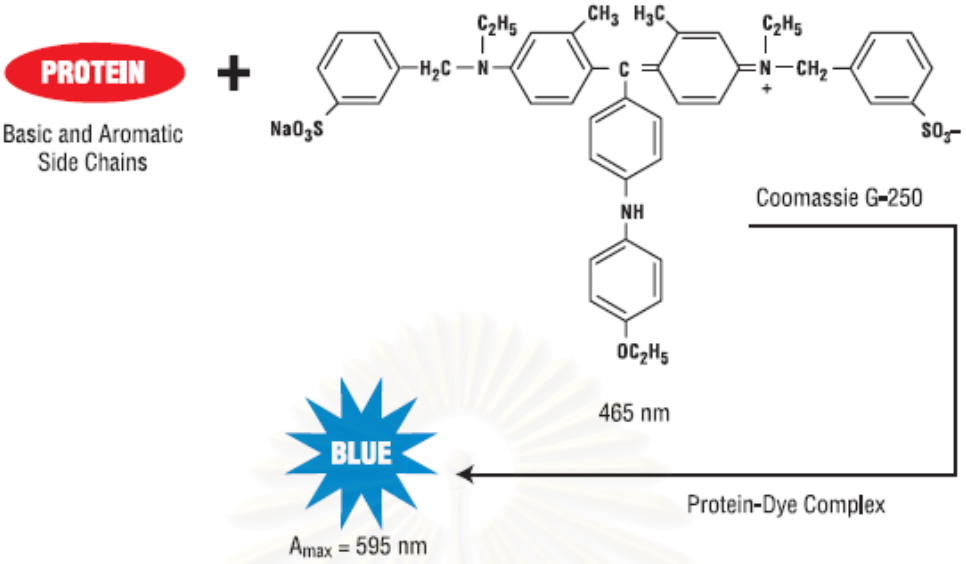

Figure 2. 8 Reaction schematic for the Bradford assay

2.4.4 Dot Blotting Assay

Protein can be immobilized to solid phase supports by vacuum slot-blotting using a commercially available apparatus. After protein solutions have been applied to an immobilizing membrane, proteins are visualized by sensitive staining with Coomassie Blue, amido black, silver, etc. The major advantage of dot blotting for assaying protein concentration is that preliminary estimates of protein can be obtained rapidly for a large number of samples. This method is particularly useful for monitoring the protein content of chromatographic fractions, especially in situation where the eluent buffer is incompatible with the colorimetric assay and a UV detector is unavailable.

2.5 Protein Purification

The unique structure and chemical composition of each protein is important for its function. It is also important for separating proteins in a protein purification strategy. The steps of purification, which vary for different proteins, usually exploit minor difference in the solubility, net charges, size, and the binding specificities of protein. Most purification techniques are performed at 0-4°C to minimize temperature-dependent processes such as protein degradation and denaturation [59].

2.5.1 Protein Extraction

The first step of protein purification techniques is disrupting the cells and releasing the proteins into an aqueous “extract”. There are many type of cell. Most cells have particular characteristics which need special attention during disintegration. The extract is prepared, after cell disintegration, by centrifuging off insoluble material. Before centrifuging, the mixture is usually described as a homogenate. Protein should be present in the liquid layer as much as possible after centrifugation [60].

Table 2.2 Cell Disintegration Techniques

Technique	Example	Principal
Hand homogenizer	Liver tissue	Cells forced trough narrow gap, Rips off cell membrane
Gentle cell lysis	Erythrocytes	Osmotic disruption of cell membrane
Blade homogenizer	Muscle tissue, Animal tissue, Plant tissue	Chopping action breaks up large cells, shears apart smaller ones
Bead mill	Cell suspension	Rapid vibration with glass beads rips cell walls off

2.5.2 Separation by Precipitation

Protein can be precipitated from solution by a wide range of agents (Table 2.3). Precipitation is a useful technique as it allows both purification and concentration in a single step. The salting-out technique of protein purification is one of the most widely used techniques in enzyme purification. It is mainly dependent on the hydrophobic character of the protein. The solubility of protein depends on hydrophilic amino acid on their surfaces that attracts water molecules and interacts with them.

The solubility of protein is affected by co-solvents (especially salts). In a low ionic strength aqueous solution, the presence of salt stabilizes the various charged group on protein molecules, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as salting-in effect. However, as the

Table 2.3 Protein Precipitation Techniques

Precipitating agent	Principle	Example
Neutral salt	Salting-out	Ammonium sulphate
Weakly polar solvent	Reduction of dielectric constant	Ethanol
Acid/alkali	Isoelectric precipitation	Sulphuric acid
Hydrophilic uncharged organic polymers	Phase-distribution/steric exclusion	Polyethylene glycol
Polyelectrolyte	Electrostatic complex formation	Polyacrylic acid
Metal ions	Charge neutralization	Calcium

salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration, the solubility of the protein decreases. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecule. This phenomenon of protein precipitation in the presence of excess salt is known as salting-out effect and the magnitude of this effect depends on the natural of the salt.

The salting out ability of anions follows the Hofmeister series, which for some common anions is $SCN^- < ClO_4^- < NO_3^- < Br^- < Cl^- < acetate^- < SO_2^- < PO_4^{3-}$. One of these salts, ammonium sulfate has been the most widely used because it has high solubility and is relatively inexpensive. However, small contaminating amounts of heavy metals, especially iron, could be detrimental to sensitive enzymes. Metal-complexing agents such as EDTA should be present in the solution before adding the ammonium salt. The optimum concentration of ammonium sulfate required to precipitate the protein of interest is determined by adding increasing amounts of the ammonium sulfate and saving the precipitate for further analysis. Ammonium sulfate can be added as a solid (Table 2.4) for amounts to be added to reach a saturation level. To remove the salt from the protein sample, dialysis or gel filtration chromatography can be used [24].

Table 2. 4 Final Concentration of Ammonium Sulfate: Percentage Saturation at 0°

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

2.5.3 Dialysis of Proteins

Dialysis is a procedure to separate smaller molecules (e.g., salt) from larger molecules (e.g., protein) by using a semipermeable membrane, such as a cellulose membrane with pore (Figure 2.9), that allows the passage of the smaller molecules but not the larger molecules. Dialysis tubing is available in variety of size. The bag is placed in a larger stirred vessel containing the desired buffer. Changing the buffer at least one is need because at equilibrium is achieved, at which point the concentration of the dialyzable material (salt) is the same on the inside and outside of the dialysis bag. If the volume outside the bag is much larger than the volume inside the bag, there will be a substantial decrease in the salt concentration within the dialysis bag. If the outside solution is changed several times during the dialysis, an even greater decrease in the salt concentration can be achieved [24].

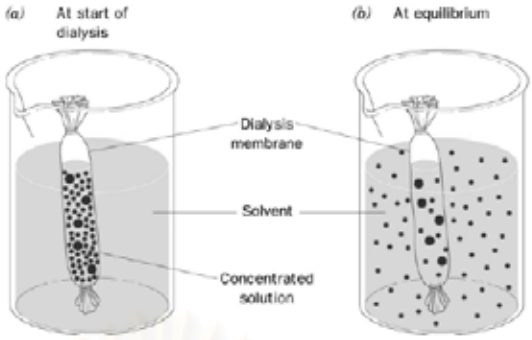

Figure 2.9 The dialysis at start and equilibrium state.

2.5.4 Separation by Chromatography

Chromatography is process used to separate molecules on the basis of a chemical property, such as molecular mass, charge, or solubility. The sample is passed trough the stationary phase the flow of the mobile phase. Molecular with different physical properties partition differently between the stationary and mobile phase, result in a separation. Molecules that are strongly attracted to the stationary phase will be retained, relative to molecules that are not attracted strongly to the stationary phase. By choosing the appropriate stationary and mobile phase, it is possible to obtain effective separations of molecules that are only slightly different from each other.

For protein purification, chromatography technique separates according to differences in specific properties, as shown in Table 2.5 [61].

Table 2.5 Protein properties used during purification.

Protein Properties	Type of Chromatography
Size and shape	Gel filtration
Net charge	Ion-exchange chromatography
Isoelectric point	Chromatofocusing
Hydrophobicity	Reversed-phase chromatography
Biological function	Affinity chromatography

2.5.4.1 Ion-Exchange Chromatography

Ion-exchange chromatography separates molecules on the basis of their charge groups, which cause the molecules to interact electrostatically with opposite charges on the stationary phase matrix. Different types of molecules will bind to the matrix with affinities that depend on both the conditions used and the types and number of individual charged groups. Proteins consist of many different amino acid, and the overall charge is caused by the composite effect of many different ionizable group. The pH at which a protein is uncharged is called the isoelectric pH (pI). The pI of most proteins is in the range of 5-9. Ion-exchange chromatography of proteins is usually performed at least 1 pH unit away from the pI of the protein of interest to assure that it is charge (see Figure 2.10). When the pH for chromatography is below the pI, protein will be positively charged and bind to a cation-exchange resin. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions. When the pH for chromatography is above the pI, protein will be negatively charged and bind to an anion-exchange resin (see Figure 2.10).

Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, Diethylaminoethane. The functional groups substituted onto a chromatographic matrix determine the charge of an ion exchange medium, a positively- charged anion exchanger or a negatively-charged cation exchanger (see Figure 2.11).

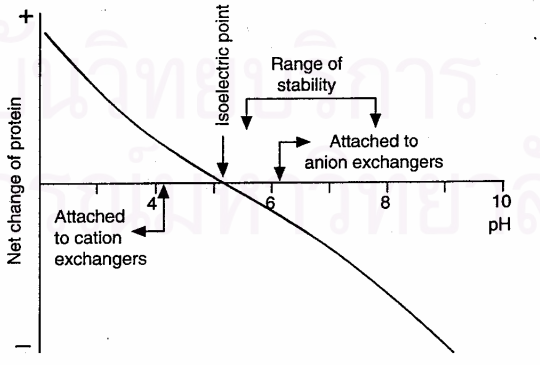

Figure 2.10 The isoelectric point of a molecule

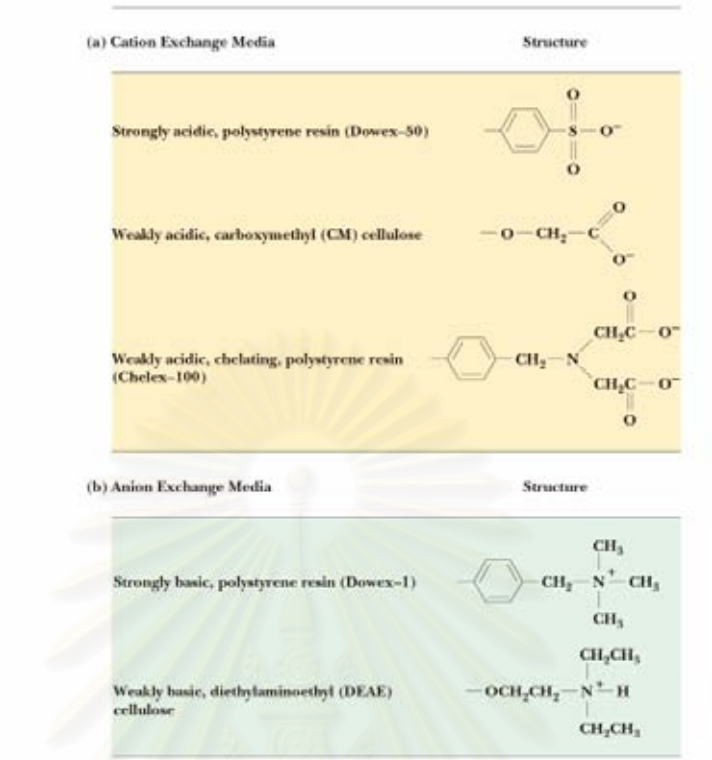

Figure 2.11 Different types of ion exchange resins (a) Cation exchanger (b) Anion exchanger.

The proteins that adsorb to the ion exchanger are eluted from the column by an increase in ionic strength, by increases salt concentration or change pH. When the concentration of the counterions is continuously varied during the elution process, it is referred to as gradient elution, whereas when the concentration of counterions is altered in a stepwise fashion during elution, this is referred to as step elution.

2.5.4.2 Gel Filtration Chromatography

Gel filtration chromatography separates molecules on the basic of their molecular mass or, more properly. The stationary phase consists of fine beads that contain pores of controlled size. The sample is applied in a narrow band and then washed through the column by the mobile phase. Large molecules in the sample which cannot pass through the pores of the beads pass around the outside of the bead and smaller molecules can enter the pore of the beads have a longer. Thus large molecules leave the column first followed by the smaller molecule in order of their size (see Figure 2.12).

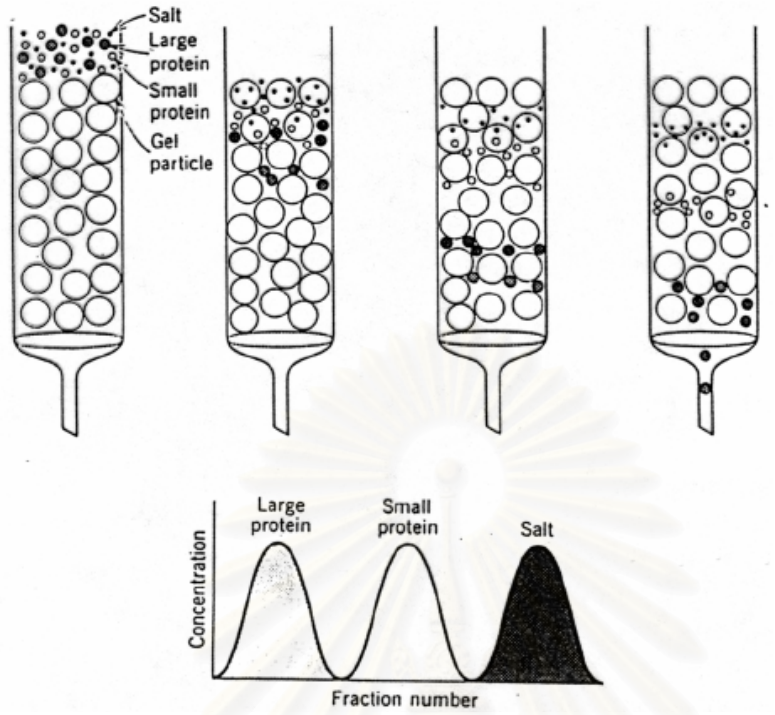

Figure 2.12 Gel filtration chromatography

The bead size and the bead distribution of the media are very important parameters for resolution of the protein peaks. The choice of media depends on the application. If high-resolution purification purification is needed, a medium with a smaller bead size (10-30 μm) and narrow particle size distribution should be chosen. For analytical purposes, silica and hydrophilic vinyl polymer-based media can be used.

2.5.4.3 Affinity Chromatography

Affinity chromatography is frequently used to purify specific biological macromolecules such as proteins and nucleic acids. In nature these biological macromolecules are often involved in highly specific interactions with other proteins or nucleic acids.

Affinity chromatography, the stationary phase is referred to use of an immobilized natural ligand, which specifically interacts with the desired protein. The ligand is immobilized on suitable particles which can pack into a column. Then a sample containing the protein is passed into the column and the specific interaction hold back the desired protein, while others pass through. After washing the non-

interacting molecules away, the desired protein is eluted with specific substances, often resulting in highly purified material.

The following are two important considerations for affinity chromatography. First, choice of ligand: the ligand must bind strongly to the target molecule to facilitate its capture from a complex protein mixture. Second, matrix selection: matrix supports (typically, a macroporous polysaccharide bead such as agarose) tether the active ligands and provide a porous structure so that these is an increased surface area to which the target molecules bind. Ligand can be covalently affixed to substituent groups within the matrix (e.g., amino, hydroxyl, carbonyl, and thiogroups) that are easily activated using conventional chemical method. The matrix, in addition to requiring activation, must also often stand up to rigorous column regeneration procedures, such as rinsing the column with sodium hydroxide and to the extremes of pH often required to recover sample molecules.

Lectin affinity column are especially useful for purifying membrane proteins and secretory proteins, which are frequently glycosylated. Table 2.6 lists the carbohydrate specificities of lectins commonly used in affinity chromatography [57].

Table 2. 6 Lectins and their carbohydrate-binding specificities

Lectins	Carbohydrate Binding Specificity
Concanavalin A (Con-A)	α-Man, α -Gla, α-GlcNAc
Wheat-germ agglutinin(WGA)	β-GlcNAc, Sialic acid
Dolichos biflorus (DBA)	α-GlcNAc
Ricinus communis(RCA-1)	β-Gal
Ulex europaeus (UEA-1)	α-Fucose
Arachis hypogaea (PNA)	β-Gal
Glycine max (SBA)	α-GalNAc, β-GlcNAc
Limulus polyphemus (LPA)	Sialic acid
Helix aspersa (HAA)	α-GlcNAc, α-GalNAc

2.5.4.4 Reversed Phase Chromatography

Reversed Phase Chromatography is based on interaction between hydrophobic ligands covalently attached to the adsorbent and the hydrophobic patches of

molecules. Whereas small molecules are separated by an equilibrium mechanism that distributes the molecules between the stationary and mobile phase during their passage through the column. Polypeptides, being too large to enter the hydrophobic stationary phase, adsorb to the hydrophobic surface and desorbs under increasing concentrations of mobile phase. The stationary phases are formed by bonding a hydrocarbon phase to the silica matrix. The hydrocarbon group forming the hydrophobic phase is usually a linear aliphatic hydrocarbon of 18(C_{18}), 8(C_8), or 4(C_4) carbons. The primary separation will be on the basis of hydrophobicity. As the eluting aqueous solvent is made less polar by dilution with acetonitrile or methanol, proteins are eluted in the order of their increasing hydrophobicity.

2.5.5 Separation by Gel Electrophoresis

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. Proteins have a net charge at any pH other than their isoelectric point (pI). Thus, when placed in an electric field, proteins will migrate toward the electrode of the opposite charge. Various substances have been used as supporting medium for electrophoresis separations such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel. Currently, almost all protein electrophoresis applications use polyacrylamide gel as supporting medium.

polyacrylamide gels are formed by the copolymerization of acrylamide (CH_2=CH-CO-NH_2), a water-soluble monomer, with a cross-linking agent to form a three-dimensional lattice. The cross-linking agent of choice for most applications is N,N'-methylene bisacrylamide (BIS). The polymerization reaction occurs by a free radical chain mechanism with ammonium persulfate and tetramethylenediamine (TEMED, $(CH_3)_2$N-CH_2-N$(CH_3)_2$) . The average size of the pores in a polyacrylamide gel can be controlled by varying the amount of monomer used or by varying the degree of cross-linking, with higher degrees of cross-linking resulting in narrower pores.

The size pores in a polyacrylamide gel is determined by two parameters: the total amount of acrylamide present (%T) and the ratio of cross-linker to acrylamide

monomer (%C). The %T is the ratio of the sum weights belong to acrylamide monomer and the cross-linker in the solution. As the total amount of acrylamide (%T) increases, the pore size decreases. With cross- linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size.

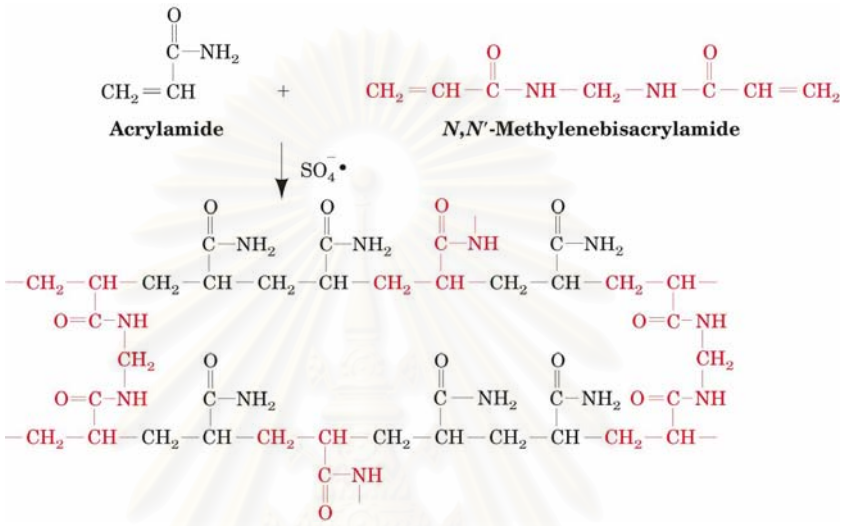

Figure 2.13 The polymerization reaction of acrylamide and methylenebisacrylamide.

$$T = \frac{(a+b)\times 100}{V}[\%]$$

$$C = \frac{b\times 100}{a+b}[\%]$$

a is the mass of acrylamide in g,

b the mass of methylenebisacrylamide in g, and

V the volume in mL.

Figure 2. 14 Determination of %T and %C for acrylamide gels.

Sodium dodecyl sulfate (SDS) is a powerful detergent, which has a hydrophobic region. It separates exclusively according to molecular weight. By loading with anionic detergent SDS, the charge of the proteins is so well masked that anionic micelles with a constant net charge per mass unit result: 1.4 g SDS per g protein. Disulfide bonds which can form between cysteine residues can be cleaved by reducing thiol agent such as 2-mercaptoethanol or dithiothreitol(DTT) (Figure 2.15).

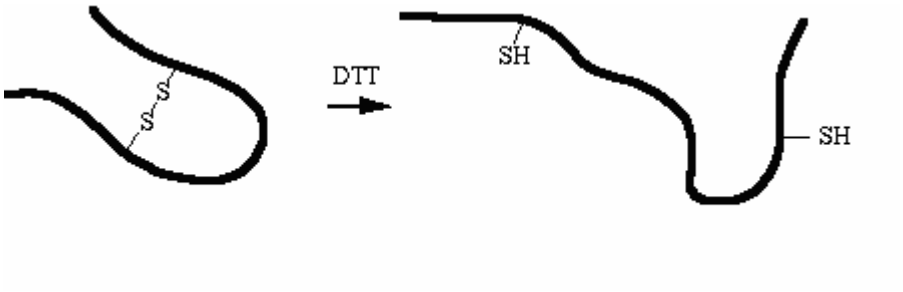

Figure 2.15 DTT reduces disulfide bounds, removing the last traces of tertiary or quaternary structure

When proteins are treated with SDS, they are denatured. They also become negatively charged because of the charge on the detergent, and the amount of detergent bound is so large that any differences in native charge are swamped. The bigger the proteins, the more SDS is bound, so that all proteins treated with SDS have the same ratio of charge to mass. The moving through the gel in the electric field depends on the size of protein. The larger the protein the more slowly it will move through the matrix of the polyacrylamide. The molecular weight of the proteins can be estimated with a calibration curve using marker proteins (Figure 2.16).

Some proteins are colored and can be seen directly on a gel, but most are colorless. To visualize most proteins, a staining procedure is needed. Coomassie blue is a general protein stain, causing the protein to be come visible as blue bands within

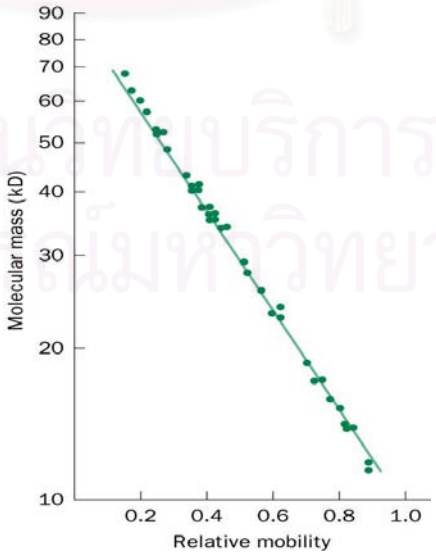

Figure 2.16 Calibration curve of protein standard.

the gel. Silver stain can detect very small amounts of proteins, causing them to turn brown-black [62,63].

2.6 Protein Identification Techniques

2.6.1 Edman Sequencing

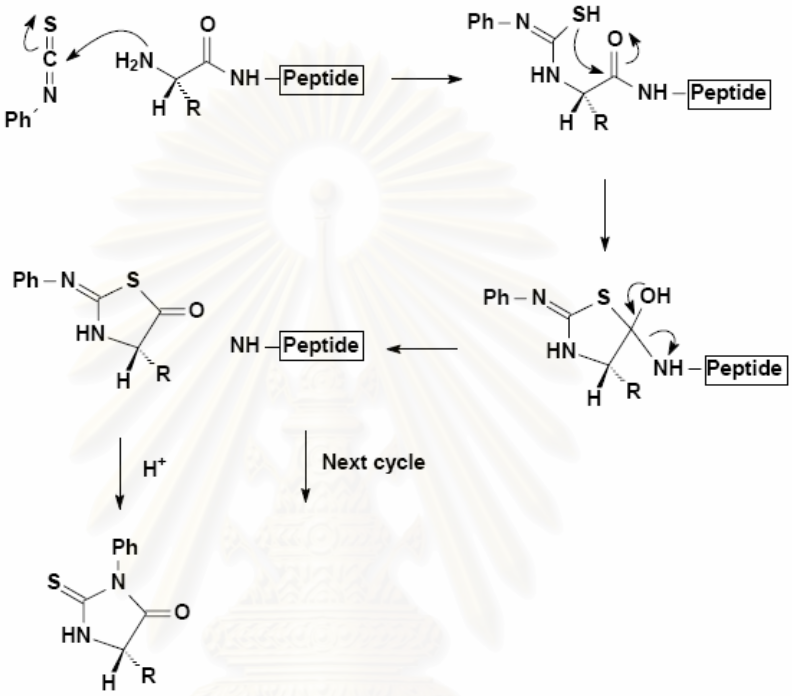

Figure 2.17 Edman degradation: phenylisothiocyanate to react with the N-terminal residue under alkaline conditions.

The utility of the Edman degradation technique is that it allows for additional amino acid sequence to be obtained from the N-terminus inward, and it is illustrated schematically in Figure 2.15. This method utilizes phenylisothiocyanate to react with the N-terminal residue under alkaline conditions. The resultant phenylthiocarbamyl derivatized amino acid is hydrolyzed in anhydrous acid. The hydrolysis reaction results in a rearrangement of the released N-terminal residue to a phenylthiohydantoin derivative. The entire sequence of reactions can be repeated over and over to obtain the sequences of the peptide. Identification is based on correlating the retention time of the eluting amino acid to a standard chromatogram. This process has subsequently been automated to allow rapid and efficient sequencing of even extremely small quantities of peptide [64].

2.6.2 Mass Spectrometry

Mass spectrometry is an instrumental approach that allows for the mass measurement of molecules. Mass spectrometers use a variety of techniques to create charged ions or charged ion fragments and separate these ions. The principal of mass spectrometry is measurement of the mass-to-charge ratio (m/z) of gas-phase ion. The ionic separation is done by using a combination of magnetic or electric field. The ions are generated in the ionization source by inducing either the loss or the gain of a charge (e.g. electron ejection, protonation, or deprotonation). Once the ions are formed in the gas phase they can be electrostatically directed into a mass analyzer, separated according to mass and finally detected. The result of ionization, ion separation, and detection is a mass spectrum that can provide molecular weight or even structural information. The resulting mass spectrogram is series of peaks, with each peak corresponding to a different mass-to-charge ratio.

Mass spectrometry enables protein structural information, such as peptide masses or amino acid sequences, to be obtained. This information can be used to identify the protein by searching protein databases. The harvesting of protein information by mass spectrometry can be devised into three stages: (i) sample preparation, (ii) sample ionization, and (iii) mass analysis [65].

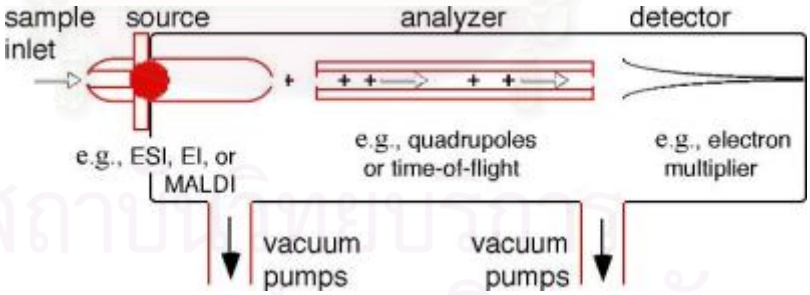

Figure 2.18 Components of a mass spectrometer.

2.6.2.1 Sample Preparation

In most of proteomics, a protein is resolved from a mixture by using a 1- or 2-D polyacrylamide gel. The challenge is to extract the protein or peptides from the gel, purify the sample, and analyze it by mass spectrometry. A protein is in-gel digested with a protease and then many of the peptides can be extracted from the gel. For many applications, in-gel digestion needs to be purified to remove gel contaminants such as

salts, buffers, and detergents which can interfere with mass spectrometry. One method of peptide purification commonly employed for this proposes is reverse phase chromatography [65].

2.6.2.2 Sample Ionization

Many ionization methods are available. The ionization method to be use should depend on the type of sample under investigation and the mass spectrometers available. Ionization methods include the following: Atmospheric Pressure Chemical Ionization (APCI), Chemical Ionization (CI), Electron Impact (EI), Electrospray Ionization (ESI), Fast Atom Bombardment (FAB), Field Desorption/Field Ionization (FD/FI), Matrix Assisted Laser Desorption Ionization (MALDI), Thermospray Ionization (TSP).

For biological samples to be analyzed by mass spectrometry, it must be ionized by some means, because mass spectrometry relies on the motion of the gas phase ion in a magnetic and/or electric field. Proteins and peptides are nonvolatile substances, and cannot be ionized without prior derivatization by the methods such as electron ionization(EI) and chemical ionization (CI). Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques most commonly used to volatize and ionize the proteins or peptides for mass spectrometric analysis [66]. In both methods, peptides are converted to ions by the addition or loss of one or more protons.

(a) Electrospray Ionization (ESI). ESI is a soft ionization technique for the mass analysis of macromolecules, because it can gently make gas-phase ion without any need for heating, impact with particles like electrons and atoms, or energy irradiation. The ESI source consists of a very fine needle and a series of skimmers. A sample solution is sprayed from the needle at high voltage into a source chamber to form droplets. The droplets carry charge when they exit the needle and, as the solvent evaporates, the droplets disappear leaving only the highly charged ions behind. This process is called nebulization. Electrospray is a desirable ionization method for

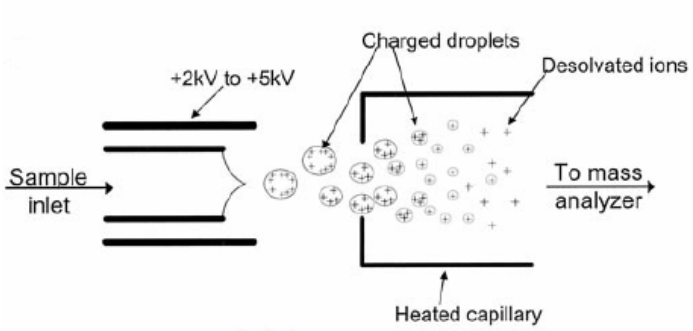

Figure 2.19 The ESI source. The liquid sample exits a capillary on which a voltage is applied. This process ionizes the sample and causes the exiting liquid to form a spray of small droplets.

coupling between liquid chromatography and mass spectrometry, because it easily transforms target molecules in solution to gas-phase ions at atmospheric pressure. An advantage of the ESI process is that it direct by produces both singly and multiply charged ions. This is useful for the mass analysis of macromolecules, such as proteins and peptides. Multiple charging can display very large molecule ions as an m/z ratio on a mass spectrum with a relatively small m/z range, allowing an accurate measurement of the molecular weight.

(b) Matrix-Assisted Laser Desorption/Ionization (MALDI). MALDI is also a soft ionization method like ESI. MALDI is based on the bombardment of sample molecules with a laser light, using a nitrogen laser at 337 nm, to bring about sample ionization. Proteins or peptides are co-crystallized within an organic matrix such as sinapinic acid or α-cyano- 4-hydroxy-cinnamic acid. Co-crystallization is achieved by mixing a solution of the sample analyst with a solution of the matrix. The mixture is then applied to a metal target plate and allowed to dry, resulting in the formation of crystals. A laser beam provides light that is absorbed by the aromatic matrix molecules. Energy is subsequently transferred to analyst that becomes desorbed into gas phase. The MALDI process is tolerant of impurities in the target sample, suh as salts. However, contamination in samples may prevent the generation of homogeneous co-crystals on the target plate and cause peak broadening by adduct

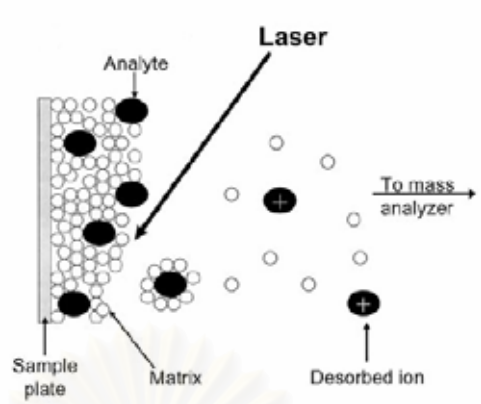

Figure 2.20 The MALDI source. A laser is pulsed at a mixture of sample and matrix molecules that have been co-crystallized.

formation. MALDI is also a soft ionization method and so results predominantly in the generation of singly charged molecular-related ions regardless of the molecular mass, hence the spectra are relatively easy to interpret. In addition, MALDI makes use of pulsed laser irradiation, and is therefore typically connected to a time-of-flight (TOF) mass spectrometer.

2.6.2.3 Mass Analysis

These mass analyzers have different features, including the m/z range that can be covered, the mass accuracy and the achievable resolution. The compatibility at different analyzers with different ionization methods varies.

When ions have been formed in the source, they are transported to the analyzer region and separated according to their mass-to-charge ratio.

(a) Quadrupole mass analyzers. The quadrupole analyzer is constructed of four electronically conducting cylindrical rods and is operated by the application of a combination of direct current (DC) and radio frequency (RF) voltages (Figure 2.19).The mass filter establishes a two-dimensional quadrupole field between the four cylindrical electrodes with the two opposite rods connected electrically. One rod pair (+) is opposite rods connected to a positive DC voltage, upon which a sinusoidal RF voltage is superimposed. The other rod pair (-) is opposite rods connected to a negative

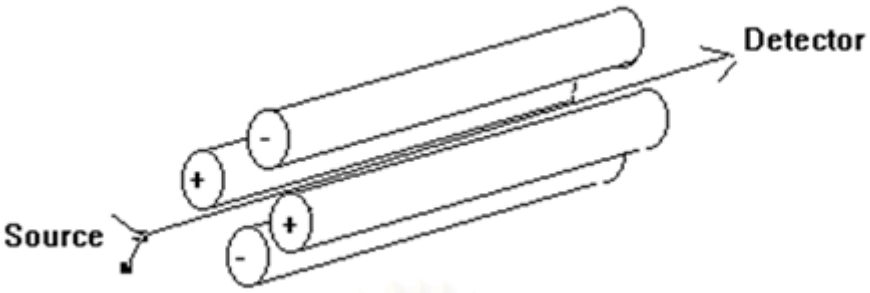

Figure 2.21 Schematic of Quadrupole Analyzer

DC voltage, upon which a sinusoidal RF voltage is superimposed. A quadrupole can act to transmit all ions or as a mass filter to allow the transmission of ions of a certain mass-to-charge (m/z) ratio. The mass filter is a continuous analyzer compared to the TOF analyser that has a pulsed nature. This feature makes the quadrupole highly compatible with continuous infusion sources such as electrospray and liquid separation techniques such as high-performance liquid chromatography (HPLC) and CE [67].

(b) Time of flight (TOF). A TOF instrument is one of the simplest mass analyzers. The principle of the TOF mass analyzer is to measure the flight time of ions accelerated out of an ion source into a field-free drift tube to a detector. The flight time is related to the *m/z* values of the ions according to the following formula:

$$TOF = L\,(2U_{acc}\,e)^{-1/2}\,(m/z)^{1/2}$$

where L is the drift length in the field-free region, U_{acc} is the potential difference in the accelerating region, e is the charge of an electron, m is the mass of the ion and z is its charge state. The TOF is usually measured from the time point at which the ions are accelerated out of the source to the time point when they reach the detector. Heavier ions have a longer flight time to the ion detector. The ions will separate in the TOF mass analyzer according to their *m/z* ratios, light ions arriving at the detector earlier than heavy ions if they carry the same number of charges [67].

(c) Ion trap. An ion trap is composed of a ring electrode and two end-cap electrodes (Figure 2.18). The ions are first captured or trapped for a certain time interval. The *rf* frequencies of the fields are then ramped up to eject ions of increasing *m*/*z*. The ions travel to the detector and, based on the frequency being used at the time

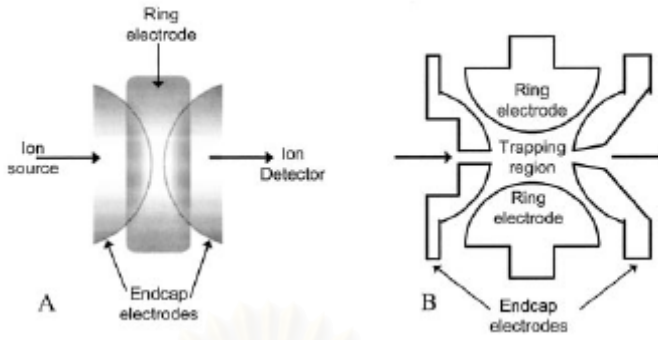

Figure 2.22 Ion trap mass analyzer. *A*;schematic of the external view showing the ring electrode and endcap electrodes. *B*;cross section of an ion trap showing the trapping region.

of detection, the *m*/*z* of the ion can be calculated. The main advantage of an ion trap mass analyzer is the ability to allow ion to be stored and then selectively ejected from the ion trap, increasing sensitivity [68]. However, there is a quantitative limitation for the ion trapping due to the instability of ion vibration caused by the repulsion between ions.

2.6.2.4 Type of Mass Spectrometer

All mass spectrometers measure the mass-to-charge ratio of analysts such as proteins, peptides, or peptide fragment. The names of the various mass spectrometers are derived from the name of their ionization source and the mass analyzer. Some of the most common mass spectrometer is reviewed. The analysis of proteins or peptides by MS can divided in peptide mass analysis and amino acid sequencing. In peptide mass analysis or peptide mass fingerprinting, the masses of individual peptides in a mixture are measured and used to create a mass spectrum. In amino acid sequencing, tandem mass spectrometry is used to fragment a specific peptide into small peptides, which can then be used to deduce amino acid sequence.

2.6.2.4.1 MALDI TOF

The principal application of a MALDI TOF mass spectrometer is peptide mass fingerprinting. The peptides are mixed with the matrix, low molecular weight compounds, which have an absorption maximum at the wavelength of the laser. The three most commonly used are 3,5-dimethoxy-4-hydroxycinamic acid (sinapinic

acid), α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. A matrix is used to protect the samples from being destroyed by direct laser beam and to facilitate vaporization and ionization. The matrix solution is mixed with the analyst such as protein sample. Sample is deposited on a metal substrate capable of holding between one and several hundred analyst spots. These are then irradiated by a laser pulse, to generate a short burst of ions. The ions are accelerated to a fixed amount of kinetic energy and travel down a flight tube. The small ions have a higher velocity and are recorded on a detector before the larger ones, producing the time-of-flight (TOF) spectrum.

2.6.2.4.2 Tandem Mass Spectrometer (MS/MS)

MS/MS are instruments that have more than one analyzer and so can be used for structural and sequences studies. Two, three and four analyzers have all been incorporated into commercially available instruments, and the analyzers do not necessarily have to be of the same type, in which case the instrument is a hybrid one. MS/MS involves multiple steps of mass sector or analysis, usually separated by some of fragment. For example, Triple-quadrupole mass spectrometers are most commonly used to obtain amino acid sequences. A particular peptide ion is selectively passed into the collision chamber. Inside the collision chamber, peptide ions are fragmented by interaction with inert gas by a process known as collision-induced dissociation (CID) or collisionally activated dissociation (CAD). The peptide ion fragments are resolved on the basis of their m/z ratio by third quadrupole.

MS/MS allows direct analysis of protein mixtures. Crude protein mixtures can be reduced to peptides, and the peptide fragmented and searched in database. In this way, large numbers of proteins, up to hundreds, can be identified all at once.

2.6.3 Peptide Sequencing by Tandem Mass Spectrometry

One peptide species out of a mixture is selected in the first mass spectrometers and is then dissociationed by collision with an inert gas, such as argon or nitrogen. The resulting fragments are separated in the second part of the tandem mass spectrometer [69]. Since peptides can undergo multiple types of fragmentation, nomenclature has been created to indicate what types of ion have been generated. These are three different types of bonds that can fragment along the amino acid

backbone: the NH-CH, CH-CO, and CO-NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. The charge can stay on either of the two fragments depending on the chemistry and relative proton affinity of the two species. Hence there are six possible fragment ions for each amino acid residue and these are labeled as in the Figure 2.21, with the a, b, and c ions having the charge retained on the N-terminal fragment, and the x, y, and z ions having the charge retained on the C-terminal fragment. The most common cleavage sites are at the CO-NH bonds which give rise to the b and the y ions. The mass difference between two adjacent b ions, or y ions, is indicative of a particular amino acid residue (see Table 2.8 residue masses of the amino acids).

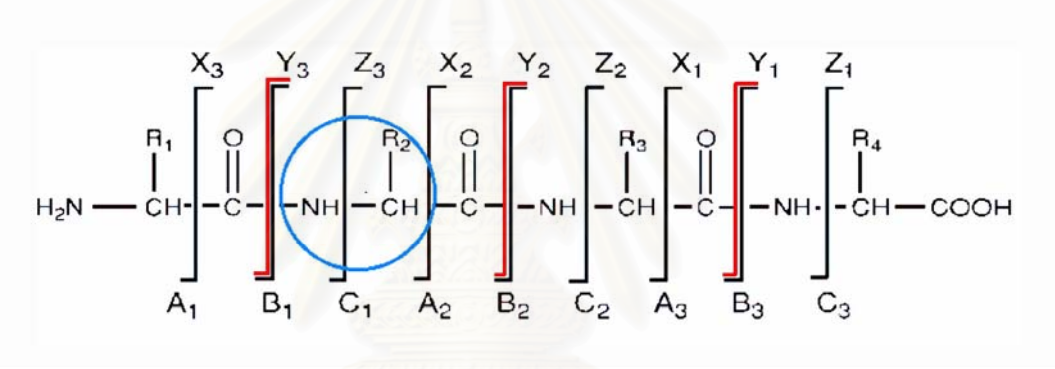

Figure 2. 23 Peptide ion fragmentation nomenclature.

Most peptide sequencing is performed on electrosprayed ions. These ions generally have a charge state corresponding to the number of positively charged amino acids plus the charge formally localized at the N terminus of peptide. Large peptides are multiply charged, and their fragmentation spectra often contain multiply charged ion series as well. Tandem mass spectra are usually interpreted with computer assistance or matched against databases directly. In very high quality spectra it is possible to interpret the fragmentation ladders (the b and the y ion series) from the low mass end through to the highest mass ion.

Table 2. 7 Residue masses of the amino acids. The residue masses of the 20 genetically encoded amino acids and selected modified amino acids.

Amino acid	One-letter code	Residue mass (Da)	Immonium ion (m/z)
Glycine	G	57.02	30
Alanine	A	71.04	44
Serine	S	87.03	60
Proline	P	97.05	70
Valine	V	99.07	72
Threonine	T	101.05	74
Cystine	C	103.01	76
Leucine	L	113.08	86
Isoleucine	I	113.08	86
Asparagine	N	114.04	87
Aspatate	D	115.03	88
Glutamine	Q	128.06	101
Lysine	K	128.09	101
Glutamate	E	129.04	102
Methionine	M	131.04	104
Histidine	H	137.06	110
Oxidized Methionine	Mo	147.04	120
Phenylanine	F	147.07	120
Arginine	R	156.10	129
Carbamidomethylcystenine	C	160.03	133
Tyrosine	Y	163.06	136
Acrylocysteine	C	174.04	147
Tryptophan	W	186.08	159

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2.6.4 Database Utilization

Information of proteins or peptides from Edman sequencing or MS are used for protein identification by database searching. The goal of database searching is to be able to quickly and accurately identify large numbers of proteins.

2.6.4.1 Peptide Mass Fingerprinting Database Searching

Peptide mass fingerprinting is usually performed using a MALDI TOF. In this method, the masses of peptides obtained from the proteolytic digestion of an unknown protein are compared to the predict masses of peptides from the theoretical digestion of proteins in database. If enough peptides from the real mass spectrum and the theoretical one overlap, protein identification can be made. The proteins can be ranked according to the number of peptide matches.

Generally, peptide mass fingerprinting is used for the rapid identification of single protein component. The analysis and database search can be fully automated. Protein identification via database searching is facilitated by accurate m/z values of the digest fragments, the specificity of the enzyme used, and accurate m/z of the intact protein, Entire scientific conferences have been devoted to this approach which has been coined “proteomics.” Table 2.7 previews some of the protein database available on the internet [70].

2.6.4.2 Amino Acid Sequence Database Searching

If the amino acid sequence of a peptide can be identified, it can be used to search database to search databases to find the protein from which it was derived. A partial amino acid sequence is obtained by interpretation of the MS/MS spectrum and this information combined with the mass of the peptide and the masses of the peptide on either side of the sequence tag where the sequence is not known. Also included in the search is the type of protease used to produce the peptides.

Table 2. 8 Protein databases available on the internet

NCBInr	A nonredundant database compiled by the NCBI by combining most of the public domain database (ESTs not included).
Swiss Prot	A curted protein sequence database which strives to provide a high level of annotation, such as the description of the function of a protein, its domain structure, post-translational modifications, variants, etc. This database offers a minimal level of redundancy and high level of integration with other databases.
OWL	A nonredundant composite of four publicly available primary source: SWISSPROT, PIR, GenBank (translation) and NRL-3D.
Genpept	Protein translation of Genbank (ESTs not included).
Unknome	A theoretical database use in de novo MS/MS spectral interpretation that is created on-the-fly and contains all amino acid sequence permutations consistent with the parent mass and amino acid composition information contained in an MS/MS spectrum.

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CHAPTER III

EXPERIMENTAL

3.1 Material

3.1.1 Plant Material

Thai bitter gourd, fresh green fruits was obtained from vegetable markets in Bangkok, Thailand.

3.1.2 Erythrocytes

Rabbit blood cells were obtained from the faculty of Veterinary medicine of Chulalongkorn University of Thailand.

3.1.3 Chemicals

Potassium hydrogen phosphate (KH_2PO_4), Disodium hydrogen phosphate ($Na_2H_2PO_4$), Sodium chloride (NaCl) and Ammonium sulphate ($(NH_4)_2SO_4$) used in extraction and precipitation step were obtained from Merck (Germany). EDTA was purchased from Fluka (Germany). Media for anion exchange resin, DEAE-cellulose, was the product of Amersham Pharmacia Biotech. For positive test control of Hemagglutinating activity, Concanavalin A (Con A) lectin, substrate and enzyme used in α-glucosidase inhibitiory activity, *p*-nitrophenyl-α-D-glucopyranoside(PNPG) and α-Glucoside, respectively were purchased from Sigma (St. Louis, MO, USA). Solvent use for in-solution digestion and preparation for mass spectrometric techniques were analytical grades obtained from Labscan (Bangkok, Thailand) and Amersham Pharmacia Biotech (Sweden).

3.1.4 Apparatus and Instruments

Column Chromatography, the protein samples were run on AKTA explorer (Amersham pharmacia biotech wikstroms, Sweden) with COLUMN XK 2.6×15 cm (Amersham pharmacia biotech wikstroms, Sweden) which the buffer system in this technique was adjust pH with pH meter (Denver Instrument USA. system). Dialysis bag (SnakeSkin Dialysis Tubing, Pierce, USA.) was used for protein desalting. To concentrate protein, Speed vacuum (MAXI dry plus, Heto vacuum centrifuge,

Denmark) and Freeze-dryer have been used. Microtiter plate reader was used in measurement absorbance of protein quantities, Bradford test, and α-glucosidase inhibitory activity. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometer (BIFLEX Bruker, Germany) was used to analyze protein mass spectra. The other apparatus and instruments used in this research were Siliconized eppendorf (Axygen sciencetific Inc.), Pipette tips (Bioline USA.), Micropipette (Pipetteman, Gilson, France.), Vortex mixer (Vortex-genie2, Sciencetific Industries.), Sonicate (DHA-100; Branson, U.S.A.), Water Bath Shaking (Memmert, Germany), and Orbital Shaker (Kika-Werke GMBH&Co., Germany).

3.2 Methods of Protein Purification

3.2.1 Protein Extraction and Precipitation

All extraction and precipitation steps were carried at 4°C, *Momordica charantia*, fresh green fruits were thoroughly washed in tap water, cut, and the seed were removed manually. Then 4.7 kg of seedless was homogenized with 14.1 liter ice-cold 50mM phosphate buffer (pH 7.2) containing 0.15M NaCl and 1mM EDTA. The homogenate was extracted with stirring overnight at 4°C. The light green homogenate was filtered through three-layer of cheesecloth to remove insoluble residue. The solution was then centrifuged at 10,000 rpm for 15 minutes. The supernatant was filtered through filter paper. The protein filtrate was fractionated by ammonium sulfate precipitated at 0-20 %, 20-40%, 40-60%, 60-80%, and 80-100% of ammonium sulfate saturation (see Table 1A, APPENDIX A) and the fractions were designated as crude protein P20, P40, P60, P80 and P100 respectively. To removed precipitated protein by centrifugation at each step before increasing the ammonium sulfate concentration. The recovered protein pellets (0-20%, 20-40%, 40-60%, 60-80% and 80-100%) were re-suspend in 50mM phosphate buffer (pH 7.2) containing 0.15M NaCl. The crude proteins were dialysis with 50mM phosphate buffer (pH 7.2) for further purified using anion exchange column chromatography.

3.2.2 Determination of Protein Concentration [58]

Protein concentrations were determined using the Bradford reagent. For the calibration curve, bovine serum albumin (BSA) was used protein standard solution.

This research used microassay method that adapted for use with a microplate. The protein sample was pipetted 10 μl into each well and then added 200 μl of the Bradford working solution (see Table 2A, APPENDIX A). After adding the protein reagent, the absorbance of each sample measure between 2-60 minutes at wavelength 595 nm.

3.2.3 Column Chromatography

Crude protein was loaded onto a column of DEAE-cellulose (2.6 x 15 cm) column equilibrated with a solution of buffer A (50 mM Phosphate Buffer pH 7.2). The column was washed with the same solution to remove impurities, and a fraction of 10 ml was collected at flow rate of 1 ml/min. Elution buffer consists buffer A that containing 1 M NaCl (buffer B). The column was eluted by stepwise gradient of NaCl at 5%, 10%, 20%, 30%, 50% and 100% of buffer B. All procedures were carried out at 4°C. The protein absorption was monitored at 280 nm. All fractions were determined total proteins and dialysised with H_2O and lyophilized.

3.3 Biological Activity Testing

3.3.1 Assay for Hemagglutinating and Hemolytic Activities [71]

The fractions from anion exchange chromatography were tested for hemagglutinating activity, a serial twofold dilution of the protein solution in microtiter U-plates (50 ml) was mixed with 50 ml of a 2% suspension of rabbit erythrocytes in phosphate-buffered saline (pH 7.2) at room temperature. The results were about 1 h when the negative control had sedimented. Hemolysis was indicated by a change in the appearance of the erythrocyte suspension from turbidity to clearness. Un-agglutinated erythrocytes formed a clear dot whereas agglutinated erythrocytes formed a diffuse mat at the bottom of the well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination unit per mg protein. Bovine serum albumin (BSA) with a concentration of 3 mg/ml was used as the negative control

3.3.2 α-Glucosidase Inhibitory Activity [72]

α-Glucosidase and *p*-nitrophenyl -α-D-glucopyranoside (PNPG) were assayed using 50 mM phosphate buffer at pH 6.7. 10 μl of protein sample at the designated concentration was premixed with 40 μl of enzyme solution (1U/ml) and incubated at 37°C for 10 min. 950 μl of 1mM PNPG as a substrate was then added to the mixture to initiate the enzyme reaction. The reaction was incubated at 37°C for 20 min and stopped by adding 1 ml of 1 M Na_2CO_3. α-Glucosidase inhibitory activity was determined by measuring release of the yellow *p*-nitrophenol at 400 nm. Calculated % α-glucosidase inhibition follow formula below :

$$\%\text{inhibition} = 100 - \left[\left(\frac{\text{Abs(sample)} - \text{Abs(blank)}}{\text{Abs(control)}} \right) \times 100 \right]$$

3.4 Method of Protein Identification [64]

3.4.1 In-Solution Digestion

1 mg of total protein sample was resuspended in 100 μl 6 M urea, 100 mM tris buffer, in a 1.5 ml eppendroff tube. Then the 5 ul of the reducing reagent was added and allowed to stand for 1 hour at room temperature. 20 μl of the alkylating reagent was mixed and allowed to stand at room temperature for 1 hour. After that, 20 μl of the reducing agent was added. The sample was mixed by gentle vortex and allowed the reaction to stand at room temperature for 1 hour. The urea was reduced concentration by diluting the reaction mixture with 775 μl of H_2O. The 100 μl trypsin solution containing 20 μg of trypsin was added and carried out the digestion overnight at 37°C. After overnight, the trypsin reaction was stopped by adding 20 μl of 2% TFA. These solutions were kept at 20°C for further analysis (see APPENDIX B for reagents).

3.4.2 Desalting Peptide

For desalting of small volumes of peptides solutions prior to MALDI mass spectrometry C18 ZipTips (Millipore) were employed. C18 ZipTips was wet by drawing 10 μl of 50% acetronitrile into the tip. This process was repeated with another 10 μl aliquot of 50% acetronitrile. Then, the tip was equilibrated with 10 μl of

0.1% trifluoroacetic acid three times. The peptides sample was loaded onto the ZipTip, washed with 10 μl of 0.1% trifluoroacetic acid three times and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid

3.4.3 Sample Preparation for MALDI-TOF

The dried sample was dissolved in 50 % ACN, 0.1% TFA and vortexed. For peptide mixtures, 2 μl of sample was mixed with 2 μl of CCA solution (10mg α-cyano-4-hydroxy-cinnamic acid, 50%ACN/0.1%TFA) in eppendorf 0.5 ml. Then, 0.5 ul of the mixture solution was spotted on MALDI target plate and allowed to dry at room temperture. Myoglobin (1mg/ml, average mass 16,951 Da), Angiotensin II (1mg/ml, average mass 2,465.1983), and Bovine serum albumin (1mg/ml, average mass 66,433.96 Da) were used as external calibration for peptide and protein molecular mass, respectively.

3.4.4 Protein Identification by Database Searching

Peptide mass spectra and amino acid sequence were acquired using MALDI-TOF MS. Peptide mass fingerprint (PMF) data, obtained from protein digestion, were used to search in databases NCBInr.20030905 for viridiplantae using Mascot program (http://www.matrixscience.com/cgi/index.pl?page=../home.html). The peptide mass fingerprinting of the proteins were scored with the Mowse score.

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CHAPTER IV

RESULTS AND DISCUSSION

The proteins from fruit pulp of *Momordica charantia* Linn. were analyzed by using all methods that described in the previous chapter. The results have been shown and discussed in each part of this chapter, respectively.

4.1 Extraction of Crude Protein from Fruit Pulp of *Momordica charantia* Linn.

Crude proteins from fruit pulp of *Momordica charantia* were extracted and precipitated by 0-20 %, 20-40%, 40-60%, 60-80%, and 80-100% of ammonium sulfate saturation. Crude proteins were designed to be P20, P40, P60, P80 and P100 respectively. The advantage of this precipitated, the proteins were not denatured that can be further tested biological activity. Crude protein yields at different stage of protein extraction and precipitation process are shown in Table 4.1. From this table, the amount of protein is highest when precipitate protein with 40-60 % ammonium sulfate saturation (crude protein P60) and lowest when precipitate protein with 80-100 % ammonium sulfate saturation (crude protein P100).

Table 4.1 The amount of proteins from precipitate fraction of 4.7 kg fruit pulp of *Momordica charantia* Linn.

Crude Protein	% $(NH_4)_2SO_4$ saturated	Volume (ml)	Conc. (mg/ml)	Total Protein (mg)	Protein Yield (%)*
P20	0 - 20	1470	0.36	529.20	23.19
P40	20 - 40	1420	0.45	639.00	28.00
P60	40 - 60	2340	0.34	795.60	34.86
P80	60 - 80	1350	0.20	270.00	11.83
P100	80 - 100	1620	0.03	48.60	2.13

Note. ** calculated from extractable proteins*

4.2 Separation and Identification of *Momordica charantia* Proteins

Crude proteins were loaded on the DEAE-cellulose column and eluted at 5%, 10%, 20%, 30%, 50% and 100% of 50 mM PB, 1 M NaCl, pH 7.2(buffer B). All fractions were collected and desalted by dialysis with H_2O and then lyophilized.

Crude proteins and all fractions were tested to check the biological activity. Two activities were performed, hemagglutination and α-glucosidase inhibition activity. All bioactivity was tested two times.

All fractions that have bioactivity were subject to trypsin digestion (in-solution digestion). The advantage of in-solution digestion, the digest that is prepared will contain peptides from all of the proteins in the mixture. Another advantage is an enhancement of the detectability of low-abundance component of the mixture [64]. The peptides were analyzed by MALDI-TOF MS. For identification of proteins, the peptide mass mapping data were used to search in database NCBInr.20070709 for viriplantae using Mascot program (http://www.matrixscience.com) by setting the following criteria: Mass tolerance is 1 Da, and maximum number of missed tryptic cleavages is 1. The modification parameters were oxidation of Met and modification of Cys. The separation and identification of *Momordica charantia* is outlined in Figure 4.1.

4.2.1 Crude Protein P20

Crude Protein P20 was loaded on DEAE-cellulose column (Figure 4.2). There were seven peaks which were designated protein 20U(unbound peak), 20B5, 20B10, 20B20, 20B30, 20B50, and 20B100. Table 4.2 shows % yield of each protein. The major protein of crude protein P20 is protein 20B30 (22.10 % yield). Proteins were dialysised with H_2O and lyophilized. And then, all proteins powder was tested bioactivities (the hemagglutination activity and α-glucosidase inhibition) (see Table 4.3). Crude protein P20 and all its proteins have no agglutination activity. Hemolysis activity shows in crude protein P20 and protein 20U. α-Glucosidase inhibition found in the protein 20U. The IC_{50} for α-glucosidase inhibition of protein 20U was found to be 6.31 mg/ml. Protein 20U was chosen to analyze by MALDI-TOF MS because protein 20U present α-glucosidase inhibition. The mass spectra are shown in Figure 4.3. And then, the mass spectra were used for performing of protein peptide mass mapping via Mascot program. The search results was shown in Table 4.4. Highest identities of protein 20U was obtained against NUMOD3, motif family protein from *Oryza sativa* (japonica cultiva-group), with 124 mowse score. Matched peptides shown in bold black in Figure 1C, Appendix C.

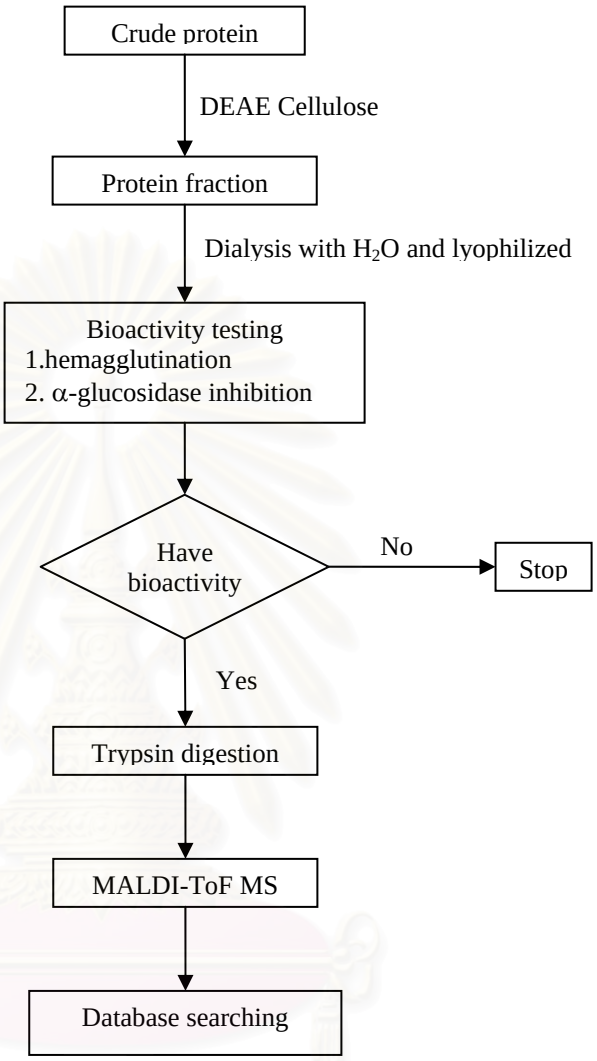

Figure 4.1 Separation and identification of protein from fruit pulp of *Momordica charantia*.

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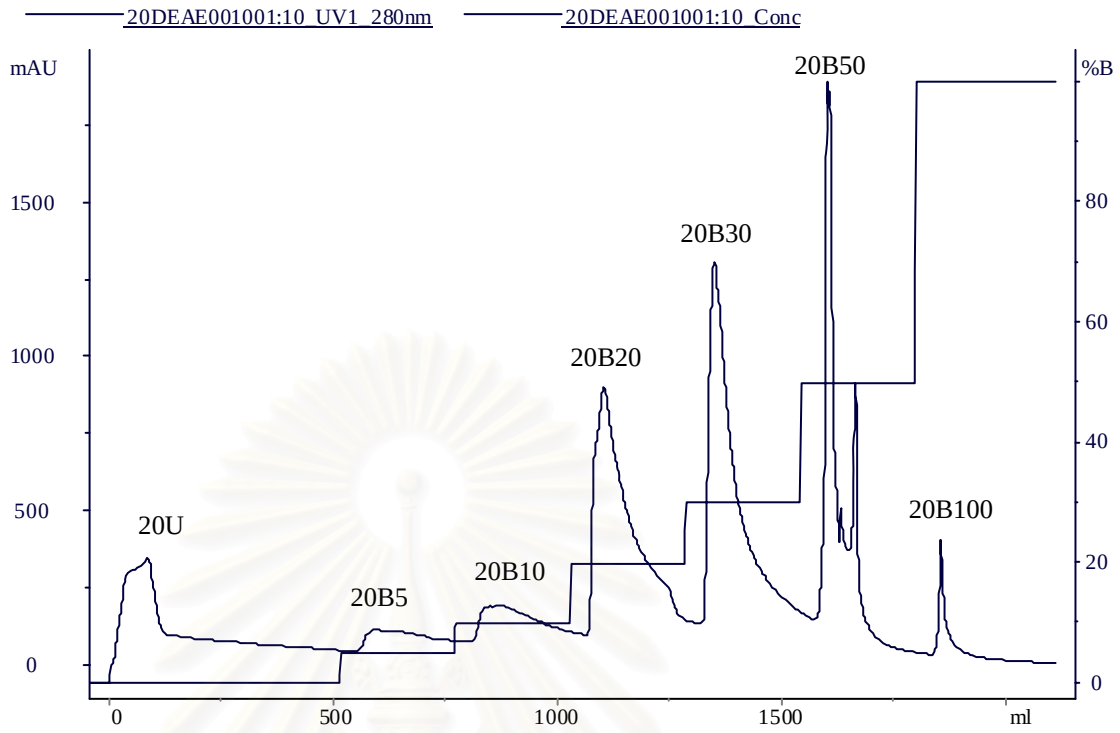

Figure 4.2 Anion exchange purification of crude protein P20. Stepwise gradient of NaCl at 5%, 10%, 20%, 30%, 50%, and 100 % of buffer B. The percent of buffer B is shown by dotted line. Flow rate 1.0 ml/min.

Table 4.2 Protein yield which separated from crude protein P20

Crude Protein (mg of loaded)	Buffer B (%)	Protein	Total Volume (ml)	Total Protein (mg)	Protein Yield (%)*
P20 (195.50 mg)	0	20U	500	30.00	15.35
	5	20B5	160	1.60	0.82
	10	20B10	260	7.80	3.99
	20	20B20	220	35.20	18.01
	30	20B30	240	43.20	22.10
	50	20B50	120	19.20	9.82
	100	20B100	20	0.20	0.10

Note. ** calculated from loaded crude protein*

Table 4.3 Bioactivity testing of crude protein P20 and protein which separated from crude protein P20

Fraction	Specific hemagglutinating activity (U/mg protein)	IC_{50} of % α-glucosidase inhibition (mg/ml)
P20	-*	-
20U	-*	6.31
20B5	-	-
20B10	-	-
20B20	-	-
20B30	-	-
20B50	-	-
20B100	-	-

Note. *-*; no agglutinate but hemolysis, - ; no agglutinate or no* α-glucosidase inhibition

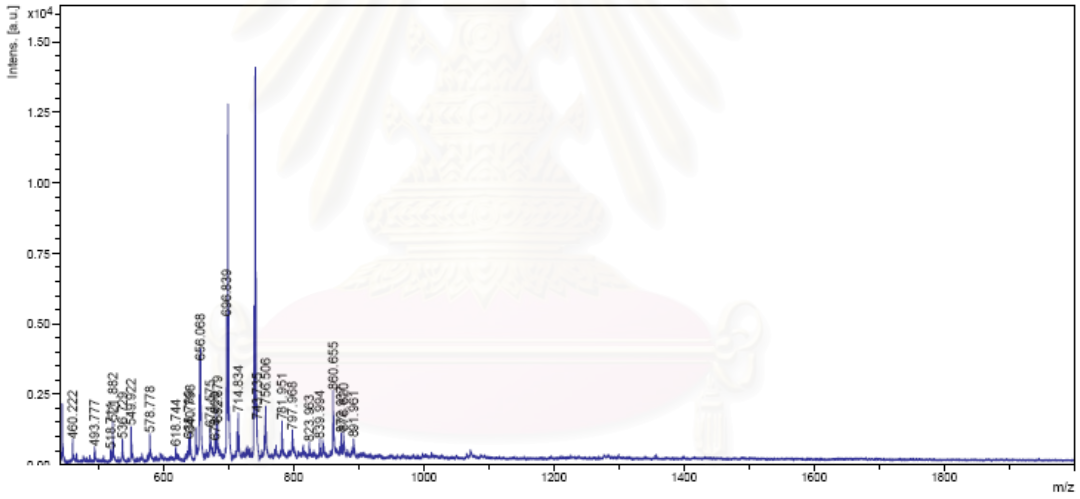

Figure 4.3 MALDI-MS spectrum of trypic fragment of protein 20U

Table 4. 4 The search result of protein 20U

Protein	MASCOT results			
	Mowse Score	Mass (Da)	Protein name	Organism
20U	124	47548	NUMOD3 motif family protein	*Oryza sativa* (japonica cultiva-group)

4.2.2 Crude Protein P40

Crude Protein P40 was loaded on DEAE-cellulose column (Figure 4.4). There were seven peaks which were designated protein 40U(unbound peak), 40B5, 40B10, 40B20, 40B30, 40B50 and 40B100. Table 4.5 shows % yield of each protein. The major protein of crude protein P40 is protein 40B20 (19.56 % yield). Proteins were dialysised with H_2O and lyophilized. And then, all protein powders were tested bioactivities (the hemagglutination activity and α-glucosidase inhibition) (see Table 4.6). Protein 40U, 40B5, 40B10, 40B20, 40B30, 40B50 and 40B100 showed agglutination activity. Three order of highest specific hemagglutinating activity are protein 40B5, 40B20, 40B50 and 40B100 were found to be 400.00, 363.64, 363.64 and 333.33 U/mg protein, respectively.α-Glucosidase inhibition found in crude protein P40, protein 40B5 and 40B50. The IC_{50} for these compounds were found to be 4.15, 1.20 and 9.13 mg/ml, respectively. Protein 40U, 40B5, 40B10, 40B20, 40B30, 40B50 and 40B100 which have bioactivity were chosen to analyze by MALDI-TOF MS. The mass spectra are shown in Figure 4.5 – 4.11 respectively. And then, the mass spectra were used for performing of protein peptide mass mapping via Mascot program. The search results were shown in Table 4.7 and matched peptides shown in bold black in Figure 2C-8C, Appendix C.

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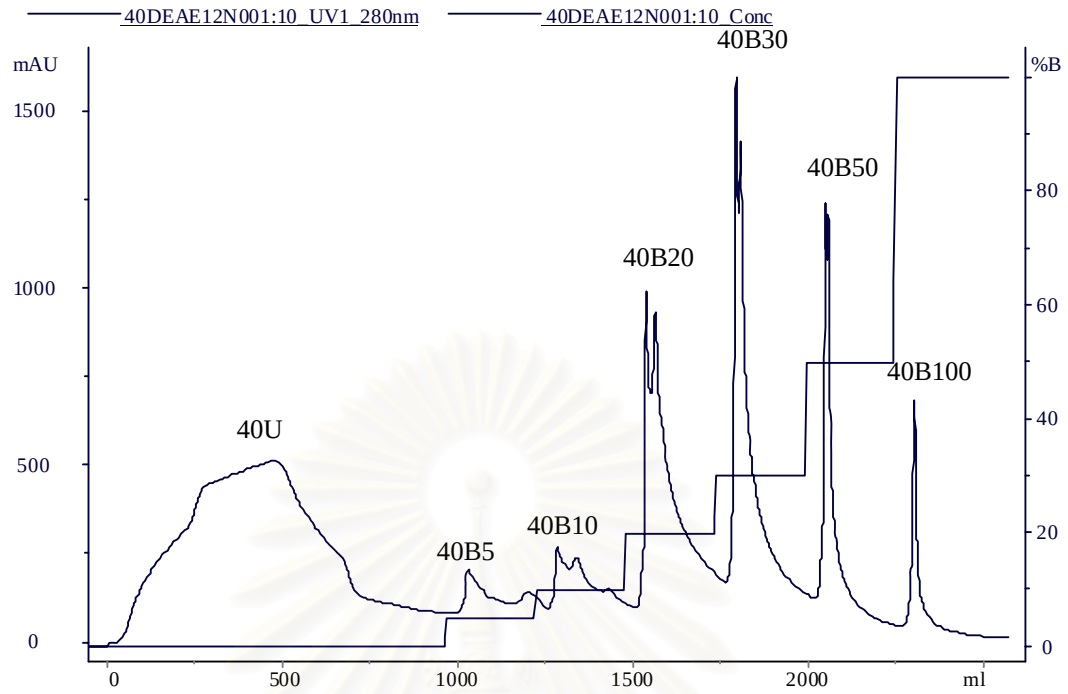

Figure 4.4 Anion exchange purification of crude protein P40. Stepwise gradient of NaCl, 5%, 10%, 20%, 30%, 50%, and 100 % of buffer B. The percent of buffer B is shown by dotted line. Flow rate 1.0 ml/min.

Table 4.5 Protein yield which separated from crude protein P40

Crude Protein (mg of loaded)	Buffer B (%)	Protein	Total Volume (ml)	Total Protein (mg)	Protein Yield (%)*
P40 (180.00 mg)	0	40U	500	20.00	11.11
	5	40B5	260	5.20	2.89
	10	40B10	240	14.40	8.00
	20	40B20	220	35.20	19.56
	30	40B30	160	30.40	16.89
	50	40B50	80	8.80	4.89
	100	40B100	60	0.60	0.33

Note. ** calculated from loaded crude protein*

Table 4.6 Bioactivity testing of crude protein P40 and protein which separated from crude protein P40

Protein	Specific hemagglutinating activity (U/mg protein)	IC_{50} of % α-glucosidase inhibition (mg/ml)
P40	-	4.15
40U	90.91	-
40B5	400.00	1.20
40B10	25.00	-
40B20	363.64	-
40B30	90.91	-
40B50	363.64	9.13
40B100	333.33	-

Note. *- ; no agglutinate or no* α-glucosidase inhibition

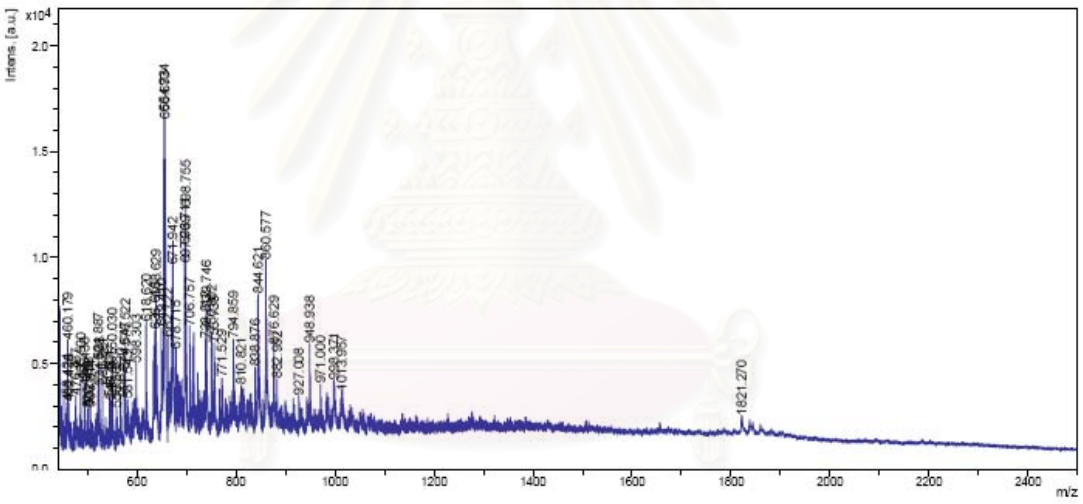

Figure 4.5 MALDI-MS spectrum of trypic fragment of protein 40U

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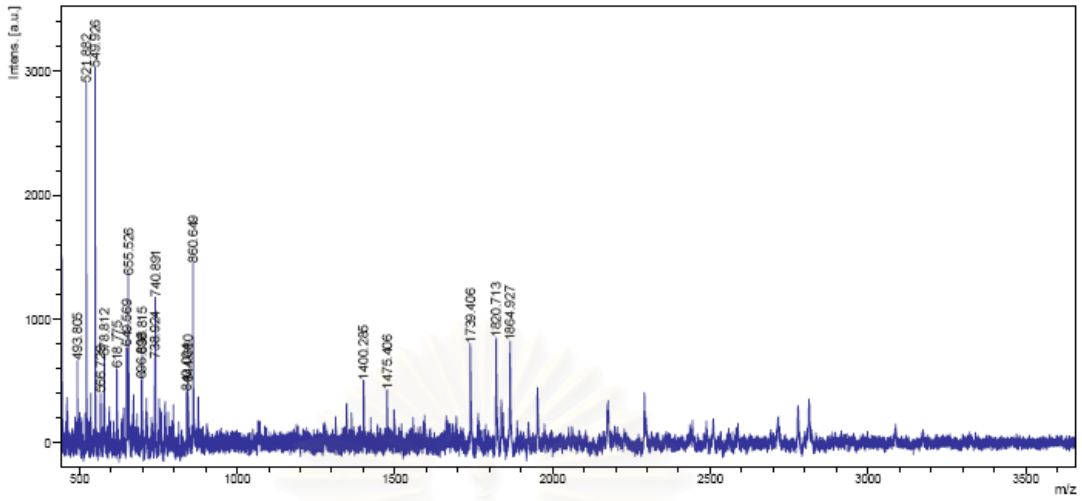

Figure 4.6 MALDI-MS spectrum of trypic fragment of protein 40B5

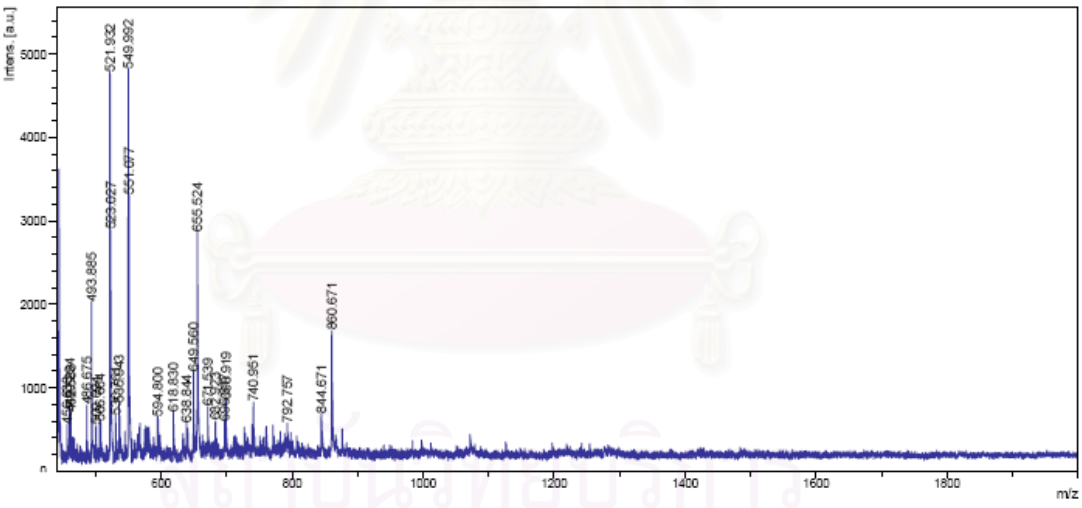

Figure 4.7 MALDI-MS spectrum of trypic fragment of protein 40B10

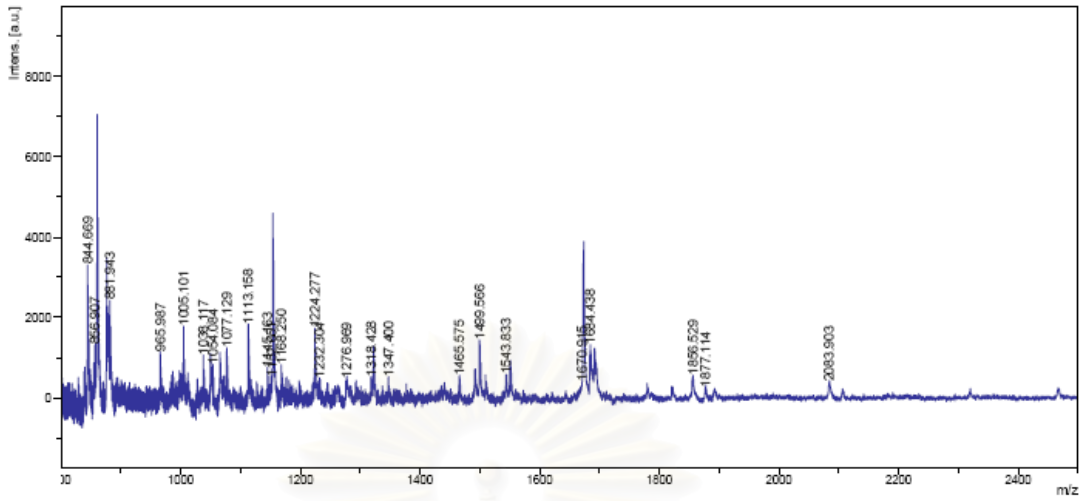

Figure 4.8 MALDI-MS spectrum of trypic fragment of protein 40B20

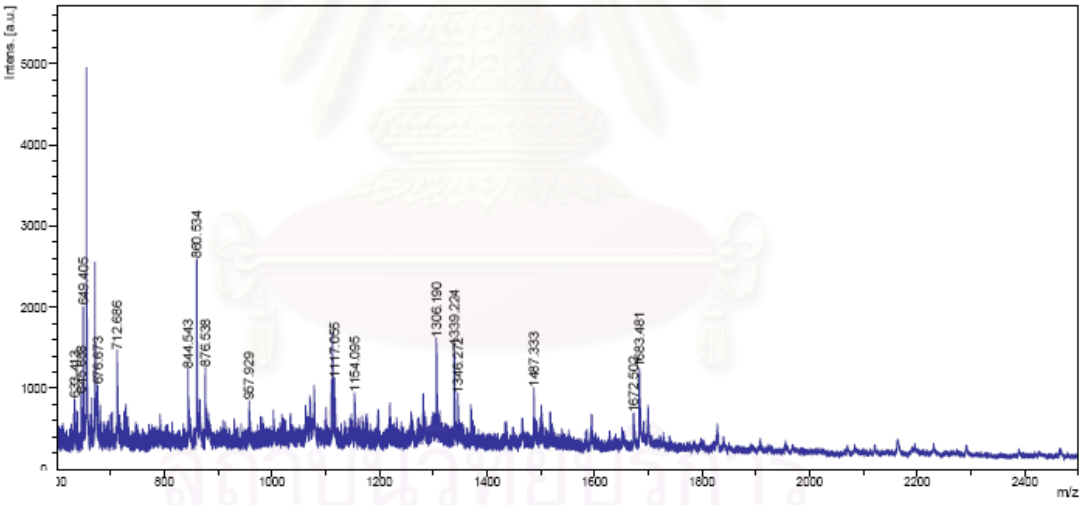

Figure 4.9 MALDI-MS spectrum of trypic fragment of protein 40B30

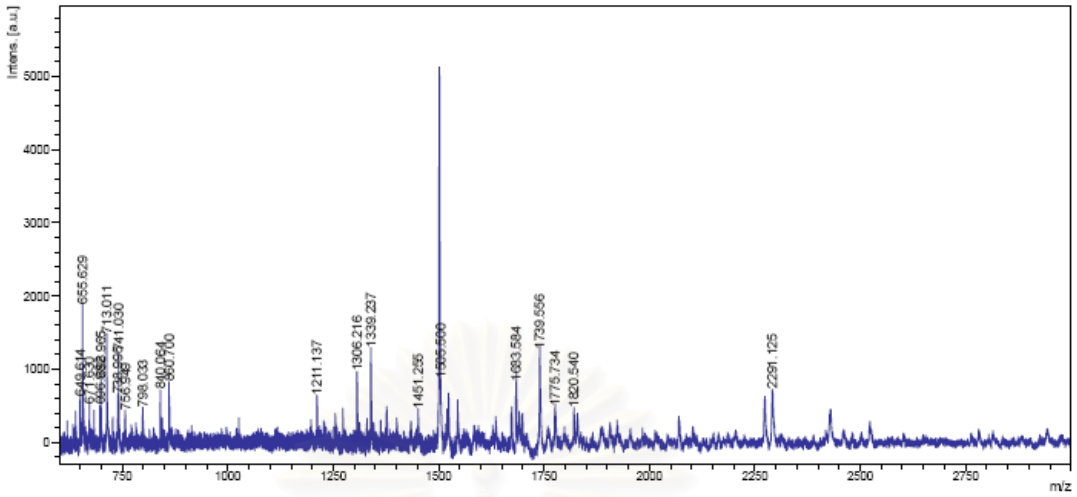

Figure 4.10 MALDI-MS spectrum of trypic fragment of protein 40B50

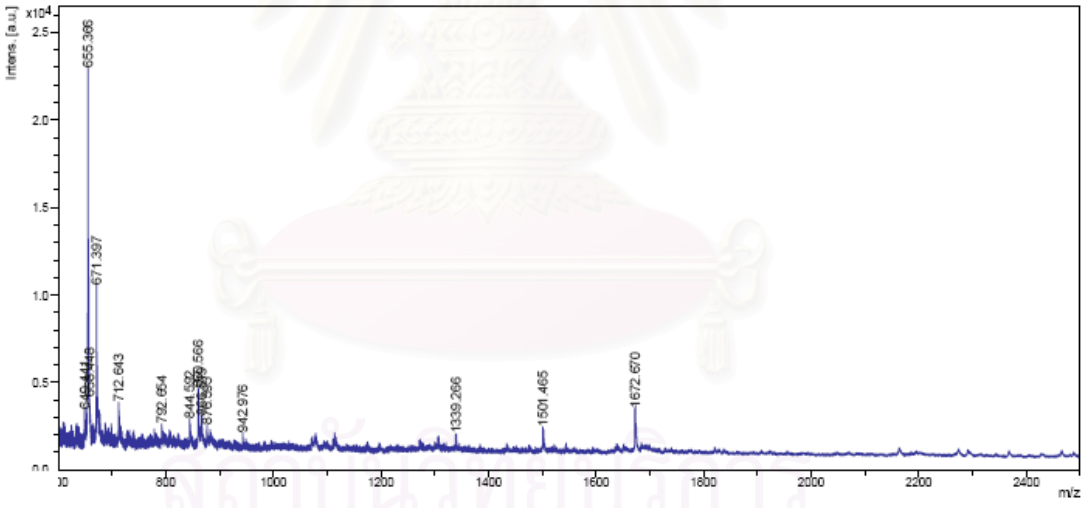

Figure 4.11 MALDI-MS spectrum of trypic fragment of protein 40B100

Table 4. 7 The search result of protein 40U, 40B5, 40B10, 40B20, 40B30, 40B50 and 40B100

Protein	MASCOT results			
	Mowse Score	**Mass (Da)**	**Protein name**	**Organism**
40U	101	61597	muturase K	*Quesnelia edmundoi*
40B5	73	39692	OSJNBa0084N21.4	*Oryza sativa* (japonica cultiva-group)
40B10	85	18925	hypothetical protein	*Vitis vinifera*
40B20	101	19370	beta-fructofuranosidase	*Hamamelis virginiana*
40B30	76	66562	Lignostilbene-alpha beta-dioxygenase (ISS)	*Ostreococcus tauri*
40B50	73	42329	Alcohol dehydrogenase superfamily, zinc-containing	*Medicago truncatula*
40B100	47	44390	TNP2-like protein	*Oryza sativa* (japonica cultiva-group)

4.2.3 Crude Protein P60

Crude Protein P60 was loaded on DEAE-cellulose column (Figure 4.12). There were seven peaks which were designated protein 60U (unbound peak), 60B5, 60B10, 60B20, 60B30, 60B50 and 60B100. Table 4.8 shows % yield of each protein. The major protein of crude protein P60 is protein 60U (15.87 % yield). Proteins were dialysised with H_2O and lyophilized. And then, all protein powders were tested bioactivities (the hemagglutination activity and α-glucosidase inhibition) (see Table 4.9). α-Glucosidase inhibition found in crude protein P60 and protein 60U. The IC_{50} of crude protein P60 and protein 60U were 0.82 and 4.14 mg/ml, respectively. Crude preparations (P60) can be considered among the most powerful α-glucosidase inhibitor than protein 60U. This suggests that, the inhibitor activity may need group of proteins. Protein 60U was chosen to analyze by MALDI-TOF MS. The mass spectra are shown in Figure 4.13. The mass spectra were used for performing of protein peptide mass mapping via Mascot program. The search results were shown in Table 4.10. A highest identity of protein 60U was obtained against

galactosyltransferase family protein from *Arabidopsis thaliana*, with 81 mowse score. Matched peptides shown in bold black in Figure 9C, Appendix C.

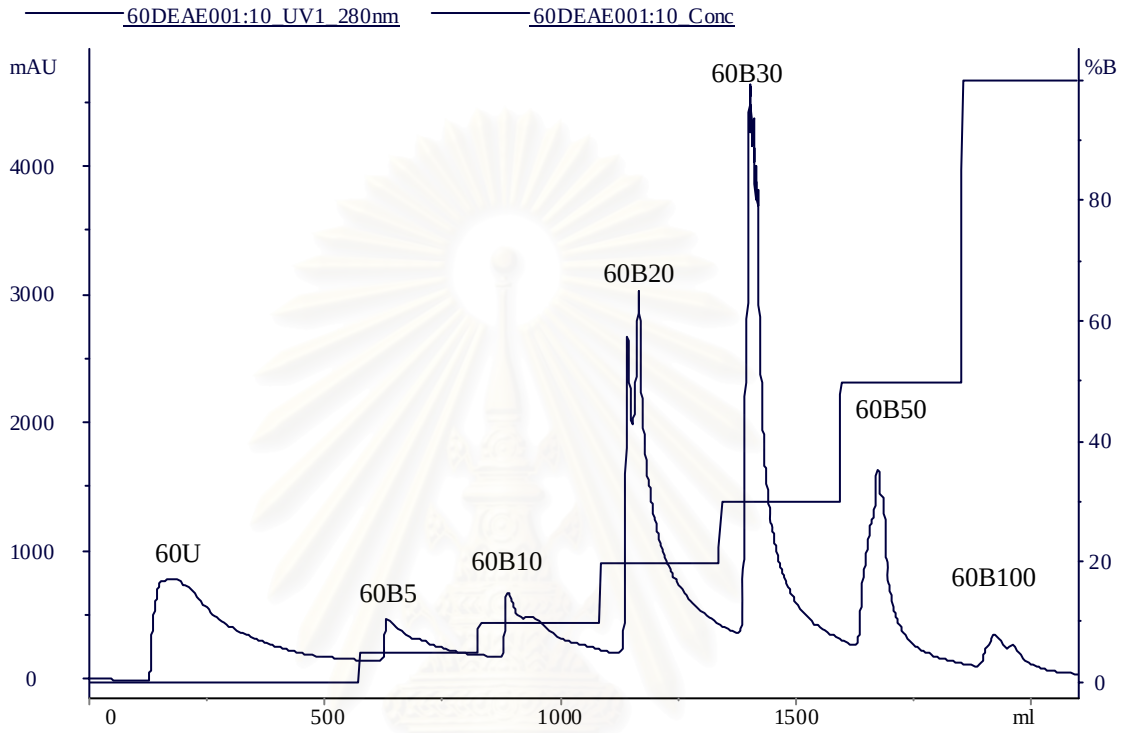

Figure 4.12 Anion exchange purification of crude protein P60. Stepwise gradient of NaCl, 5%, 10%, 20%, 30%, 50%, and 100 % of buffer B. The percent of buffer B is shown by dotted line. Flow rate 1.0 ml/min.

Table 4.8 Protein yield which separated from crude protein P60

Crude Protein (mg of loaded)	Buffer B (%)	Protein	Total Volume (ml)	Total Protein (mg)	Protein Yield (%)*
P60 (504.00 mg)	0	60U	500	80.00	15.87
	5	60B5	140	21.00	4.17
	10	60B10	90	16.20	3.21
	20	60B20	140	39.20	7.78
	30	60B30	210	67.20	13.33
	50	60B50	130	22.10	4.38
	100	60B100	120	1.20	0.24

Note. ** calculated from loaded crude protein*

Table 4.9 Bioactivity testing of crude protein P60 and protein which separated from crude protein P60

Protein	Specific hemagglutinating activity (U/mg protein)	IC_{50} of % α-glucosidase inhibition (mg/ml)
P60	-	0.82
60U	-	4.14
60B5	-	-
60B10	-	-
60B20	-	-
60B30	-	-
60B50	-	-
60B100	-	-

Note. *- ; no agglutinate or no* α-glucosidase inhibition

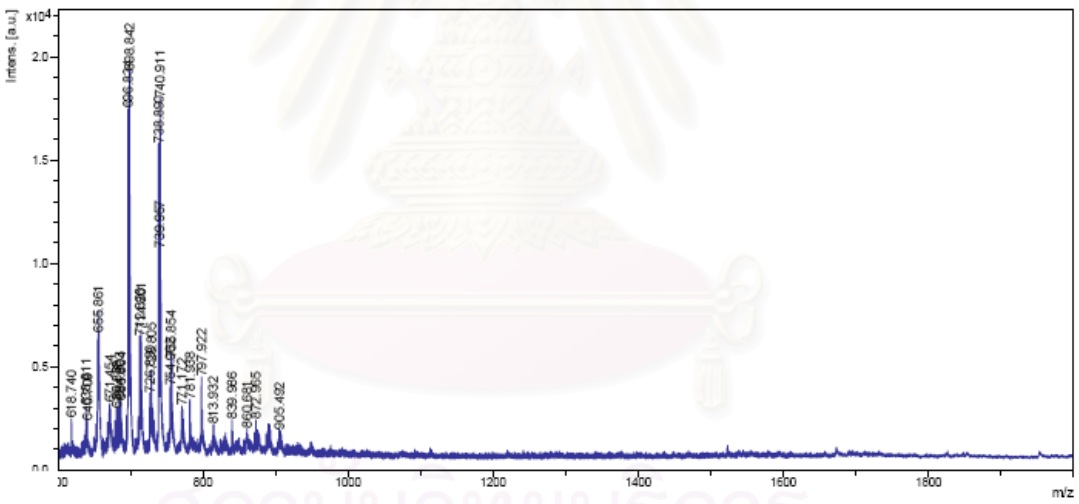

Figure 4.13 MALDI-MS spectrum of trypic fragment of protein 60U

Table 4. 10 The search result of protein 60U

Protein	MASCOT results			
	Mowse Score	Mass (Da)	Protein name	Organism
60U	81	39096	galactosyltransferase family protein	*Arabidopsis thaliana*

4.2.4 Crude Protein P80

Crude Protein P80 was loaded on DEAE-cellulose column (Figure 4.14). There were seven peaks which were designated protein 80U (unbound peak), 80B5, 80B10, 80B20, 80B30, 80B50 and 80B100. Table 4.11 shows % yield of each protein. The major protein of crude protein P40 is protein 80U. Proteins were dialysised with H_2O and lyophilized. And then, all protein powders were tested bioactivities (the hemagglutination activity and α-glucosidase inhibition) (see Table 4.12). Hemagglutination activity was found in crude protein (P80) and protein 80U that specific hemagglutinating activity are 5.28 and 12.27 U/mg protein, respectively. Crude protein P80, protein 80U, 80B20, 80B50, and 80B100 have α-glucosidase inhibition activity. The IC_{50} for these compounds were 48.19, 7.66, 1.50, 0.24 and 0.02 mg/ml, respectively. All proteins that have bioactivity were chosen to analyze by MALDI-TOF MS. The mass spectra are shown in Figure 4.15 – 4.18 respectively. And then, the mass spectra were used for performing of protein peptide mass mapping via Mascot program. The search results were shown in Table 4.13 and matched peptides shown in bold black in Figure 10C – 12C, Appendix C.

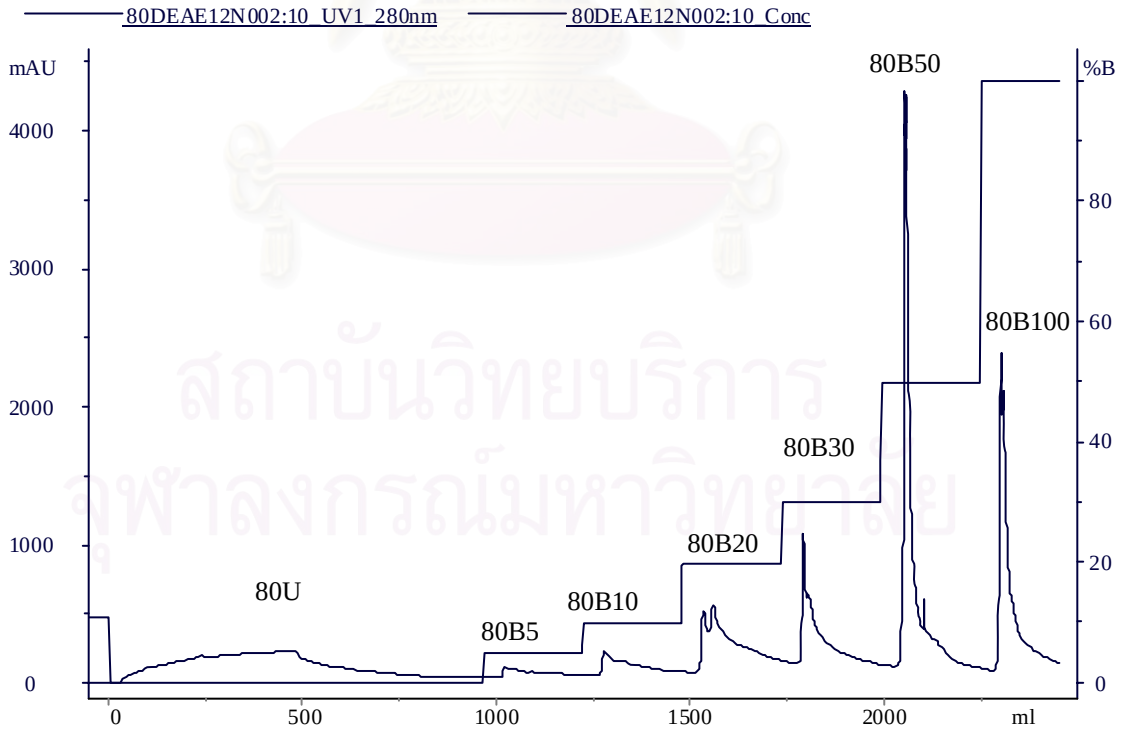

Figure 4.14 Anion exchange purification of crude protein P80. Stepwise gradient of NaCl, 5%, 10%, 20%, 30%, 50%, and 100 % of buffer B. The percent of buffer B is shown by dotted line. Flow rate 1.0 ml/min.

Table 4.11 Protein yield which separated from crude protein P80

Crude Protein (mg of loaded)	Buffer B (%)	Protein	Total Volume (ml)	Total Protein (mg)	Protein Yield (%)*
P80 (103.50 mg)	0	80U	500	35.00	33.82
	5	80B5	170	5.10	4.93
	10	80B10	40	3.60	3.48
	20	80B20	250	15.00	14.49
	30	80B30	70	7.00	6.76
	50	80B50	180	7.20	6.96
	100	80B100	70	16.80	16.23

Note. ** calculated from loaded crude protein*

Table 4.12 Bioactivity testing of crude protein P80 and protein which separated from crude protein P80

Protein	Specific hemagglutinating activity (U/mg protein)	IC_{50} of % α-glucosidase inhibition (mg/ml)
P80	5.28	48.19
80U	12.27	7.66
80B5	-	-
80B10	-	-
80B20	-	1.50
80B30	-	-
80B50	-	0.24
80B100	-	0.02

Note*. - ; no agglutinate or no* α-glucosidase inhibition

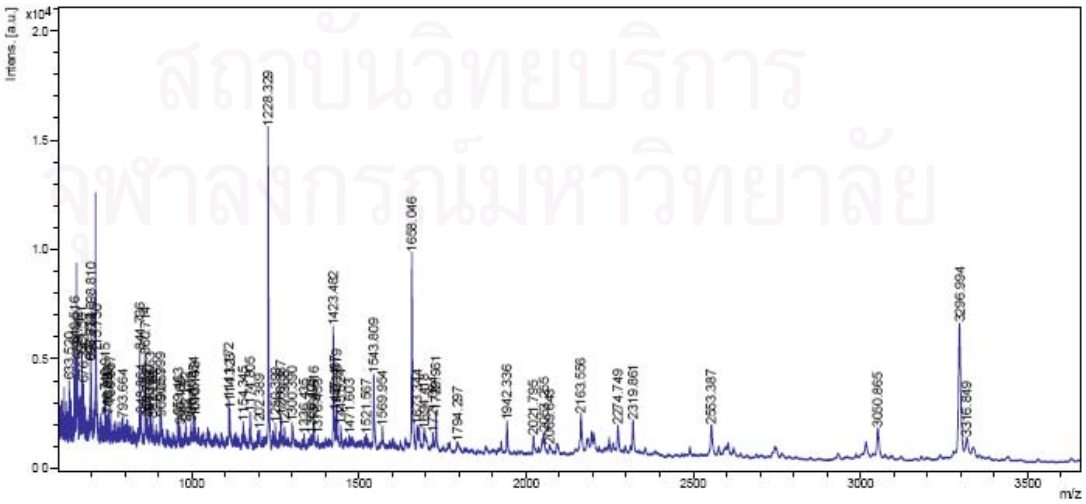

Figure 4.15 MALDI-MS spectrum of trypic fragment of protein 80U

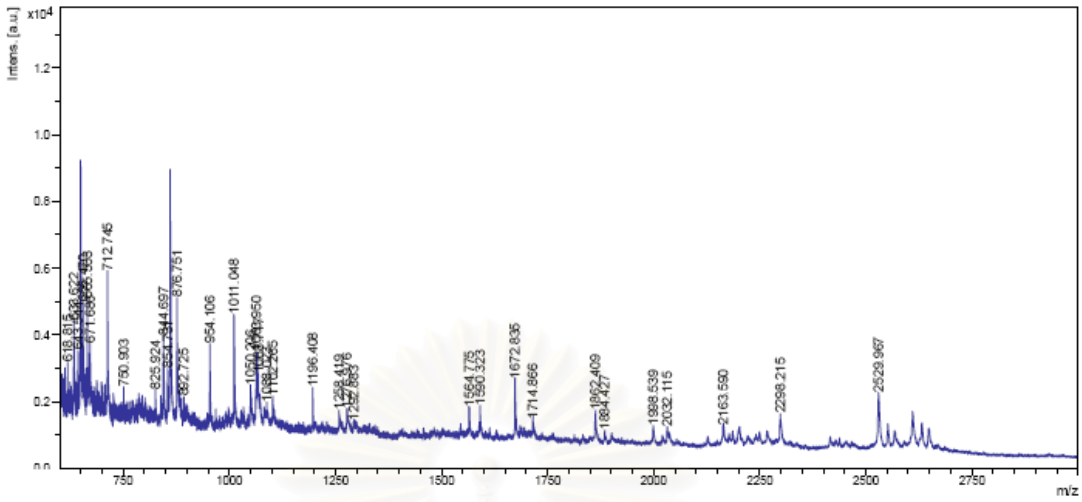

Figure 4.16 MALDI-MS spectrum of trypic fragment of protein 80B20

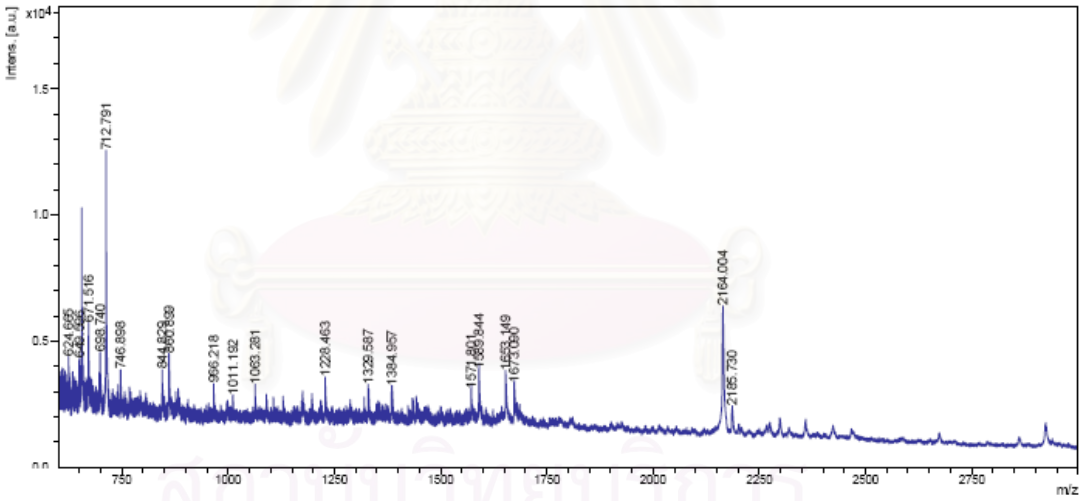

Figure 4.17 MALDI-MS spectrum of trypic fragment of protein 80B50

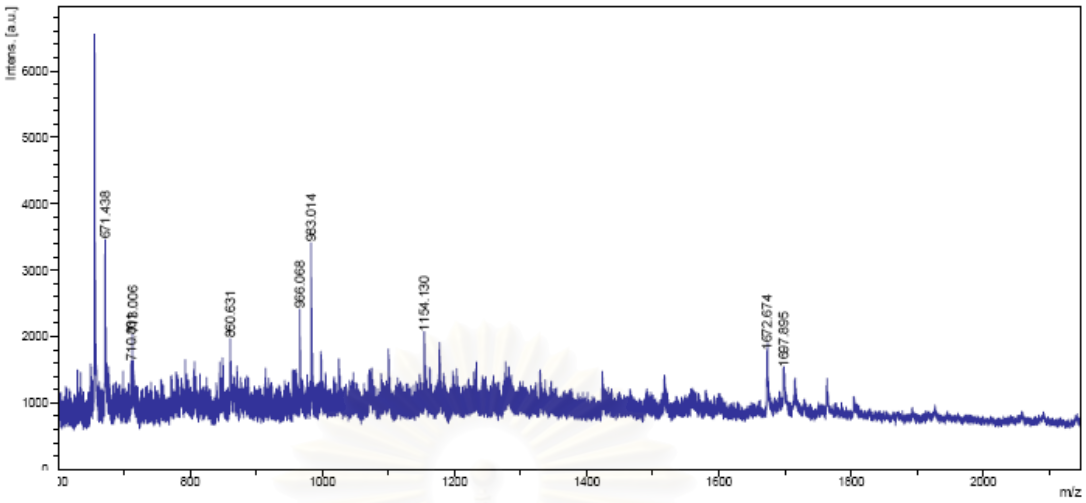

Figure 4.18 MALDI-MS spectrum of trypic fragment of protein 80B100

Table 4.13 The search result of protein 80U, 80B20, 80B50 and 80B100

Protein	MASCOT results			
	Mowse Score	**Mass (Da)**	**Protein name**	**Organism**
80U	92	2442	galactinol synthase	*Momordica charantia*
80B20	79	6603	hypothetical protein	*Vitis vinifera*
80B50	49*	61081	hypothetical protein	*Arabidopsis thaliana*
80B100	74	7589	F8M12.18 gene product	*Arabidopsis thaliana*

Note*.* there are no signification between* match between the results and proteins in database.

4.2.5 Crude Protein P100

Crude Protein P100 was loaded on DEAE-cellulose column (Figure 4.19). There were seven peaks which were designated protein 100U (unbound peak), 100B5, 100B10, 100B20, 100B30, 100B50, and 100B100. Table 4.14 shows % yield of each protein. The major protein of crude protein P100 is protein 100B20 (23.81 % yield). Proteins were dialysised with H_2O and lyophilized. And then, all protein powders were tested bioactivities (the hemagglutination activity and α-glucosidase inhibition) (see Table 4.15). Protein 100U and 100B5l have hemagglutination activity which

were found to be 13.33 and 13.70 U/mg protein, respectively. α-Glucosidase inhibition was found in crude protein P100, protein 100U and 100B100. The IC_{50} for these compounds were found to be 0.78, 5.06 and 0.12 mg/ml, respectively. All fractions that have bioactivity were chosen to analyze by MALDI-TOF MS. The mass spectra are shown in Figure 4.20 – 4.22. And then, the mass spectra were used for performing of protein peptide mass mapping via Mascot program. The search results were shown in Table 4.16 and matched peptides shown in bold black in Figure 13C-14C, Appendix C.

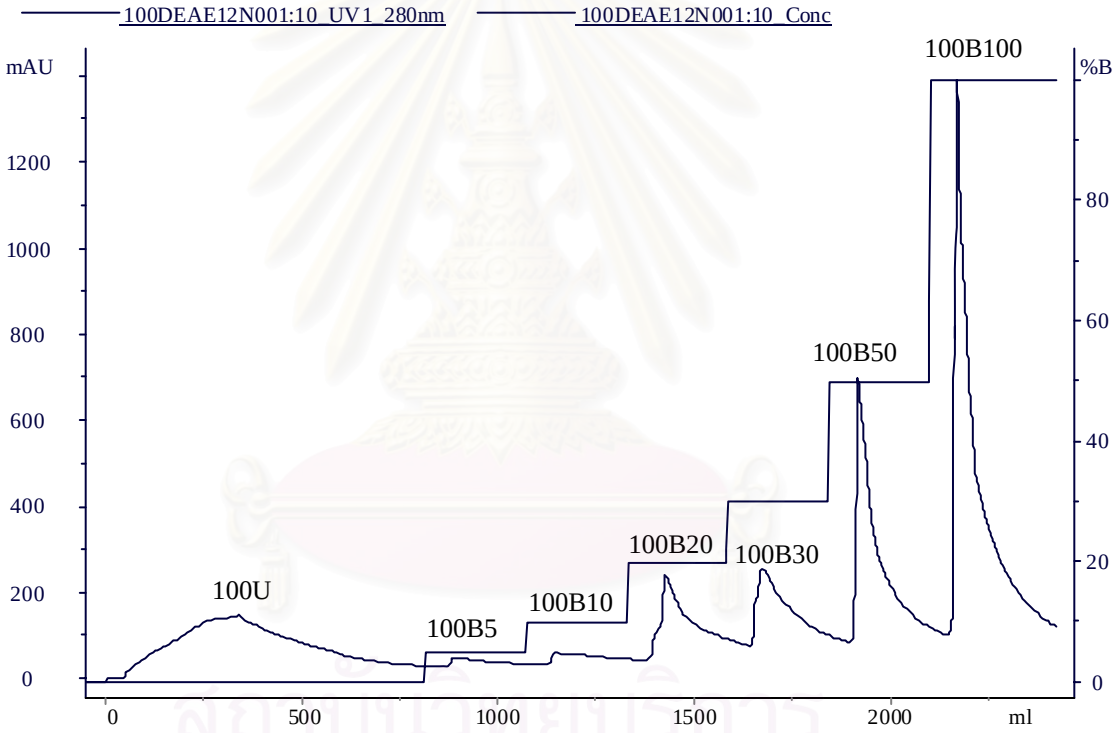

Figure 4.19 Anion exchange purification of crude protein P100. Stepwise gradient of NaCl, 5%, 10%, 20%, 30%, 50%, and 100 % of buffer B. The percent of buffer B is shown by dotted line. Flow rate 1.0 ml/min.

Table 4.14 Protein yield which separated from crude protein P100

Crude Protein (mg of loaded)	Buffer B (%)	Protein	Total Volume (ml)	Total Protein (mg)	Protein Yield (%)*
P100 (21.00 mg)	0	100U	500	5.00	23.81
	5	100B5	100	1.00	4.76
	10	100B10	140	1.40	6.67
	20	100B20	50	5.00	23.81
	30	100B30	110	2.20	10.48
	50	100B50	150	1.50	7.14
	100	100B100	260	2.60	12.38

Note. ** calculated from loaded crude protein*

Table 4.15 Bioactivity testing of crude protein P100 and protein which separated from crude protein P100

Protein	Specific hemagglutinating activity (U/mg protein)	IC_{50} of % α-glucosidase inhibition (mg/ml)
P100	-	0.78
100U	13.33	5.06
100B5	13.70	-
100B10	-	-
100B20	-	-
100B30	-	-
100B50	-	-
100B100	-	0.12

Note*. - ; no agglutinate or no* α-glucosidase inhibition

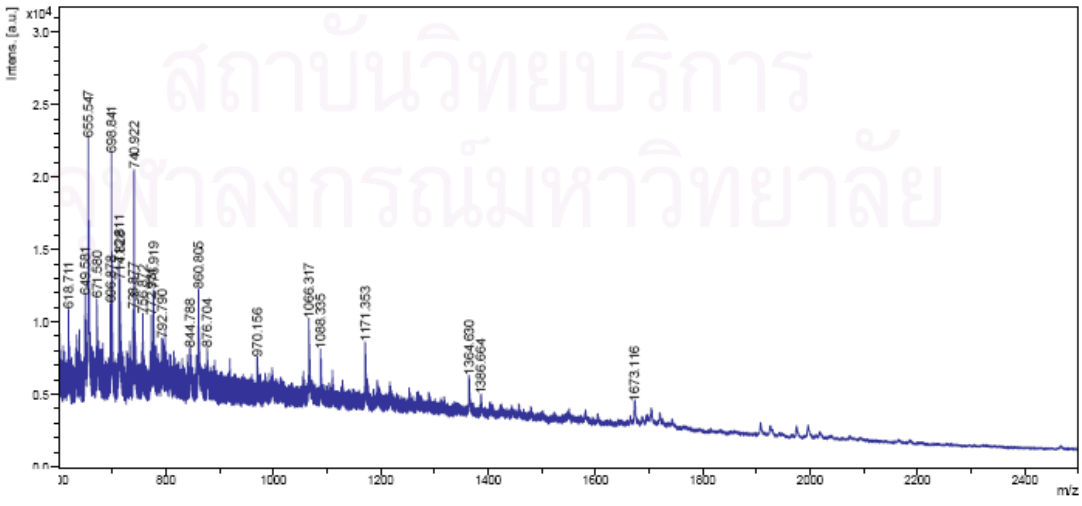

Figure 4.20 MALDI-MS spectrum of trypic fragment of protein 100U

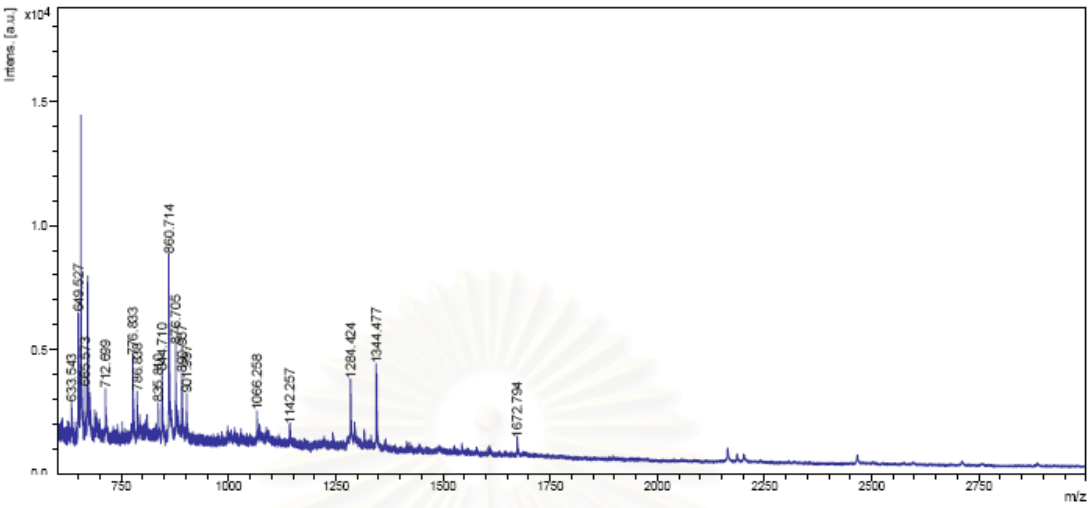

Figure 4.21 MALDI-MS spectrum of trypic fragment of protein 100B5

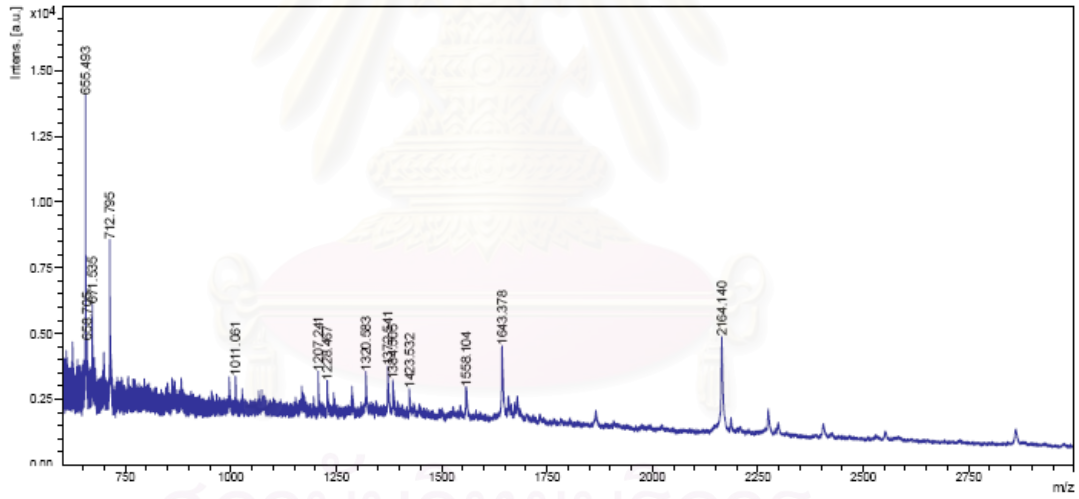

Figure 4.22 MALDI-MS spectrum of trypic fragment of protein 100B100

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Table 4.16 The search results of protein 100U, 100B5 and 100B100

Protein	MASCOT results			
	Mowse Score	**Mass (Da)**	**Protein name**	**Organism**
100U	45*	51130	putative MAP kinase phosphatase	*Oryza sativa* (japonica cultiva-group)
100B5	71	23677	ribosomal protein S4	*Anthoceros formosae*
100B100	77	32782	hypothetical protein	*Vitis vinifera*

Note*.* there are no signification between* match between the results and proteins in database.

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CHAPTER V

CONCLUSION

Crude proteins from fruit pulp of *Momordica charantia* were obtained by aqueous solution extraction and ammonium sulfate precipitation. Crude proteins were applied on to DEAE-cellulose ion exchange column. All proteins which separated were tested bioacitivity, hemagglutination and α-glucosidase inhibition activity. Proteins that have bioactivity were identified by MALDI-TOF MS and searched by MASCOT program.

The highest of the amount of crude protein was met when precipitated with 40-60 % ammonium sulfate saturated (P60). The amount of protein was lowest at 80-100% ammonium sulfate saturated (P100). % Yields of these crude proteins were 34.86 and 2.13, respectively.

Crude proteins were purified in only one step by using DEAE-cellulose anion exchange chromatography and collected each protein. Protein 40U, 40B5, 40B10, 40B20, 40B30, 40B50, 40B100, 80U, 100U, and 100B5 have hemagglutinating activity with rabbit red blood cell. The highest specific heamagglutination activity is protein 40B5 (400 U/mg protein). From peptides mass mapping by MALDI-TOF MS and database searching found that protein 40B50 similar to partial amino acid residue of OSJNBa0084N21.4 protein from *Oryza sativa*. α-Glucosidase inhibition activity found at protein 20U, 40B5, 40B50, 60U, 80U, 80B10, 80B20, 80B50, 80B100, 100U and 100B100. The preliminary screening showed that protein 80B100 show highest the inhibitory activity. The IC_{50} for these compounds were found to be 0.02 mg/ml. From peptides mass mapping and database searching found that protein 80B100 similar to partial amino acid residue of F8M12.18 gene product from *Arabidopsis thaliana.*

From these results, it suggests that fruit pulp of *Momordica charantia* Linn. contains many proteins and many bioactive components of different mechanisms, so this plant should be further studies.

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APPENDICES

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Appendix A

Table 1A Final Concentration of Ammonium Sulfate: Percentage Saturation at 0°C

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Adapted from "Data for Biochemical Research" (R.M.C. Dawson, D.C. Elliott, and K.M. Jones, eds.), 2nd Ed. Oxford Univ. Press,
London, 1969.

Table 2A Bradford solution

Solution	Chemical	Amount
Bradford Stock solution	95% ethanol	100 ml
	88% phosphoric acid	200 ml
	Coomassies Blue G-250	350 mg
Bradford Working solution	DI-H_2O	425 ml
	95% ethanol	15 ml
	88% phosphoric acid	30 ml
	Bradford Stock solution	30 ml

Appendix B

Reagents for in-solution digestion

All of the reagent are prepared immediated prior to use.

1. Tris stock: 12.1 g of Tris base in 200 ml of water. Adjust the pH of the solution to pH 7.8 with 6 M HCl. Add water to give a final volume to 250 ml.

2. 6 M urea, 100 mM Tris buffer : Place 2.0 g of urea in a 15 ml centrifuge tube. Add 1.25 ml of the Tris stock. Adjust the total volume to 5 ml with water.

3. Reducing agent : Dissolve 30 mg of DTT in 750 ul of water. Add 250 ul of Tris stock.

4. Alkylating reagent : Dissolve 36 mg of iodoacetamide in 750 ul of water Add 250 ul of Tris stock.

5. Typsin solution : Add 25 ul of ice-cold Tris stock and 75 ul of ice-cold water to 20 ug of trypsin. The solution is kept on ice until use.

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Appendix C

Match to: **gi|110288797** Score: **124** Expect: **1.5e-07**

NUMOD3 motif family protein, expressed [Oryza sativa (japonica cultivar-group)]

Nominal mass (Mr): **47548**; Calculated pI value: **8.72**

Number of mass values matched: **17**

Sequence Coverage: **17%**

Matched peptides shown in **Bold Black**

1	MHLGHAQSEE	TR**IKISMGVR**	**RGWNLR**LQKL	MIQDGCFVEW	R**DMIADAAR**K
51	GFAGGISLQW	NSYKILTEQM	RQEWLEKVQK	RRSMPRPTGN	RRAPKSPEQR
101	RKIAEAIAAK	WLDKEYRERV	CSGIASYHGT	SSGTKVPRKP	RSAREPGSKR
151	DTVKKKPIQS	RSAGLEDACG	TTPTVKRKKS	ATPYKDPMAG	EK**LEMITKIR**
201	**AQRAALEIEK**	**K**EAIKRAR**SL**	**IAEAEK**AANA	LETVASTSPF	AQASLIEAR**K**
251	**LVTEAR**LSLQ	HVDDEGPADS	ASDDASQDSG	ASDLHNHDMA	NQNDVIKQEN
301	KPVNGMELPP	SNVNGRDFYF	DVSTLTETDH	LR**DYQRIENS**	**MER**AYLLPSA
351	SSAIQDVNEN	HRMKDFNAHQ	LMVNDESITI	DQIASEVAEI	YPDEPQEDDT
401	LPVQKSKMR**W**	**VRGR**LVEVEE			

Figure 1C. Match peptide of 20U compare with NUMOD3 motif family protein, *Oryza sativa* (japonica cultiva-group)

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Match to: **gi|63078419** Score: **101** Expect: **3.1e-05**

maturase K [Quesnelia edmundoi]

Nominal mass (Mr): **61597**; Calculated pI value: **9.39**

Number of mass values matched: **15**

Sequence Coverage: **14%**

Matched peptides shown in **Bold Black**

1 MEELQGYLEK DRSRQQHFLY PLLFQEYIYA FAHDHGLNDS IFYEPVEIIG
51 YDK**KSSSLLV K**RLIIRMYQQ NYLINSVNYS NQNRFVGHNT YFYSHFFSQM
101 ISEGFAIIVE IPFSLR**LVSF PEEKEIPK**CQ NLRSIHSIFS FLEDKLSHLN
151 YVSDILIPYP IHLEILVQIL QCRIQDVPSL HLLRFFLHEY HNWNSLITPK
201 **KSIYVFSKEN KR**LFWFLYNS YVSECEFVFV FLRKQSSYLR LTSSGTFLER
251 **IQFYGK**IEHL IVVYR**NYFQK** TLWFFTDPFM HYVR**YQGK**AI LASK**GTHLLM**
301 **KK**WKCYLVNL WQYYFHFWSQ PHRIHINQLS NYSFYFLGYL SSVLR**NPLVV**
351 **R**NQMLENSFL IETGIKKFDT IVPVIPLIGS LSKAKFCTVS GHPISKPIWT
401 DLSDCDIIDR FGRICRNLSH YHSGSSEKRS LYRIKYILRL SCAR**TLAR**KH
451 K**STVRSFLRR** LGSVLLEEFF TEEEQVLSLI FPKPTPFSLH GSRREHIWYL
501 DIIRINNLVN H

Figure 2C. Match peptide of 40U compare with muturase K, *Quesnelia edmundoi*

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Match to: **gi|38347466** Score: **73** Expect: **0.018**

OSJNBa0084N21.4 [Oryza sativa (japonica cultivar-group)]

Nominal mass (Mr): **39692**; Calculated pI value: **6.58**

Number of mass values matched: **10**

Sequence Coverage: **13%**

Matched peptides shown in **Bold Black**

1 **MAMSNK**FTAK PHVFDGTDFS HWYSR**MQSYI MAENYDIWR**K VSHPYVIPEA
51 INTTALKTEF ELNCKACNIL LSGFSR**SDYD R**VAHLQTAHE IWVALNNFHQ
101 GTNNIIELRR **DLFK**KEYIKF EMKPEEALDD YLSRFNKILS DLRSVDSSYD
151 ANYPQSEISR HFLNGDSKSS ALVSSSTSLD VGASSSKSSV LALINAMFDD
201 QPEQFEEEDL VLLSNK**FSRA MK**NVRNRKRG EPNRCFECGA LDHLRSHCPK
251 **LRR**GKKKDDG RVKDDDMNKK K**NMKEK**KKKK HYEDETKGKQ VVDLAFIARN
301 ASSDVDESDD DNEEKLSYDQ LEYAAYK**FAK K**LQTCSIALD E

Figure 3C. Match peptide of 40B5 compare with OSJNBa0084N21.4, *Oryza sativa* (japonica cultiva-group)

Match to: **gi|147771285** Score: **85** Expect: **0.0013**

hypothetical protein [Vitis vinifera]

Nominal mass (Mr): **18925**; Calculated pI value: **7.77**

Number of mass values matched: **8**

Sequence Coverage: **20%**

Matched peptides shown in **Bold Black**

1 M**AFFSFVKEY QR**YKKK**FAQR KVVLGRRAFR** TDGMIANSDD FQADLPNSGG
51 LITSTVRGVE IRLSPESICR IFDISSVELW MYESKAWPTV LGFESTTLVL
101 PYDYFLTRVF K**DVGVDLSR**E IDFEAFTIYD TYDEQPLGQM KFEKAPNGSW
151 IRRVERPLAQ ARG

Figure 4C. Match peptide of 40B10 compare with hypothetical protein, *Vitis vinifera*

Match to: **gi|4092516** Score: **101** Expect: **3.1e-05**

beta-fructofuranosidase; fructosidase; invertase [Hamamelis virginiana]

Nominal mass (Mr): **19370**; Calculated pI value: **9.43**

Number of mass values matched: **10**

Sequence Coverage: **37%**

Matched peptides shown in **Bold Red**

1 FTYSTSTIHM VQFTWAHSTS TDLVNWIPHE YAIYMSQPSD INGCWSGSAT

51 MLPTGNPVIL YTGINTQNQQ VQNLAVPKNL SDPFLREWVK **SPNNPLMAPT**

101 **IMNKINASSF RDPTTAWLGP DRLWR**VIIGS KR**NRRGLAIL YMSKDFLRWT**

151 **KAQHPLHSSK** NTGMWECPD

Figure 5C. Match peptide of 40B20 compare with beta-fructofuranosidase, *Hamamelis virginiana*

Match to: **gi|116000672** Score: **76** Expect: **0.0095**

lignostilbene-alpha beta-dioxygenase (ISS) [Ostreococcus tauri]

Nominal mass (Mr): **66562**; Calculated pI value: **5.21**

Number of mass values matched: **8**

Sequence Coverage: **10%**

Matched peptides shown in **Bold Black**

1 MPPARAVRTP MKPVEEVAGD ASGVGGGLSQ QTPFERQYKD YATGYASVPG

51 LAYENMGWVT DVEGEIPKEM EGTLLRNGPA MYERGGFVKS YLDGDGMVTS

101 IAVKDGKAYF RNKFVRTEHF DQEEEQDKYI MPSIFTASDP RPFPFFSRLF

151 GDIIGGDLRR KQNGAYNKPY ALNPDTLETI GHGACDLSSA MHTSHYR**TVT**

201 **EPDGSRR**CVA FLNEVDWRTE TTHAVFYEFD ENGK**EVSRR**A YDYPSSYVHD

251 LIVTENYYIL FDCPVKIDFP AVFTKYIFEK SCLSELICED TSRRPLFRIF

301 PRRGDSRDVK TAPADYWCYA YHHVNGFEDK DGNVVFDTCT WDKFTLYFTD

351 ICNPNGVDNY PR**MKLSRFII DMDKLEAK**HY LLSDTPCELP ITSWDYTGLP

401 YEHMYLSTSV GRTEDGVNGP MQALSKASLK IDEQK**LYYEE QWVPGDRK**FA

451 MEPFFVPRKG GTDEDDGWVV ALVHDAAAEK SNFDGRGTEM VIIDAKKFSE

501 GPVARLRLPS YVPFGVHGSW SPK**YVAGPPK EDELK**R**LQEM R**SKNDGKPIS

551 LGASAAEPVI HATPSPQAIG VGVASLLMGI TALSSILG

Figure 6C. Match peptide of 40B30 compare with Lignostilbene-alpha beta-dioxygenase (ISS), *Ostreococcus tauri*

Match to: **gi|92888659** Score: **73** Expect: **0.02**

Alcohol dehydrogenase superfamily, zinc-containing [Medicago truncatula]

Nominal mass (Mr): **42329**; Calculated pI value: **6.03**

Number of mass values matched: **7**

Sequence Coverage: **14%**

Matched peptides shown in **Bold Black**

1 MAATSSEGEG KPIRCKAAVA RRPGESLVIE EIMVAPPMPR **EVRIR**IICSS
51 ICHIDLTFSD MQDPPGFFPR ILGHEAIGVV ESVGKNVTEV TKGDVVIPIF
101 LPDCGECIDC KSTKSNLCTN FPFKVSPWMP R**HENTR**FTDL NGEIIHHFMY
151 VSSFSEYTVV TFANVTKIDP EIPPNGACLL SCGIGAGVGA AWRTAGVEPG
201 STVAIFGLGS IGLAVAEGAR **LCGATK**IIGV DVNPEKFEVG KKFGLTDFVH
251 AGECGSKPVI TEMTDGGADY CFECVGMASL VQDAYASCRK **GWGKTIVLGL**
301 **DKPGSR**ISLS CSEVLHGGKT IQGNLFGGLK PKSHVSILLK **RYMDKELQLD**
351 **EFVTHEVSFK DINK**AFDLLR NGQCLRCMIW MDN

Figure 7C. Match peptide of 40B50 compare with Alcohol dehydrogenase superfamily, zinc-containing, *Medicago truncatula*

Match to: **gi|23266303** Score: **47** Expect: **7.5**

TNP2-like protein [Oryza sativa (japonica cultivar-group)]

Nominal mass (Mr): **44390**; Calculated pI value: **6.54**

Number of mass values matched: **8**

Sequence Coverage: **13%**

Matched peptides shown in **Bold Black**

1 **MYADR**RSKEF IDGVHYFLR**V AEANRQR**GFI CCPCNKCKNQ K**EYSASR**TIH
51 FYLFESGFMP SYNCWTSYGE QGVEMEDDEV EDDNIPDFAQ YVGFEGNQTG
101 EEEIAADGND VADDLGQMLQ DAREDCESEK EAHKLDKMLE DHRISLYPGS
151 EQGHKKLDTT LEFLQWKAKN EVSDKAFGDL LKLVKNILPG GNKLPETMYE
201 AKKIVCPLGL EVHKIHACPN DCILYRGEEY ENLEACPVCK **ALRYK**IRRDD
251 PGEVNGQLTK KRIPAKVMWY FPIIPRLRRL FRNKGNARML R**WHAEERQQD**
301 **GMLR**HPADGS QWRNIDRNFK EFGKDARNIR **FGLSTDGMNP SGR**STWLVTM
351 CIYNLPPWLC MKRKYIMMPI IIQGPK

Figure 8C. Match peptide of 40B100 compare with TNP2-like protein, *Oryza sativa* (japonica cultiva-group)

Match to: **gi|15232447** Score: **81** Expect: **0.0033**

galactosyltransferase family protein [Arabidopsis thaliana]

Nominal mass (Mr): **39096**; Calculated pI value: **8.07**

Number of mass values matched: **6**

Sequence Coverage: **23%**

Matched peptides shown in **Bold Black**

1 MSSSPKLFHA RPSFFTRRST PLIVFTSLAI GLTGFLFGLS TILFPGLRLS
51 GRSCLTNLPP KTVKIVWDVA GNSIVNGEVK RHKVMGFVGI QTGFRSAGRR
101 RALRNTWMPS DPEGLRR**LEE STGLAIRFII GK**TKDEAKMV ELRSEVAMYD
151 DFILLDIEEE YSKLPYKTLA FFKAAYALYD SEFYVK**ADDD IYLRPDR**LSL
201 LLAKER**GHSQ TYLGCMKK**GP VFTDPKLKWY EPLADLLGK**E YFLHAYGPIY**
251 **ALSADVVTSL VALK**NNSFRM FSNEDVTIGA WMLAMNVNHE NLHTLCEPEC
301 SPYSIAVWDI PK**CSGLCNPE KR**MLELHMLE SCSK**SPTLPS DDE**

Figure 9C. Match peptide of 60U compare with galactosyltransferase family protein, *Arabidopsis thaliana*

Match to: **gi|34550078** Score: **92** Expect: **0.00027**

galactinol synthase [Momordica charantia]

Nominal mass (Mr): **2442**; Calculated pI value: **4.55**

Number of mass values matched: **4**

Sequence Coverage: **100%**

Matched peptides shown in **Bold Black**

1 **MSPAAATETI DSTDAPKRAY VTF**

Figure 10C. Match peptide of 80U compare with galactinol synthase, *Momordica charantia*

Match to: **gi|147780948** Score: **79** Expect: **0.005**

hypothetical protein [Vitis vinifera]

Nominal mass (Mr): **6603**; Calculated pI value: **4.97**

Number of mass values matched: **5**

Sequence Coverage: **80%**

Matched peptides shown in **Bold Black**

1 **MAEEAGKASG IEKFDGTDFA YWR**MQIEDYL YGRK**LHLPLL GTKPESMKAE**

51 **EWALLDR**

Figure 11C. Match peptide of 80B20 compare with hypothetical protein, *Vitis vinifera*

Match to: **gi|3513728** Score: **74** Expect: **0.016**

F8M12.18 gene product [Arabidopsis thaliana]

Nominal mass (Mr): **7589**; Calculated pI value: **10.01**

Number of mass values matched: **4**

Sequence Coverage: **45%**

Matched peptides shown in **Bold Black**

1 **MAGPAQAAKQ SSEVLGQR**KS LGICPLRAAA VGAVIIGGIG YVVLYSKKKP

51 EASAGDVAK**V MSGVGGTPEN TRPRN**

Figure 12C. Match peptide of 80B100 compare with F8M12.18 gene product, *Arabidopsis thaliana*

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Match to: **gi|28202174** Score: **71** Expect: **0.029**

ribosomal protein S4 [Anthoceros formosae]

Nominal mass (Mr): **23677**; Calculated pI value: **10.47**

Number of mass values matched: **7**

Sequence Coverage: **23%**

Matched peptides shown in **Bold Black**

1 **MSRYR**GPRVR IIR**RLGTLPG LTSK**TPESKP SYINQSTSSR K**ISQYRIRLE**
51 **EK**QKLRFHYG ITERQLLK**YV RIAR**KAKGST GQVLLQLLEM R**LDNIIFR**LG
101 LAPTIPGARQ LVNHRHVLVN DCTVDIPSFR CKPQDVITIR DRQK**SQNLMK**
151 **R**SRDSYEKYG IPNHLTFNSV QNIGLVNETI DRDWIGLKIN ELLVVEYYSR
201 QA

Figure 13C. Match peptide of 100B5 compare with ribosomal protein S4, *Anthoceros formosae*

Match to: **gi|147818082** Score: **77** Expect: **0.0083**

hypothetical protein [Vitis vinifera]

Nominal mass (Mr): **32782**; Calculated pI value: **9.34**

Number of mass values matched: **7**

Sequence Coverage: **18%**

Matched peptides shown in **Bold Black**

1 **MEDTMKEK**GV LKLVHPGRFV EIHTEPITAA EVLRKNPR**HS ITRPDVFR**NP
51 WVVVQPEAVL TPGRVFFIVP NQTIYRLLK**A SGHCK**QSAPL PQYDSPKATH
101 DLCWFPK**QIS PLR**AWAGITP KHQNPKQILQ HQVQTMPRNG VMSSWDQDSD
151 KNLRGHSLVE PWGRKFGKHR HSHQEFEQEP LLESMGETTS YHAEENSSMI
201 NNCNVSQPKT KEGELKYRXR K**QATMLKSCL R**K**QDSVRKAL SPR**DSYGHSD
251 DPGIQKIFSY QDNLESGNLG SEWEHKVQHK QII

Figure 14C. Match peptide of 100B100 compare with hypothetical protein, *Vitis vinifera*

BIOGRAPHY

Miss Supaporn Nammoonnoy was born on February 6, 1975 in Khonkaen, Thailand. She graduated with Bachelor Degree of Science, from Department of Chemistry, Faculty of Science, Khonkaen University in 1997. She works at Radioisotope Laboratory, National Institute of Health, Department of Medical Science, Ministry of Public Health. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2004.

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