CHAPTER II

LITERATURE REVIEW

Botanical characteristic of bitter gourd (*Momordica charantia* L.)

Bitter gourd has many common names such as African cucumber, ampalaya, assorossie, bitter melon, balsam apple, balsam pear, bitter cucumber, bitter pear melon, karela, and papayilla (Ross, 1999, Morgan and Midmore, 2002 and Taylor, 2002). Bitter gourd belongs to the Cucurbitaceae family, which includes squash, watermelon, muskmelon, pumpkin and cucumber. It is an annual vine. The stem is slender and more or less pubescent with 5 angles and furrows. The lobes are mostly blunt but have small marginal points up to about 12 cm long (Ross, 1999). The tendrils are simple or forked. The leaves are palmate, 5-9 lobes and 5-17 cm in diameter. The leaf blades are broadly ovate-reniform (Siemonsma and Piluek, 1994). The margins are sinuate with apiculate points. Prominent sessile bracts are entirely at or near the base of slender peduncle (Purseglove, 1968). The flowers are rotate-companulate (Saralamp *et al.*, 1996). Solitary male and female flowers borne in the leaf axils (Taylor, 2002). Female peduncles are short and male peduncles are long (Ross, 1999). Male flowers appear first and exceed the number of female flowers by about 25 to 1. The flowers open at sunrise and remain open for only one day (Natural Health Center, 2003). The fruit is pendulous, fusiform and 5-25 cm long (Purseglove, 1968). It is narrow to both ends and ribbed with prominent tubercles (Ross, 1999). Fruit skin is covered with blunt warts. It splits from apex downward to base and the pericarp is bitter (Saralamp *et al.*, 1996) exposing the scarlet arils (Bailey, 1951). At maturity, fruits tend to split open, revealing orange flesh and a bright red placenta to which the seeds are attached. Seeds are tan and oval, with a rough etched surface and are about 5-7 seeds/gram (Natural Health Center, 2003). Seeds are about 10 mm long, light gray or brown with prominent patterns (Bailey, 1951).

Siemonsma and Piluek (1994) reported that *M. charantia* has been classified in cultivated and wild groups. The cultivated group is *M. charantia* ssp. or var. *charantia.* and the wild group

is *M. charantia* ssp. or var. *abbreviata* (Ser.) Grebensc. India and South-East Asia divided *M. charantia* into 2 groups (Siemonsma and Piluek, 1994) as follows:

1. *M. charantia* var. *minima* Williams & Ng. Fruits in this variety have diameters less than 5 cm, all immature fruits are green and ripe seeds are 13-14.5 mm × 6.8-8.5 mm. Cultivars of this variety are divided into 3 groups: short fruit (6-7.5 cm), medium fruit (8-12 cm) and long fruit (12-22 cm).

2. *M. charantia* var. *maxima* Williams & Ng. Fruits in this variety have diameters larger than 5 cm, immature fruits are white or green and ripe seed are 14.8 mm × 8.5 mm. Cultivars of this variety were divided into 2 groups: medium fruit (12-17 cm) with white fruit and long fruit (about 20 cm) with green fruit when immature.

Fruit length was used for the classification of wild and cultivated *M. charantia*. The wild *M. charantia* is about 5 cm long and the cultivated *M. charantia* is up to 25 cm long (Robinson and Decker-Walters, 1997). Yang and Walters (1992) divided bitter gourd into three horticultural groups or types: 1) small fruit type, 10-20 cm long, 0.1-0.3 kg/fruit in weight, usually dark green, and very bitter. 2) long fruit type, most commonly grown commercially in China, 30-60 cm long, 0.2-0.6 kg/fruit in weight, light green in color with medium size protuberances, and only slightly bitter; and 3) triangular fruit type, cone-shaped, 9-12 cm long, 0.3-0.6 kg/fruit in weight, light to dark green with prominent tubercles, moderately to strongly bitter.

In Thailand, bitter gourd is divided into 2 groups by using fruit size. Large fruit size is called "Chinese bitter gourd" and the smaller one is called "Mara Khee Nok or Thai bitter gourd" (Wattanapiromsakul, 2002).

Momordica anti-viral protein of 30 kilodalton (MAP30)

M. charantia is a tropical plant currently distributed across the globe because it is adapted to a wide variation of climates (Ng, 1993). It is a well-recognized source of secondary metabolites. All parts of the plant are used on an economic basis. The uses of this species were described in detail by Nayar and More (1998) and Ross (1999). It has potential biological and medicinal uses.

Because of its widespread uses and economic importance, the plant has long been a research focus for scientists (Wettberg, 1998). Recently, proteins with ribosome-inhibiting properties have been isolated from several cucurbit species (Ng *et al.*, 1991). Some of these species have been

used for anti-HIV activity, because its ribosome-inhibiting properties have been shown to be effective in inhibiting the replication of human immunodeficiency virus (HIV) in infected lymphocyte and phagocyte cells, indicating potential as a therapeutic agent for AIDS (McGrath *et al.*, 1989).

Bitter gourd contains an array of novel and biologically active phytochemical that have been documented with cytotoxic activity included a group of ribosome-inactivating proteins (Talor, 2002). Ribosome-inactivating proteins have been utilized in the construction of immunotoxin (Fong *et al.*, 1996, Go *et al.*, 1992, Mock *et al.*, 1996 and Ng *et al.*, 1992). The physiological role of ribosome-inactivating proteins in plants may be related to defense against pathogens (Lodge *et al.*, 1993) and some of their specific proteins might be an essential proteins of sex differentiation in bitter gourd (Wang *et al.*, 1998).

MAP30 is a ribosome-inactivating protein. It was first isolated from seed of bitter gourd by researchers at the University of New York around 1989. It is believed to have multiple functions that could be beneficial for anti-HIV activity (Lee-Huang *et al.*, 1990 and Schreiber *et al.*, 1999).

Activities and potentialities of MAP30

MAP30 is a protein found in bitter gourd that is believed to have the ability to inhibit HIV-1 integrase, which is an enzyme essential for gene expression of the virus. It has been suggested that MAP30 protein could inhibit HIV-1 infection in T lymphocytes and monocytes as well as replication of HIV-1 in infected cells. It has not been found to be toxic to normal uninfected cells. (Lee-Huang *et al.*, 1995b). It is claimed that bitter gourd could stop the production of reverse transcriptase, protease and integrase of HIV (Weaver, 2000). Two proteins, α- and β-momorcharin, in the seeds of bitter gourd appear to modulate the activity of both T- and B-lymphocytes and could suppress macrophage activity, but were non-cytotoxic (Leung *et al.*, 1987 and Michael, 2002).

The report of potent anti-tumor activity human cancer cell lines and inhibition of HIV-1 infection in lymphocytes and monocytes and viral replication in HIV-infection cells (Lee-Huang *et al.*, 1995a and 1995b) have stimulated interest in MAP30. Another clinical study showed that MAP30 was relative to herpes virus infections (Bourinbaiar and Lee-Huang, 1996) and human breast tumor (Lee Huang *et al.*, 2000).

Effect on motility and vitality of human spermatocytes

Schreiber *et al.* (1999) investigated the effect of MAP30 obtained from *M. charantia* on the motility and vitality of human spermatocytes. Human sperm was treated with the anti-HIV agent, MAP30. Nonoxynol-9, a commonly used spermicide and phosphate-buffered saline were used as the positive and negative controls. Results indicated that MAP30 did not inhibit the motility or vitality of human sperm cells over a dose range of 100-0.1 μg/ml, whereas nonoxynol-9 demonstrated spermicidal action on all samples over the same dose range. It was concluded that MAP30 was not toxic to human sperm cells at the dose which it inhibits HIV-1 and herpes simplex virus. It had no effect on the motility of spermatozoa, even at a dose of 1,000 times the maximum effective concentration. MAP30 may be useful as nonspermicidal protection against sexually transmitted diseases.

Effects on syncytium formation, viral protein p24 expression and HIV reverse transcriptase activity

HIV virus consists of a complex protein. The outer shell of the virus is known as viral envelope. Within the viral envelope is a HIV protein, which is called matrix (p17). Within this matrix is the viral core or capsid, which is made of a core antigen (viral protein p24). The major structures contained within the viral core are two single strands of HIV RNA, protein p7 (nucleocapsid) and three enzyme proteins. Three enzyme proteins are reverse transcriptase (p51), protease (p11) and integrase (p32) (Avert, 2003). The viral protein p24 assembles with identical proteins to form a cone-shaped structure that encloses genetic material of HIV in a mature virus particle. In addition to its structural function, scientists speculate that the viral protein p24 plays other important roles in the HIV life cycle (Summers, 1996). HIV infection was measured by syncytial-forming microassay (Nara and Fischinger, 1988, Nara *et al.*, 1987 and Laal *et al.*, 1993). HIV replication was measured by viral protein p24 expression (Nara *et al.*, 1987) and viral-associated reverse transcriptase activity (Hoffman *et al.*, 1985).

Lee-Huang *et al.* (1990) isolated and purified MAP30 from ripe fruits and seeds of *M. charantia*. MAP30 exhibited dose-dependent inhibition of cell-free HIV-1 infection. Result found that dose required for 50% inhibition (ID_{50}) of MAP30 was 0.83, 0.22 and 0.33, respectively. The inhibition was measured by viral protein p24 expression and viral-associated

reverse transcriptase activity in HIV-1 infected H9 cells. No cytotoxic or cytostatic effects were found under the assay conditions. These data suggest that MAP30 may be in the treatment of HIV-1 infection. Lee-Huang *et al.* (1995a) examined MAP30 by measuring quantitative focal syncytium formation on CEM-ss cell monolayers, viral core protein p24 expression and viral-associated reverse transcriptase activity in HIV-1 infected H9 cells. The enhancement of weak HIV antagonists, dexamethasone and indomethacin, by MAP30 has been examined by measuring the reduction in p24 expression in acutely infected MT-4 lymphocytes. MT-4 is a cell line from human, peripheral blood, leukemia and T cells. The morphology of MT-4 is lymphoblast-like and is susceptible to HIV (Pinaev, 2003). The use of MAP30 combines with low pharmacological doses of dexamethasone and indomethacin may improve the efficacy of anti-HIV therapy (Bourinbaiar and Lee-Huang, 1995). The cloning and expression of the gene encoding biologically active recombinant MAP30 (re-MAP30) provides an abundant source of homogeneous material for clinical investigations, as well as structure-function studies of this novel antiviral and anti-tumor agent (Lee-Huang *et al.*, 1995a).

Effects on integrase of HIV-1 activity

Integrase is a product of HIV which inserts and removes DNA from host genomes. Intregration of viral DNA into the host chromosome is a vital step in the replicative cycle of retroviruses, including the AIDS virus. Lee-Huang *et al.* (1995b) investigated the effects of MAP30 on HIV-1 integrase. Their results showed that MAP30 exhibited dose-dependent inhibition of HIV-1 integrase. The inhibition of HIV-1 integrase by MAP30 suggests that impediment of viral DNA integration may play a key role in the anti-HIV activity of this plant protein. Besides the inhibition of HIV-1 integrase, MAP30 possessed a topological activity on plasmid and viral DNAs including HIV-1 long terminal repeats which long terminal repeats are essential sites for integration of viral DNA into the host genome by viral integrase (Lee-Huang *et al.*, 1995b).

Effects on herpes simplex virus *in vitro*

Herpes simplex virus type 2 is as a cofactor in the sexual transmission of HIV and its infection is almost always sexually transmitted and causes genital ulceration (Mbopi-Keou *et al.*,

2003). Human lung WI-38 fibroblasts are susceptible to herpes simplex virus (Incafci and Balconi, 2003). The effects of the anti-HIV protein MAP30 from *M. charantia* on the infection and replication of herpes simplex virus was examined. Human lung WI-38 fibroblasts were cultured in the presence of tenfold dilutions of MAP30 and were exposed to herpes simplex virus and viral yield was measured at 24-48 hours by ELISA. The effective concentration for 50% inhibitions (EC_{50}) for MAP30 was 0.1-0.2 μM. In comparison, the EC_{50} for acyclovir, a commonly used anti-herpes simplex virus drug, was 0.2 μM. The antiherpetic activity of MAP30 against acyclovir-resistant strains was more potent than acyclovir. These results suggested that MAP30, previously shown to be active against HIV, might also useful for the therapy of herpes simplex virus infections (Bourinbaiar and Lee-Huang, 1996).

Effects on MDA-MB-231 human breast tumor

A study was conducted to investigate the efficacy of MAP30 on the estrogen-independent human breast tumor MDA-MB-231 both *in vitro* and *in vivo*. MDA-MB-231 is a cell line that is morphologically epithelial-like and is estrogen receptor negative (Nicolo', 2003). MDA-MB-231 human breast cancer cells were treated with MAP30 and the effects of MAP30 on the expression of breast tumor antigen HER2 was also examined. Results indicated that MAP30 inhibited the expression of HER2 gene *in vitro* and inhibited cancer cell proliferation. When MDA-MB-231 human breast cancer cells were transferred into severe combined immunodeficiency (SCID) mice, all mice succumbed to tumors after 46 days. Treatment of human breast cancer bearing SCID mice with MAP30 at 10 μg/injection for 10 injections. Results showed that there were significant increases in survival of the mice. 20-25% of the mice remained tumor free for 96 days. MAP30 is effective against human breast cancer MDA-MB-231 *in vitro* and *in vivo* and may be of potential therapeutic use against breast carcinomas (Lee-Huang *et al.*, 2000).

Toxicity of MAP30

The anti-HIV activity of MAP30 was found out to be a specific virus. The effect of MAP30 on cellular DNA and protein synthesis was determined in uninfected cells, H9 which is human lymphoblast-like cell line and susceptible to HIV-1 (Chicco-Bianchi, 2003). The results found that MAP30 ranging from 0.334 to 33.4 nM did not effect cellular incorporation of labeled

thymidine or leucine, while the majority of viral protein p24 and HIV reverse transcriptase activity productions were inhibited. MAP30 at 334 nM reduced cellular DNA yield of 25% and protein synthesis of 28% when compared with the productions of viral protein p24 and HIV reverse transcriptase. Cytotoxicity of MAP30 to uninfected cells in culture may be expressed at toxic dose 50 (TD_{50}). TD_{50} is a dose level which cellular protein and DNA synthesis is inhibited by 50%. The TD_{50} divide by the inhibitory dose at 50% inhibition (ID_{50}) is defined as therapeutic index of anti-HIV agent. The therapeutic indices are in the range of 1,000 to 10,000. They are used for the three assays of antiviral activity. These assays are microtiter syncytial-forming, p24 expression and viral-associated reverse transcriptase (Lee-Huang, 1994). The therapeutic index of MAP30 has a value of at least 1,000 (Lee-Huang *et al.*, 1990). Furthermore, the cytotoxicity of MAP30 on the infection and replication of herpes simplex virus was negligible and comparable when compare with acyclovir which is a commonly used anti-herpes simplex virus drug (Bourinbaiar and Lee-Huang, 1996).

The toxicity of this anti-HIV agent to healthy animals was studied on 6-8 week old Canada Foundation for Innovation (CFI) mice. Filter-sterilized anti-HIV agent in buffer A were injected intraperitoneally at doses of 0.1, 1, 10 and 100 mg/100 g body weight every 3 days. Control animals received similar injections of sterile buffer A. Animals were weighed and examined for gross pathological alterations. The animals appeared alert and fed normally. They were sacrificed and examined. Results showed that no lesions were observed in any organ in MAP30 treated animals. MAP30 showed toxicity to healthy mice at a lethal dose 50 (LD_{50}) of 56-62 mg/kg (Chu and Cutler, 1992).

Activities and toxicity of other ribosome-inactivating proteins in bitter gourd

In addition to MAP30, there are several isolated ribosome-inactivating proteins with molecular weights at the range of 24-30 kDa from seeds and fruits of bitter gourd such as momordin, α-momorcharin, β-momorcharin, δ-momorcharin, ε-momorcharin, momorcharin I and II and Thai bitter gourd protein, Mara Khee Nok at 29 kDa. There is also a small ribosome-inactivating protein, which is called γ-momorcharin, which has much lower molecular weight (11.5 kDa) than the other ribosome-inactivating proteins. These ribosome-inactivating proteins

have a number of biological effects including abortion, inhibition of tumor growth and anti-HIV. Their activities and toxicity:

1. Momordin was isolated from seeds of bitter gourd. Its molecular weight is 28.69 kDa (Minami and Funatsu, 1993). Minami *et al.* (1998) investigated an effect of chemical modification of momordin on inhibiting protein-synthesis. When tryptic peptides from modified momordin were analyzed, the modified residues were identified as His140, Tyr165 and Lys231. Furthermore, the amounts of three modified momordin which binds to rat liver ribosome were reduced to about half or less than half of that of native momordin. These results suggested that His140, Tyr165 and Lys231are highly exposed on the surface of momordin molecules and are involved in its protein-synthesis inhibitory (PSI) activity, probably by binding to ribosomes. Besides studying PSI activity of momordin, there have been studied about cytotoxicity of anti-CD5-momordin immunotoxin in normal and tumour cells of peripheral blood mononuclear cells (PBMC) and human T cell leukaemias. In a short-term assay, this immunotoxin displayed different cytotoxic activities on normal and tumor cells but in a long-term clonogenic assay (15 days); the immunotoxin demonstrated a comparable efficacy of clonogenic cell killing for both cell types (Porro *et al.*, 1995).

2. α-momorcharin (29 kDa) and β-momorcharin (28 kDa) have shown to exhibit abortifacient, immunosuppressive and antitumor effects (Leung *et al.*, 1987 and Ng *et al.*, 1994). They are also called abortifacient proteins. Researchers in Hong Kong discovered these proteins in bitter gourd seed. The proteins were shown to exert an immunosuppressive effect but were non-cytotoxic. They appeared to modulate the activity of both T- and B-lymphocytes and significantly suppressed macrophage activity. Very small doses of the protein injected into lab mice was able to produce significant suppression in the proliferation of lymphocytes but slightly larger doses produced abortion in pregnant mice (Leung *et al.*, 1987). These proteins were tested for a possible effect on ovulation and plasma levels of ovarian steriods in mice. The mice were induced to superovulate by using pregnant mare' s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG). Both proteins diminished the number of oocytes ovulated when they were given on the day prior to or on the day of PMSG treatment. Both proteins did not diminish the number of oocytes ovulated when they were given after the gonadotropin injection. Animals that have previously been treated with the plant protein, after mating underwent

pregnancy resulting in a litter size similar to that of the controls (Ng *et al.*, 1988). In addition, α-momorcharin had the most potent inhibitory effect when it was exerted on mouse monocyte-macrophage (P388) cells, but had the least effect on sacoma cells. α-momorcharin also enhanced the tumoricidal effect of mouse macrophages on mouse mastocytomal (P815) cells (Ng *et al.*, 1994). Furthermore, the anti- HIV activities of α-momorcharin were examined by using HIV-1 III B which induced the inhibition of syncytia formation and using the reduction of p24 core antigen expression level and decrease the number of HIV antigen positive cells in acutely and chronically infected cultures. The results were concluded that α-momorcharin is a unique component of momorcharin with anti- HIV activity and markedly inhibited HIV-1 replication in acutely but not chronically HIV-1-infected T-lymphocytes (Zheng *et al.*, 1999).

3. γ-momorcharin was purified from the seeds of bitter gourd. It inhibited protein synthesis in the rabbit reticulocyte cell-free system with ID_{50} of 55 nM. γ-momorcharin which acted on a specific adenosine in 28S ribosomal RNA of rat liver which is in a highly conserved loop of 28S ribosomal RNA (Pu *et al.*, 1996).

4. δ-momorcharin (30 kDa) and ε-momorcharin (24 kDa) were isolated from the seeds and fruits of bitter gourd. These proteins inhibited cell-free translation in rabbit reticulocyte lysate with an IC_{50} of 0.15 and 170 nM, respectively (Paul *et al.*, 1999).

5. Momorcharin I and II were basic glycoproteins with molecular weight 26 and 28 kDa, respectively. They strongly inhibited the protein synthesis in a cell-free system with IC_{50}< 0.1 ng/ml. The LD_{50} in mice of momorcharin was 2.54 mg/kg and for momorcharin II it was 9.14 mg/kg. The inhibitory effects on protein synthesis were similar to bitter gourd inhibitors, α- and β-momorcharin. In addition, momorcharin II may be more suitable for the preparation of immunotoxins because of its lower toxicity (Zeng *et al.*, 1992).

6. Thai bitter gourd protein, Mara Khee Nok at 29 kDa (MRK29) was isolated from bitter gourd ripe fruit and seeds. The 20 amino acid sequence from N-terminus of MRK29 differed from that of MAP30 in the amino acid residue numbers 5, 8, 10, 11, 12, 13, 14, 15 and 16. MRK29 differed from momordin and momorcharin in the amino acid residue number 3, 8, 13 and 19 (Jiratchariyakul *et al.*, 2001). MRK29 inhibited the HIV-1 reverse transcriptase with 50 percentage of relative inhibitory ratio (50% IR) at the concentration of 18 μg/ml. The

concentration of MRK29 at 0.175 μg/ml exerted 82% reduction of viral core protein p24 expression in HIV-infected cells. MRK29 might had a moderating effect on immune cells because it increased 3-fold the tumor necrosis factor (TNF) activity (Jiratchariyakul *et al.*, 2001). Buchakul (2001) studied the toxicity test of this protein on acute and subchronic toxicity. In acute toxicity tests, this protein was administered intravenously and intraperitoneally into mice and rats. The results showed that the LD_{50} of this protein in mice and rats was approximately 1 mg/kg. Abnormal signs and symptoms found in mice were bronchoconstriction, depression and seisure. The abnormal signs and symptoms found in rats were bronchoconstriction, depression, seisure and a red-black discharge from eyes. In subchronic toxicity tests, this protein was administered daily intrarectally into rats for three months at doses of 0.25, 0.5, 1 and 2 mg/kg/day. The abnormal signs and symptoms found were diarrhea and death caused by diarrhea. Moreover, 10% of the rats treated with this protein at a dose of 0.25 mg/kg/day had an elevation in liver enzymes. In high doses of this protein, the level of these enzymes was reduced. This protein also had an irritating effect on the rectum at the site of drug administration.

Extraction and isolation of *Momordica* seed proteins

Proteins with abortifacient, ribosome inactivating, antitumor and anti-HIV activities from bitter gourd plants were designated momorcharin, momordin, MAP30 and MRK29. They were isolated from seeds of *M. charantia*. α-momorcharin and β-momorcharin were isolated from *M. charantia* seeds. Their isolation procedures are based on aqueous extraction, acetone fractionation and ion exchange chromatography. Ammonium sulfate precipitation and gel filtrations steps that may used to improve their purification (Ng *et al.*, 1992). They were purified by an improved procedure using affinity Affi-gel Blue gel and ion exchanged Mono-s FPLC column (Go *et al.*, 1992). Furthermore, Momordin II, from *M. charantia* seeds, was purified by a procedure which involved a series of chromatographies on S- Sepharose, Sephadex G-50, CM-Sepharose and Red Sepharose columns (Valbonesi *et al.*, 1999).

δ-momorcharin , a new ribosome-inactivating protein was isolated from dried ripe seeds of *M. charantia* (Paul *et al.*,1999). Using a Mortar and pestle to powder the seeds. 5 gram of powder was homogenized in 10 mM sodium dihydrogen phosphate (NaH_2PO_4) (pH 7). The supernatant was collected as the unadsorbed fraction. The gel was then washed with buffer and

packed into a column. After all the unadsorbed proteins were removed, the column was eluted with 0.5 M NaCl in the buffer. The 0.5 M NaCl elute was concentrated by ultrafiltration and dialyzed against 2 mM NaH_2PO_4 (pH 7.5), filtered and then applied to a Mono S HR 5/5 FPLC column (Pharmacia) previously equilibrated with the dialyzing buffer. The column was washed with the same buffer and then eluted with a linear gradient of 0-60 mM NaCl of the same buffer. The fractions were pooled, concentrated accordingly and then dialyzed against distilled water. Furthermore, ε-momorcharin was isolated from *M. charantia* fruits without seeds nor pulp (Paul *et al.*, 1999). The precipitate was dissolved in and dialyzed against 10 mM NaH_2PO_4 (pH 7) before affinity chromatography in an Affi-gel blue column. The adsorbed proteins were eluted with 0.5 M NaCl. After dialysis, the proteins were resolved by FPLC on the cation exchange Mono S column. Protein concentration was determined using the method by Lowry *et al.* (1951) SDS-PAGE was performed according to the procedure of Laemmli and Favre (1973).

An inhibitor of HIV, MAP30 was isolated and purified from matured seeds of *M. charantia* (Lee-Huang *et al.*, 1990). *M. charantia* seeds were cracked and crushed into a powder. It was then extracted with ice cold 0.15 M NaCl (solution A) by homogenizing with a ratio of 6 ml of solution A/1 g of seeds. The pH of the extract was adjusted to 3.6 with 1 M hydrogen chloride (HCl). The mixture was stirred gently at 4°C for 15 minutes. The supernatant was fractionated by precipitation with ammonium sulfate [$(NH_4)_2$ SO_4] or acetone (-20°C). The fraction in the concentrated $(NH_4)_2$ SO_4 or two volumes of acetone was retained for anti-HIV activity. The precipitate was dissolved in sodium phosphate, pH 6.3 (solution B) and dialyzed against the solution. This material was referred to as step 1 sample. The step 1 sample was loaded onto a column of CM-Sepharose CL 6B equilibrated with solution B. MAP30 bound to CM-S and retained on the column. The elution was monitored by absorbance at 280 nm. The column was then eluted with a linear gradient consisting of 240 ml of solution B and 240 ml of solution B containing 0.2 M NaCl. MAP30 was found in peak 2. This material was designated as the step 2 sample. Step 2 sample was further purified by gel filtration on a column of Sephadex G75 in 20 mM sodium phosphate buffer, pH 6.3. Homogenous MAP30 was eluted as a single peak at about 0.45 column volume. The size, homogeneity and subunit structure of MAP30 was determined by SDS-PAGE in the presence and absence of 2-mercaptoethanol. A single band with a molecular

mass corresponding to 30 kDa was obtained for MAP30 both in the presence and absence of the reducing agent.

For the extract of MAP30 from ripe fruits, 2-5 kg of them were used. Thai bitter gourd contains an important protein named MRK29. MRK29 was isolated from seeds of *M. charantia* as raw material for extraction (Jiratchariyakul *et al.*, 2001). 100 grams of frozen seeds was crushed and 50 g of the endosperm was homogenized with 300 ml of ice cold 0.15 M NaCl solution. The pH of the suspension was adjusted to 3.6-4.0 with 2 N HCl, then gently stirred at 4 °C for 15 minutes. The protein filtrate was fractioned by $(NH_4)_2$ SO_4 precipitation to remove the impurities. The protein fraction precipitated at 30-60% $(NH_4)_2$ SO_4 saturation was further purified using a gel filtration column which was connected to a HPLC system. The purified protein, MRK29, had a retention time at 29.93 minutes. MRK29 is characterized by molecular weight measured by time of flight mass spectroscopy (TOFM), isoelectric point and amino acid sequencing. Protein concentration was measured by using bovine serum albumin as a standard. The assay mixture contained 20 µl of protein solution and 1 ml of diluted dye reagent. The reagent was filtered through a Whatman no.1 paper. After mixing and incubating at room temperature, the absorbance was measured at 595 nm using a Shimadzu UV 160 spectrophotometer. Using the BioRad Mini Protein apparatus was performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A number of researchers had reported and isolation and purification using various techniques of precipitations and chromatographies (Suwannaroj, 1997, Buchakul, 2001 and Tsang and Ng, 2001).

Methods for increasing protein levels in plants

Progenies of six crosses of soybean; F_2, F_3 and F_4 populations were selected and evaluated for high protein content. It was indicated that directional selection on high protein in soybean crosses could provide more genetic advances for protein content than random selection (Qui and Wang, 1992). Selection based on F_2 and F_3 single-plant protein content increased protein content in advanced generations and did not greatly affect yield but reduced oil content (Meng *et al.*, 1990). Effective selection for protein content did not have negative effect on seed yield. The protein content was estimated on the basis of protein plus oil content. The protein plus oil content could be used as a selection criterion for increasing protein content without decreasing yields

(Meng *et al.*, 1991). Morphological characteristics, yield component, number of days of various growth stages and oil content in populations of F_2 to F_4 generations of soybean crosses were not suitable to be used in indirect selection for high protein. Breeding for high yield and high protein may be carried out simultaneously (Qui *et al.*, 1991). There was heritability of protein yield per plant among crosses. Grain and protein yields were different among parents and hybrids. There was correlation between protein yield and as well as between grain and protein yields (Gu *et al.*, 1991). Some quantitative and qualitative traits of F_4 generation of hybrid soybean from intervarietal crosses involving native and foreign varieties over 2 years were analyzed. Protein content was negatively correlated with oil content (Prodanovic, 1993). Xinhai *et al.* (1999) studied the effect of selection methods on the association of yield and seed protein with agronomic characters in an interspecific cross of soybean. Progenies of F_2 to F_4 generations of an interspecific cross were made for selection by following methods: pedigree, pick-pod and mass selection under 5%, 10% and 25% selection intensities with similar selection purpose. 5%, 10% and 25% selection intensities were 25, 50 and 125 plants of desirable traits, which were selected out of total 500 plants, respectively. F_4 progenies were evaluated in replicated tests. High yield was positively associated with early maturity and decreased plant height. High protein percentage was related to later maturity and increased plant height. Because of a strong negative relationship of both seed yield and protein content with lodging and pod shattering index, increasing resistance to lodging and shattering was positively associated with high seed yield and high protein content. Genetic correlations were large and negative for branch number with both seed yield and protein content. Yield components closely associated with seed yield were number of seed weight, pods and seeds per plant and node number. Of these, node number was also positively correlated with protein content. Selection for plants with high numbers of nodes and low numbers of branches should result in the development of base populations or materials where the negative correlation between yield and protein percentage would be negligible. The analysis indicated that progenies combining high seed yield and high protein content could be readily obtained from the population influenced by mass selection of 10% intensity.

Wilcox (1998) studied increasing seed protein on soybean cultivars with eight cycles of recurrent selection. This study evaluated the changes in seed protein and seed oil concentrations of cultivars. Plants from random mating in F_2 populations segregating were evaluated in each

cycle for plant maturity, seed protein and oil concentrations. Results showed that 20% of plants with the highest seed protein in cycles 3 and the 10% of plants with the highest seed protein in cycle 4 to 7. Recurrent selection increased mean seed protein and decreased mean oil content of seeds per cycle. An average of 53% of the plants in cycles 6 to 8 had high amounts of seed protein. In spring canola (*Brassica napus)* populations, protein percentage declined slightly after eight cycles of recurrent selection (Patel *et al.*, 1999). In yellow mustard (*Sinapis alba* L.) which is an important crop for animal feed and human consumption, two cycles of recurrent selection have resulted in a significant increase in protein content simultaneously (Katepa-Mupondwa *et al.*, 1999). The response to selection for increased seed weight in the first selection cycle was positively correlated with protein content. Selecting for high protein content in the second cycle of selection was correlated with increased seed size. Oil content decreased as a correlated response to selection for increased protein content. These results indicate that there was a good potential to increase seed protein content and seed size. In addition to the increase in seed proteins by recurrent selection, Wilcox and Cavins (1995) studied the relationship between seed yield and seed protein by backcrossing high seed protein to a soybean cultivar. Parents of soybean lines that had high protein were backcrossed. High protein from the donor parent cultivar Pando was backcrossed to cultivar Cutler 71 to determine if the yield of Cutler 71 could be recovered in addition to the high protein from Pando. F_4 lines were selected randomly from the population plus three lines with the highest seed protein concentration, from the initial cross, BC_1 and the BC_2 populations were separately evaluated for agronomic traits. Seed from replication composites were evaluated for protein concentration. The parent line for each backcross was selected for high seed protein, then for yield and agronomic similarity to Culter 71. The data demonstrated that high seed protein could be backcrossed to a soybean cultivar, fully recovering the seed yield of the cultivar.

Pedigree method could be used for increasing protein in plant seeds. Pearl or Andean lupin (*Lupinus mutabilis* Sweet) is an ancient species originated from South America. It has been cultivated as a protein source for human nutrition. The seed of *L. mutabilis* contains a lot of protein and fat and could be cultivated in areas where it is not possible to plant soybean. Nineteen crosses of *L. mutabilis* were obtained by the dialel-cross of 12 lines. By using the pedigree method the four generations of progenies were planted and selected for high protein and

fat content and a low content of alkaloids in seeds. Results found that protein content of selected lines was high only after the first cycle of selection. The content of fat was increased and the content of alkaloids was decreased in selected lines, which relative to unselected lines (Hrstkova, 1998).

Factors affecting the quality and yield of seed protein

1. Varieties

Huang and Tong (1989) analyzed of 352 accessions of wild soybean (*Glycine soija*) and 16 accessions of semi-wild soybean. Protein content was positively correlated with fat content in both wild (r = 0.14) and semi-wild accessions (r = 0.62). It is concluded that selection for high protein and fat contents should be easier in semi-wild than in wild genotypes.

2. Agronomic characteristics

Escalante and Wilcox (1993) studied variation in seed protein among nodes of normal and high-protein soybean genotypes. Seeds from a single pod at all fruiting nodes and from three positions within pods of normal and high protein genotypes were analyzed for protein content in every two years. Seed protein content increased linearly from the sixth lowest fruiting node to the seventeenth highest fruiting node of both normal and high protein strains. There were no differences in protein content among seeds within pods for any strain. The data demonstrated that analyses of seed samples representative of the entire plant are essential to accurately determine seed protein of individual plants.

3. Cultural practices

Imsande (1992) measured and calculated various N-dependent growth characteristics and determined their relation to seed yields and seed protein levels in soybean. Each well-nodulated plant fixed approximately 180 mg of N during pod fill. Soybeans were grown hydroponically in a growth chamber with or without No_3-N during pod filling. When well nodulated plants were provided some N fertilizer during pod fill, the highest seed yields and the highest seed N contents

were obtained approximately 10 g/plant and 560 mg/plant respectively. Correlations between each pair of the 20 N-dependent growth-yield characteristics were generally positive except for N content (%) of the dried plant. In the absence of N fertilizer during pod fill, N content (%) of the seeds did not correlate with either harvest index or N harvest index. This suggested that in sufficient N during pod filling interfered with the orderly mobilization of foliar N to the developing seeds. New physiological parameters (seed yield merit, N yield merit and merit of genotype) were proposed for identification of genetic lines that produced both a high seed yield and a high seed protein content.