# TRYPSIN INHIBITORS FROM THREE VARIETIES OF LEGUMES: CHARACTERIZATION AND PROTEOLYTIC INHIBITION IN FISH MUSCLE SYSTEM

**RICHA KUSUMA WATI**

**MASTER OF SCIENCE**

**IN FOOD TECHNOLOGY**

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**THESIS SUBMITTED TO**

**MAE FAH LUANG UNIVERSITY IN PARTIAL FULFILLMENT OF**

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EXAMINING COMMITTEE

.....CHAIRPERSON

(Assist. Prof. Dr. Ekachai Chukeatirote)

..... MEMBER

(Assist. Prof. Dr. Lalida Shank)

..... MEMBER

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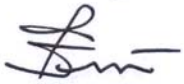

.....MEMBER

(Dr. Theerapong Theppakorn)

.....MEMBER

(Dr. Saroat Rawdkuen)

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Richa Kusuma Wati

**Thesis title** Trypsin inhibitors from three varieties of legumes: characterization and proteolytic inhibition in fish muscle system

**Author** Miss. Richa Kusuma Wati

**Degree** Master of Science (Food Technology)

**Supervisory committee** Dr. Saroat Rawdkuen Chairperson

Dr. Theerapong Theppakorn Member

# ABSTRACT

Trypsin inhibitor was isolated from navy bean (*Phaseoulus vulgaris)*, red kidney bean (*Phaseoulus vulgaris L)* and adzuki bean (*Vigna angularis*) from the Royal Project Foundation, Thailand. Extraction of navy bean and red kidney bean with 0.02 M NaOH showed the highest recovery of trypsin inhibitor while water was the best extractant for adzuki bean. The extraction time significantly affected the recovery of trypsin inhibitor ($p$<0.05). Two hours of extraction provided the best yield of trypsin inhibitor for three types of legumes. Fractionation of trypsin inhibitor was conducted by using heat treatment (70°C for 10 min), ammonium sulfate (AS) precipitation (60-80% saturation) and three phase partitioning (TPP) methods. The AS precipitation with 60-80% saturation resulted in 41, 89 and 34-fold of the purity and 74, 349 and 53% of inhibitor recovery for navy bean, red kidney bean and adzuki bean, respectively. TPP provided the purity of 5, 14, and 7-fold with 315, 441, and 228% recovery for navy bean, red kidney and adzuki bean, respectively. The SDS-PAGE revealed the major inhibitor band of navy bean with the molecular weight (MW) of 132 kDa. Meanwhile, the protein bands with the MW of 118 and 13 kDa were observed in red kidney bean and adzuki bean. The fractions from navy bean and adzuki bean showed higher pH stability compared to that of the red kidney bean and have the

optimum pH range of 7 to 9. The highest relative inhibitory activity of the fractions of navy bean and red kidney was found at 50°C and all fractions were relatively stable at 90°C for 60 min.

Increasing the concentration of salt (up to 3% w/v) did not significantly decrease the inhibitory activity of all fractions ($p>0.05$). The trypsin inhibitor fractions were capable to inhibit proteolysis of Nile tilapia. However, the autolysis of Pacific whiting and arrowtooth flounder was not inhibited by trypsin inhibitor fractions.

**Keywords**: adzuki bean / legume / navy bean / proteolytic / red kidney bean / three phase partitioning / trypsin inhibitor

# TABLE OF CONTENTS

# TABLE OF CONTENTS (Cont.)

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF FIGURES (Cont.)

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Plant seeds contain several biologically active proteins, which play various specialized functions. The most represented molecules were hydrolytic enzymes, inhibitors, lectin and the ribosome inactivating proteins (Duranti, 2003; Domoney, 1999; Brinda *et al.,* 2004). Proteins such as amylase inhibitors, lectin and trypsin inhibitor are likely to protect legume seeds against attack by predators. Protease inhibitors in legume seeds can have a major impact on nutritional value as they inhibit pancreatic serine proteases, thus impairing protein digestion (Guillamon *et al*., 2007).

The plant protease inhibitors are diverse in number and in specificity and competitiveness towards various proteolytic enzymes. They are widely distributed in plant seeds and particulary in legumes and in other storage organs (Birk, 2003). Serine protease inhibitors isolated from leguminous seeds can be classified into three families, the Bowman-Birk type inhibitors (BBIs), the Kunitz type inhibitors and the potato inhibitor I families (Zhang *et al.,*2008). The Kunitz-type inhibitors have approximate molecular weight (MW) more than 20 kDa, with low cysteine residue content and a single reactive site, meanwhile those the Bowman-Birk type have MW of 8-10 kDa with high cysteine content and two reactive sites (Laskowski and Qasim, 2000).

Fractionation is one methodology used to separate proteins into the number of smaller quantities with different specific property of the individual components. Preparative fractionation is usually performed by salting out, followed by further fractionation using ion exchanges or

affinity chromatography (Dennison, 2002). Ammonium sulfate precipitation is a widely known method used to fractionate some enzymes or proteins. Recently, separation and purification of enzymes has been developed to be more efficient, economical and scalable to isolate a target protein (Roy and Gupta, 2002). Three phase partitioning (TPP) is an emerging technique that has been used in this recent years for the extraction and purification of biomolecules. TPP uses ammonium sulfate with certain saturation to precipitate the protein and *t*-buthanol was added to make three phase layers. TPP involved some techniques like salting out, isoionic precipitation, cosolvent precipitation, osmolytic and kosmotropic precipitation of protein (Dennison and Lovrien, 1997).

Sometimes proteolysis is an unwanted process in food processing especially in the muscle foods. Martinez and Gildberg (1988) reported that the breakdown of connective tissue and myofibrillar protein might be caused by stomach and intestines bursting in fish leading to the leakage of proteolytic enzymes from digestive tract into the surrounding tissue. Endogenous serine proteases were found to cause continual degradation of myofibrillar proteins in muscle model system (Gomez-Guillen *et al.,* 2002; Benjakul *et al.,* 2003; Ohkubo *et al.,* 2008; Yarnpakdee *et al.,* 2008). The serine type proteinases were reported to be involved in proteolysis of tropical tilapia surimi (Yongsawatdigul *et al.,* 2000). In Pacific whiting, cathepsin L was the most active cysteine protease that hydrolyzed myofibrils, myosin and native and heat denatured collagen (An *et al*., 2006; Greene and Babbitt, 2006). The use of proteinase inhibitor for controlling proteolysis in muscle foods has been extensively reported. Ramiréz *et al.* (2002) reported that crude extracts from kidney bean, pea, chickpea, lentil and soybean could inhibit proteolytic activities of Mexican flounder and Atlantic croaker. Proteinase inhibitor from black cowpea and soybean seeds showed inhibitory activity against viscera proteinases of threadfin bream as indicated by reducing of modori-inducing proteinase activity (Benjakul *et al.,* 1999a).

Natural inhibitors can protect the quality of many foods more effectively than that of synthetic. The economic benefits provided by improved storage qualities are seen in reduced packaging cost, broader distribution and consumer acceptance (Shahidi*,* 1997). Ayensa *et al.* (2002) reported that the addition of bovine plasma and potato powder enhance the gel forming properties of squid mantle better than sodium pyrophosphate and ethylendiaminetetraacetic acid (EDTA). Potato powder is recommended to improve the water holding capacity of squid mantle.

Several protease inhibitor such as soybean trypsin inhibitor which are readily available from commercial sources or conveniently prepared in relatively large quantities at low cost, have been successfully used for the affinity purification of their inhibited proteases from a wide variety of sources (Fan and Wu, 2005).

Considering the demand of high activity and low cost of protease inhibitors, there is a need to find another sources of protease inhibitor like those from other legumes. varieties of legumes are important in the diet and economy of Thailand. Navy bean, red kidney bean and adzuki bean were some legumes produced by The Royal Project Foundation, Thailand. However, very little information is available on protease inhibitor in cultivar of legumes grown in Thailand. Thus this research aims to find the effective method to remove protease inhibitor as the antinutritional component for human consumption and use those legumes as potential sources of protease inhibitor as the active component which may be beneficial to the food industry.

## 1.2 Objectives

1. To isolate the trypsin inhibitor from three varieties of legume seeds from The Royal Project Foundation, Thailand.
2. To characterize the biochemical properties and its utilization for proteolytic inhibition.

## 1.3 Scope

The trypsin inhibitor in legumes consist of navy bean, red kidney bean and adzuki bean from The Royal Project Foundation were isolated (by using heat treatment, ammonium precipitation, and three phase partitioning) and characterized in term of molecular weight, inhibitory activity staining, glycoprotein staining, thermal profile and stability, pH profile and stability and salt stability. Finally, the effect of isolated protease inhibitor fraction on muscle model system was also investigated.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Legumes

The grain of food legumes is the second in importance only to cereals as source of human and animal food. The average protein content of legume grain is about 26% (w/w of dry weight), though some have up to 60%. Some legume seeds are deficient in certain essential amino acids (cysteine and methionine in particular) (Winch, 2007). Cereals that normally rich of essential amino acids are often eaten together with the legume seeds. Many legume seeds also contain certain alkaloids that must be broken down by cooking before they are eaten (Winch, 2007). Food legume seeds may contain constituents which have adverse effect on digestive enzyme activity, digestibility, nutrition and health. Some constituents were identified as protease inhibitor, lectins, saponins, goitrogenic, glycoside, cyanogenic glycoside, vicine and convicine (Sosulski and Sosulski., 2006).

Leguminous plants, after cereals, include the most important species of agricultural interest considering area cultivated and total production. The *Leguminosae* family is subdivided into three subfamilies, of which the subfamily Papilionoideae comprises most of the economically important legumes including the genus *Phaseolus*. Among the 50 described *Phaseolus* species, five (*P. vulgaris*, *P. lunatus*, *P. coccineus*, *P. acutifolius*, and *P. polyanthus*) are grown for human consumption; of these, the species *P. vulgaris* is the most cultivated worldwide accounting for 75% of the food legumes traded in the world. According to Food and Agriculture Organization data (FAO, 2006), the global production of common bean (*P. vulgaris* L.) was approximately 19 million tons in 2005. The majority of the world's legumes are used by stock feed manufacturers for animal feed. It is estimated that less than 4% of global legume production is currently consumed as human food (Grisi *et al*, 2007).

Major grain legume crops, which are commercially important to be grown in Thailand, are soybean (*Glycine max* L. Merrill), mungbean (*Vigna radiate* L. Wilczek) , blackgram (*Vigna mungo* L. Hepper) and peanut (*Arachis hypogaea* L). Soybean is the most important grain legume used mostly for oil extraction. The annual grain production was about 300,000 tons; and able to supply only 20% of the country demand. Only 12% of grain soybean demands were used for domestic consumption while others were used for feed industry (Srisombun, 2003). The FAO (2000) reported that 46.85% of world legumes were supplied by Asia, followed by Africa and North America.

**2.1.1 Composition of legumes**

Legumes consist of proteins, enzymes, proteinase and amylase inhibitors, lectins, carbohydrates, cyanogenic glycosides, lipids, vitamins and minerals, coumestrol and saponins. Fractionation of legume proteins using solubility procedures yields three major proteins: albumins, globulins and glutelins. The carbohydrates which are present in the legumes are glucose, saccharose, raffinose, stachyose, verbacose and starch. With the exception of soybeans and peanuts the lipid content is so low that they can not be considered as a source of fats and oils (Belitz and Grosch, 2002). The chemical compositions of several legumes are listed in Table 2.1.

**2.1.2 Legumes production in The Royal Project Foundation, Thailand**

**2.1.2.1 Navy bean (*Phaseolus vulgaris*)**

Navy bean or common bean (*Phaseolus vulgaris)* is now grown worldwide for its edible bean. Botanically, the common bean is classified as a dicotyledon. Before they are eaten, the raw bean seeds should be boiled for at least ten minutes to degrade a toxic compound found in the bean which would otherwise cause severe gastric upset. There are several varieties of navy bean such as snap or string beans, shell beans, pinto or mottled beans, white beans, red kidney beans, black beans, pink beans and yellow beans (Smartt, 1990). Navy bean contains 25.90% crude protein on dry weight basis and albumins accounted for 11.30% of the total crude proteins. The 88% of the total crude proteins were recovered employing acid/alkali extraction (Sathe and Salunkhe, 2006).

**Table 2.1.** Chemical composition of legumes[a]

| Name | Systematic name | Crude protein (%) | Lipid (%) | Digestible carbohydrate (%) | Crude fiber (%) | Minerals (%) |
|---|---|---|---|---|---|---|
| Soybeans | *Glycine max* | 39.0 | 19.6 | 7.6 | 16.6 | 5.5 |
| Peanuts | *Arachis hypogaea* | 27.4 | 50.7 | 9.1 | 7.5 | 2.7 |
| Peas | *Pisum sativum* | 25.7 | 1.4 | 53.7 | 18.7 | 3.0 |
| Garden beans | *Phaseolus vulgaris* | 24.1 | 1.8 | 54.1 | 19.2 | 4.4 |
| Runner beans | *Phaseolus coccineus* | 23.1 | 2.1 | n.a | n.a | 3.9 |
| Black gram | *Phaseolus mungo* | 26.9 | 1.6 | 46.3 | n.a | 3.6 |
| Green gram ( Mung beans) | *Phaseolus aureus* | 26.7 | 1.3 | 51.7 | 21.7 | 3.8 |
| Lima beans | *Phaseolus lunatus* | 25.0 | 1.6 | n.a | n.a | 3.9 |
| Chick peas | *Cicer arietinum* | 22.7 | 5.0 | 54.6 | 10.7 | 3.0 |
| Broad beans | *Vicia faba* | 26.7 | 2.3 | n.a | n.a | 3.6 |
| Lentils | *Lens culinaris* | 28.6 | 1.6 | 57.6 | 11.9 | 3.6 |

[a] The result are average values given as weight-%dry matter ; n.a : not analyzed.

Source : Belitz and Grosch (2000).

The seed of *P. vulgaris* has been the most studied in the regard of all cultivated *Phaseolus* species. Two types of anti metabolite or toxin have been found, namely protease inhibitors and phytohaemagglutinins or lectins. Both of them are inactivated by heating and beans are rendered innocuous by adequate cooking (Smartt, 1990). Phytohemagglutinins (PHA) or lectins are isolated from *Phaseolus vulgaris* using 20-35% saturation of ammonium sulfate followed by a DEAE-Sephadex A-50 column (Takhirova and Kasymova, 2001). Tormo *et al*., (2006) reported purification of pancreatic α-amylase inhibitor (α-AI) from white bean

(*Phaseoulus vulgaris*). The inhibitor significantly reduced glycaemia that occurred in patients with diabetes.

**2.1.2.2 Adzuki bean (*Vigna angularis*)**

The adzuki bean is believed to have originated in Japan and China, where it has been cultivated for many centuries. Dried adzuki beans have about the same nutritional value as dried common beans. The calcium content is only about one-fourth of the phosphorus content. The beans are rich in iron and potassium. These beans, like most other dried legumes and cereal grains, are almost totally lacking in vitamins A and C. The quality of the protein in the beans is lower than that in most animal foods because of deficiencies of some amino acids, methionine and cysteine (Audrey & Marion, 1994). Angularin, the antifungal peptide was reported to be isolated from adzuki bean by affinity chromatography on Affi-gel blue gel and ion exchange chromatography of CM-Sepharose (Ye and Ng., 2002).

**2.1.2.3 Red kidney bean (*Phaseolus vulgaris* L)**

The kidney bean with its dark red skin is named for its visual resemblance to a kidney. The kidney bean is also known as the red bean, although this usage can cause confusion with other red beans. Wu and Whitaker (1991) analyzed and characterized three trypsin-chymotrypsin inhibitors from red kidney bean (*Phaseolus vulgaris* L). These inhibitors inhibited one trypsin and one chymotrypsin/mol simultaneously. High homology was shown among these inhibitors and significant homology with lima bean and soybean Bowman-Birk inhibitors.

Nastares *et al*. (2001) found that the amino acid compositions in red kidney bean are limited of cysteine, methionine and threonine. Effect of soaking the bean into moderately acid pH (5.4) will decrease the loss of cysteine and threonine. Herzig *et al.* (1997) shows the effect of red kidney bean on inducing pancreatic growth of rat. Ingestion of red kidney bean containing high concentrations of lectin can lead to intestinal and pancreatic growth. Lectin concentration can be reduced by cooking the bean properly. The antinutritional factors such as tannins, phytic acid, polyphenols and hydrocyanic acids of red kidney bean were reduced by soaking in sodium bicarbonate solutions followed by cooking (Yasmin *et al.,* 2008). Momma (2006) investigated the digestibility of proteins in red kidney bean and a highly thermostable 20 kDa was found to be a basic subunit of legumin that resistant is to pepsin. A picture of three varieties of legumes is shown in Figure 2.1

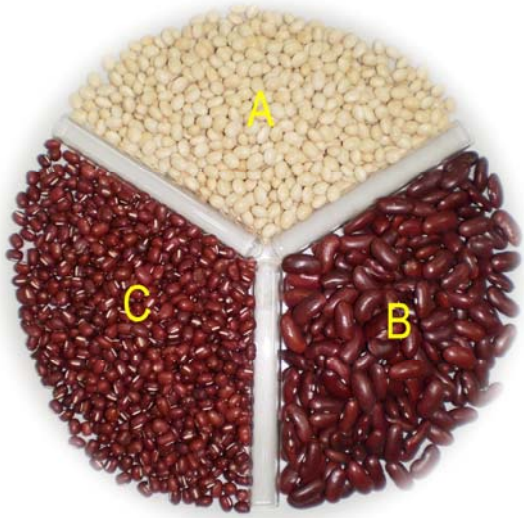


**Figure 2.1** Three varieties of legume seeds. A : navy bean; B: red kidney bean; C: adzuki bean

## 2.2 Proteolytic enzymes

Proteolytic enzymes, also called proteases, are the enzymes that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins. These enzymes are widely distributed in nearly all plants, animals and microorganisms (Joanitti *et al.*, 2006).

### 2.2.1 Classification of proteases

The standard method of classification proposed by the Enzyme Commision (EC) of the International Union of Biochemists (IUB), is based on the mode of catalysis. Protease can be classified into four groups on the basis of their reactive sites (Belitz & Grosch, 2000) as follows:

#### 2.2.1.1 Serine proteases

These enzymes have in common the presence of a serine and a histidine residue in their active sites. Typical representatives from animal sources are trypsin, chymotrypsin, elastase, plasmin and thrombin. Serine proteinases are produced by a great number of bacteria and fungi. Dahlmann *et al.* (1985) reported that serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. The MEROPS system for protease

classification, reported that some proteases belong to serine protease family such as trypsin, chymotrypsin and elastase (Beynon and Bond, 2001). Bovine trypsin 3-D structure is shown in Figure 2.2.

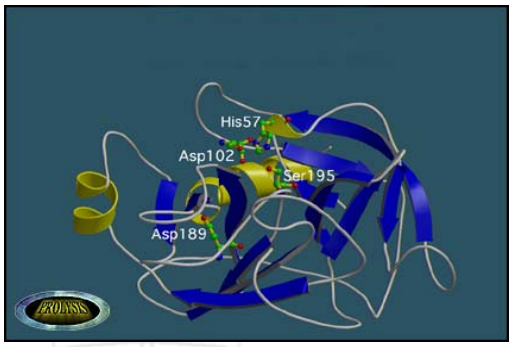


**Figure 2.2** Bovine trypsin 3-D structure

Source: Moreau (2005).

#### 2.2.1.2 Cysteine proteases

This family includes the plant proteases such as papain, ficin and bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. Papain is the archetype and the best studied member of the family (Turk *et al*., 1997). Cysteine proteases have a catalytic mechanism that involves a cysteine sulfhydrl group. Deprotonation of the cysteine sulfhydryl by an adjacent histide residue is followed by nucleophilic attack of the cysteine S on the peptide carbonyl carbon (Diwan, 2008). Papain 3-D structure is shown in Figure 2.3.

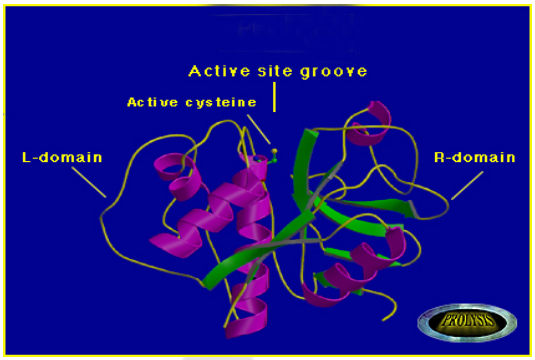


**Figure 2.3** Papain 3-D structure

Source: Moreau (2005).

**2.2.1.3Aspartic proteases**

Typical representatives of this group are enzymes of animal origin, such as pepsin and rennin, active in pH range of 2-4, and cathepsin D which has a pH optimum between 3 and 5 depending on the substrate and on the source of the enzyme. At pH 6-7 rennin cleaves a bond of k-casein with great specificity, thus causing curdling of milk. Aspartic proteases are also found in microorganism. HIV protease 3-D structure is shown in Figure 2.4.

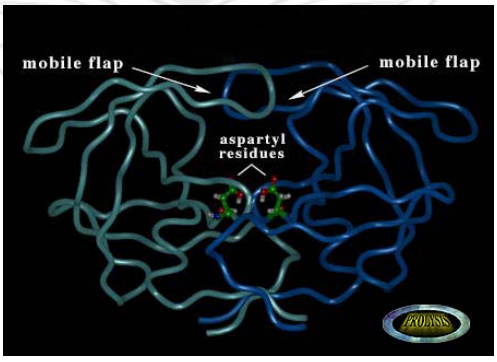


**Figure 2.4** HIV protease 3-D structure

Source: Moreau (2005).

#### 2.2.1.4 Metalloproteases

The metalloproteases include enzymes from a variety of origins, such as collagenases from higher organisms, toxins from snake venoms, and thermolysin from bacteria. They differ widely in sequence and structure, but the great majority contain a zinc atom which is catalytically active (Barret, 1995). Astacin is one example of metalloprotease found the freshwater crasyfish *Astacus astacus* Moreau (2005). Astacin 3-D structure is shown in Figure 2.5.

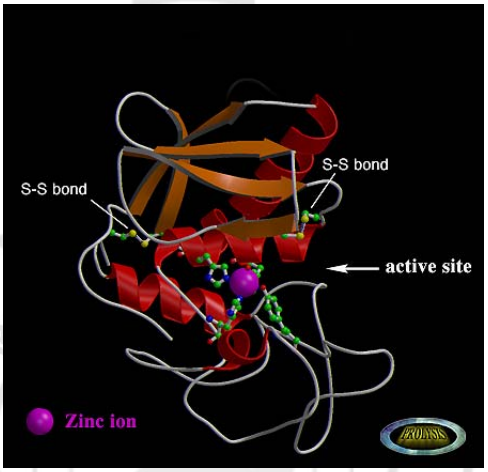


**Figure 2.5** Astacin 3-D structure

Source: Moreau (2005).

### 2.2.2 Effect of proteolytic enzymes on food products

Protease activity can be beneficial or deleterious in food production, processing and preparation. Continued activity of enzyme post mortem in mammalian muscle can result in the degradation of meat texture. This reaction can be desirable in red meats, because they are generally more tough in texture and therefore must undergo this enzymatic tenderization in order to develop and acceptable sensory quality (Rondanelli, 2002). Proteolysis is also an important metabolic activity that occurs during milk fermentation for ensuring the good environment of lactic acid bacteria in yoghurt (Farnworth, 2003). In production of cheese, proteolysis was

contributed for separated the casein curd from the milk protein. The protease from some psychotropic microorganism also can increase plasmin, the major native protease in milk that important for cheese ripening in the casein curd (Nielsen, 2002).

But post mortem softening of seafood meats due to endogenous proteases is a serious problem. Muscle softening or mushiness can be caused by endogenous proteases to the muscle as well as by digestive or organ proteases that may seep into the muscle post mortem. Some fish proteases are mainly activated at the moment of cooking, causing myosin degradation and subsequent textural destruction (Rondanelli, 2002).

Fish muscle softening by protease enzyme is one problem of surimi industry. Myofibrillar protein degradation by proteases reduces the gel strength and elasticity of surimi. The protein degradation is caused by endogenous proteases of fish muscle (Ayensa *et al.*, 2002). Yongsawatdigul *et al.,* (2000) reported the high autolytic activity of surimi made from tilapia was inhibited by soybean and leupeptin, implying that the proteinase involved was serine proteinase. Leupeptin as a specific inhibitor for calpain, substantially inhibited the autolysis of tilapia m-calpain in the presence of 1mM calcium. The m-calpain is controlled by calcium, phospholipids, calpastatin and activators, and only selectively cleave myofibrillar proteins into fragments (Jiang, 2000). One major problem for fish muscle is the occurrence of parasites, which lead to high proteolytic activity by exogenous enzymes from parasites or as an 'immune response' from the fish to the presence of parasites (Mazzarro-Manzano *et al.,* 2008). The major proteinase involved in high proteolytic activity of Pacific whiting and arrowtooth flounder was identified as cathepsin L (Visessanguan *et al.,* 2003). Rawdkuen *et al.,* (2007) reported the cysteine proteinase inhibitor from chicken plasma successfully inhibited the degradation of myosin heavy chain of Pacific whiting and arrowtooth flounder.

## 2.3 Protease inhibitors

An enzyme inhibitor is any substance that reduces the rate of an enzyme catalyzed reaction (Whitaker, 1994). Protease inhibitors mimic the protein substrate by binding to the active site of protease. Figure 2.6 shows the mechanism of protease inhibitor to mimic the substrate of the enzyme, thus will prevent the substrate to bind the active site of the enzyme. Protease

inhibitor can be broadly separated into two general categories based upon their source: synthetic and natural protease inhibitors.

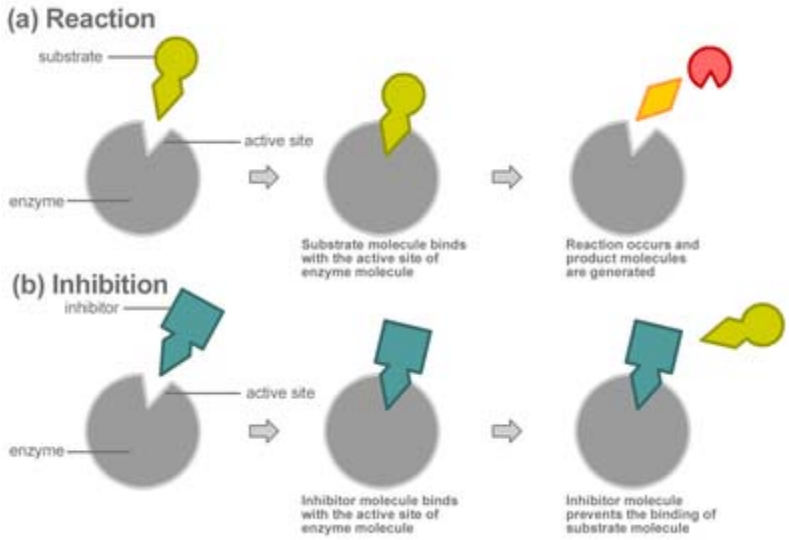


**Figure 2.6** Mechanism of inhibition of protease inhibitor
Source: Rubin (2007).

### 2.3.1 Synthetic protease inhibitors

Synthetic protease inhibitors have been made by chemical industry as alternative protease inhibitor that have specific activity for several proteases and have its own advantages and disadvantages (Lankish *et al.*, 2005). The rational design of synthetic protease inhibitors require an understanding both of the catalytic mechanism and the substrate specify of the enzyme under study. A list of some synthetic protease inhibitors is shown in Table 2.2.

**Table 2.2** Synthetic low molecular weight protease inhibitors

| Protease inhibitor | Abbr | Protease inhibited (examples) |
|---|---|---|
| Diisopropylflurorophosphate | DFP | Nearly all serine proteases |
| Phenylmethanesulfonyl fluoride | PMSF | Most serine proteases |
| (*p-Amidinophenyl*) methanesulfonyl fluoride | pAPMSF | Several serine proteases |
| Tosylphenylalanylchloromethyl ketone | TPCK | Chymomotrypsin, chymase I, bromelain, ficin, papain (not trypsin) |
| Tosyllysylchloromethyl ketone | TLCK | Trypsin, thrombin, enterokinase, plasmin, acrosin, endoproteinase LYs-C, endoproteinase Arg-C |
| Benzamidine, *p*-aminobenzamidine | PAB | Trypsin and many trypsin like proteases |
| 3,4- Dichloroisocoumarin | DCI | Many serine proteases |
| Iodoacetate | IAA | Many cysteine proteases |
| *N*-Ethyl maleimide | NEM | Most cysteine proteases |
| *p*-Hydroxymercuriphenylsulfonate | pMBS | Most cysteine proteases |
| Ethylenediamine tetraacetic acid | EDTA | Most metalloproteases |

Source: Seetharam and Sharma (1991).

**2.3.2Natural protease inhibitor**

Natural protease inhibitors commonly accumulate in high quantities in plant seeds, bird eggs, blood plasma and various body fluids (Rondanelli, 2002). Their accounts are more than 10% of the total protein from mammalian and marine animal blood plasma (Ylonen *et al*., 1999; Tahtinen *et al*., 2002).

**Table 2.3** Natural protease inhibitor in plant

| Common name | Type example | Source | Target proteases |
|---|---|---|---|
| Kunitz (plant) | Soybean Kunitz trypsin inhibitor | *Glycine max* | Trypsin, Chymotrypsin |
| MEROPS Family | Barley subtilisin inhibitor | *Hordeum vulgare* | Subtilisin, α- amylase |
| 13 A | Winged-bean chymotrypsin inhibitor | *Psophocarpus tetragonolobus* | α-chymotrypsin |
| | Kunitz cysteine peptidase inhibitor 1 | *Solanum tuberosum* | Cysteine proteases |
| Kunitz (plant) | Proteinase inhibitor A inhibitor unit | *Sagittaria sagittifolia* | Trypsin , Chymotrypsin, Kallikerin |
| MEROPS Family | Kunitz subtilisin inhibitor | *Canavalia lineate* | Subtilisin-type microbial serine proteases |
| 13 B | Cathepsin D inhibitor | *Solanum tuberosum* | Cathepsin D, trypsin |
| | Trypsin inhibitor | *Acacia confusa* | Trypsin, α-chymotrypsin |
| Cereal | Ragi seed trypsin/ α-amylase inhibitor | *Eleusine coracana* | α- amylase |
| | Barley trypsin/ factor XIIa inhibitor | *Hordeum vulgare* | α- amylase, trypsin |
| | Wheat trypsin/alpha-amylase inhibitor | *Triticum aestivum* | α- amylase, trypsin |
| | Maize trypsin/ factor XIIa inhibitor | *Zea mays* | mammalian trypsin, activated hageman factor |

**Table 2.3** Natural protease inhibitor in plant (Cont.)

| Common name | Type example | Source | Target proteases |
|---|---|---|---|
| Squash | Trypsin inhibitor MCTI-1 | *Momordica charantia* | Pancreatic elastase |
| | Trypsin inhibitor MCTI-II | *Momordica charantia* | Trypsin |
| | Macrocyclic squash trypsin inhibitor | *Momordica cochinchinensis* | Trypsin |
| | Trypsin inhibitor CSTI-IV | *Cucumis sativus* | Trypsin |
| Potato type 1 | Chymotrypsin inhibitor I | *Solanum tuberosum* | Chymotrypsin, trypsin |
| | Glutamyl peptidase II inhibitor | *Momordica charantia* | Glu S.griseus protease, subtilisin |
| | Subtilisin-chymotrypsin inhibitor CI-1A | *Hordeum vulgare* | Subtilisin, chymotrypsin |
| | Wheat subtilisin/chymotrypsin inhibitor | *Triticum aestivum* | B.lichenoformis subtilisin, α-chymotrypsin |
| Mustard | Mustard trypsin inhibitor | *Sinapsis alba* | Beta-trypsin |
| | Mustard trypsin inhibitor -2 | *Brassica hirta* | Bovine beta-trypsin, α-chymotrypsin |
| | Rape trypsin inhibitor | *Brassica napus* | Trypsin, chymotrypsin |
| Cystatin | Onchocystatin | *Onchocerca volvulus* | Cysteine proteinase |
| | Ovocystatin | *Gallus gallus* | Thiol proteinase |
| | Oryzacystatin | *Oryza sativa* | Cysteine proteinase |

**Table 2.3** Natural protease inhibitor in plant (Cont.)

| Common name | Type example | Source | Target proteases |
|---|---|---|---|
| Kininogen | Metalloprotease inhibitor | *Bothrops jararaca* | Atrolysin C, jararhagin |
| | Sacrocystatin | *Sarcophagi peregrine* | Cysteine proteinase |
| Bowman-Birk | Bowman-Birk plant trypsin inhibitor unit 1 | *Glycine max* | Trypsin, chymotrypsin |
| | Bowman-Birk trypsin/chymotrypsin inhibitor | *Arachis hypogea* | Trypsin, chymotrypsin |
| | Sunflower cyclic trypsin inhibitor | *Helianthus annuus* | Trypsin, cathepsin G, elastase, chymotrypsin |

Source: Habib and Fazili (2007).

Natural inhibitors of proteases are normally small proteins, with structures highly complementary to their target enzymes substrate-binding clefts. They can, therefore, bind the target enzymes with high affinity, resulting in efficient blocking of substrate binding to the enzyme. This is an inhibition of enzymatic activity. Some natural protease inhibitors are listed on Table 2.3. The protease inhibitor was classified into 4 groups as follows:

**2.3.2.1 Serine protease inhibitors (Serpin)**

The serpin family is the largest and the most widespread superfamily of protease inhibitor. Plant serpins have been purified and characterized from cereal seeds (Jones, 2005; Yoo *et al*., 2000; Tsybina *et al*., 2004) and potato tuber (Cv. Elkana) (Pouvreau *et al.,* 2003). A feature of all the protease inhibitors in the serpin superfamily is a particular peptide bond, located in a C-terminal domain, which is susceptible to attack by serine proteases (Carlson, 1996).

Trypsin inhibitors that have been isolated consist of two types, the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBI). KTI consists of 181 amino acid residues and includes two disulfide bridges. The molecular weight of KTI is about 20-24 kDa. KTI is primarily an inhibitor of trypsin, but also weakly inhibits chymotrypsin. It is inactivated by heat and by gastric juice (Birk, 2003). KTI are widespread in plants and have been found in legumes, cereals and in solanaceous species (Ishikawa *et al*., 1994).

BBI servers as the prototype for a family of inhibitors those are predominant inhibitor in legumes (Birk, 2003). BBI has a molecular weight of about 8 kDa with a high content of half cystines forming seven disulfide bridges (Odani & Ikenaka, 1973). These inhibitors interact independently, but simultaneously with proteases (Raj *et al*., 2002). The reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase (Qi *et al*., 2005).

**2.3.2.2 Cysteine protease inhibitors (Cystatin)**

The cystatin superfamily is composed of several families and includes proteins that are related in structure and function to an inhibitor of cysteine proteinase. The members of these families inhibit the activity of cysteine proteases and are called cysteine proteinase inhibitor or cystatins. The cystatin superfamily contains three families of proteins that are related functionally as cysteine protease inhibitors and evolutionarily by their amino acid sequence identity. They are widely distributed in plants, animals and microorganisms (Oliveira *et al*., 2003).

*Family I* (Stefin family): the members of this group have a molecular mass of about 11 kDa. They are generally present in the cytosol and are devoid any carbohydrate groups and disulfide bonds (Stato *et al*., 1990).

*Family II* (Cystatin family): these inhibitors consist of proteins with 120-126 amino acids and the molecular mass 13.4-14.4 kDa. These inhibitors contain two disulfide bonds but are devoid any carbohydrate groups (Grzonka *et al*., 2001).

*Family III* (Kininogen family): these inhibitors are glycoproteins and are of three different types. High Molecular Weight kininogens (HMW) with a molecular mass of 120 kDa and Low Molecular Weight kininogens (LMW) with molecular mass ranging between 60 and 80 kDa are known (Otto & Schirmeister, 1997).

**2.3.2.3 Aspartic protease inhibitors**

Aspartic proteinase inhibitors can be found in many sources such as potato, sunflower, barley, caroon (*Cyanara cardunculus*) flowers, yeast, the nematode *Ascaris*, and squash (García-Carreño & Hernández-Cortéz, 2000). An aspartyl protease inhibitor described in potato tubers shares considerable amino acid sequence homology with soybean trypsin inhibitor. It is a 27 kDa protein and inhibits serine proteases trypsin and chymotrypsin in addition to the aspartyl protease cathepsin D (Lawrence & Koundal, 2002).

**2.3.2.4 Metalloprotease inhibitors**

Any substrate that complexes with and/or removes an essential cation from an apoenzyme will be an inhibitor of that enzyme (Whitaker, 1994). Most of the design of class specific inhibitors of metalloproteases has focused on attempts to chelate or bind the catalytic zinc atom. Synthetic inhibitors, therefore, commonly contain a negatively-charged moiety to which is attached a series of other groups designed to fit the specificity pockets of a particular protease (Whitaker, 1994).

A metallocarboxypeptidases inhibitor is found to accumulate in potato tuber tissues during development, along with the potato inhibitor I and II families of serine protease inhibitor. These inhibitors are small peptide inhibitors consisting of 38-39 amino acid residues and have the molecular mass of about 4.2 kDa (Hass *et al.*, 1975)

## 2.4 Antinutritional compounds in legumes

In addition to the widely studied inhibitors of proteolytic and amylolytic enzymes and lectins, legumes also contain several other antinutritional/antiphysiological factors. Salukhe and Deshpande (1991) reported that those antinutritional factors of legumes seeds received little attention because they occur in small amounts and under normal condition do not pose a serious health hazard. The commonly occurring antinutrients in food legumes are lectins, proteinase inhibitor, amylase inhibitor, flatus-causing raffinose oligosaccharides, phytates, condensed tannins, goitrogens, cyanogenic glycosides, saponins, allergens, lathyrism, favism, alkaloids, and toxic amino acids (Salunkhe & Deshpande, 1991).

Vadivel and Janardhanan (2001) found the antinutritional factors such as free phenolics, tannins, L-DOPA, trypsin inhibitor, chymotrypsin inhibitor and haemagglutinating in *Cassia floribunda* seed. Their research group found that the levels of those antinutritional factors were not considered a threat to human health if the seeds were properly processed. Ahmed *et al*. (2006) also found the antinutritional factor in guar gum seeds and studied the effect of soaking followed dehulling treatments. Soaking and dehulling significantly increased protein content. This suggests that those treatments reduce the amounts of antinutritional in the seeds.

Raw legume with high contents of toxic compound such as protease inhibitors, alkaloids and tannins can reduce intake or the ability to digest feed, resulting growth and production reduction. Alkaloids and lectins can cause scouring or death at high level. These antinutritional factors also have major effects on monogastric but have less effect on ruminants as microbial fermentation in rumen can breakdown some toxic compounds (Moss, 2005). Lathyrism and favism are diseases in man which are associated with the consumption of specific legumes, *Lathyrus sativus* and *Vicia faba*, respectively. The toxic principles have been isolated from several species of lathyrus which can produce neurotoxic symptoms in animals; however the exact etiology of this disease in man is not known (Liener, 1962). With the deleterious effect for human, the antinutritional factors in legume should be removed by proper process both physical and chemical treatment.

### 2.4.1 Enzyme inhibitors in legumes

The first known plant inhibitor was that from soybean, which was discovered in 1944. Another inhibitor was later found in lima beans, as well as in many of the Leguminosae. Investigation of the 1960's suggested that all of the Leguminosae contain proteinase inhibitors, and that the earlier negative results probably due to analytical shortcomings. A partial list of the distribution of protease inhibitors present in legumes, as compiled by Liener (1989), is given in Table 2.4.

Protease inhibitor found in legumes seeds proven to lead reversible pancreatic blistering for chicken and rats. This inhibitor also causes the inhibition growth of rats due to some amino acid deficiency. The function of this inhibitor in seeds is known to be the inhibitors against damage to plant by higher animal such as insects and microorganism. For human, this inhibitor is not dangerous in certain amount, because it will be inactivated by human gastric juice. Amylase inhibitor is also found in legumes but it does not influence the digestion of starch of human due to its relatively low amounts ingested with the food is compared with to amylase activity present in human body (Belitz & Grosch, 2000).

### 2.4.2 Other compounds in legumes

Lectin or hemagglutinins is a compound that attached themselves to erythrocytes and caused agglutination or precipitation of blood. Therefore, the toxic properties of lectin is not caused by the hemagglutination activity, but several research suggest that it caused by the binding of lectins to epithelial cells on the intestinal wall, causing a deleterious nutritional effect by interfering the absorption of nutrition, while the other research show the act of lectins as inhibitor of protein biosynthesis (Belitz & Grosch, 2000).

Phytates are known to be a chelating agent that reduces the bioavailability of divalent cations. They bind with mineral such as iron, calcium, and zinc; this will be effect the absorption of those minerals (Lestienne *et al*., 2005). Some research suggest that phytates may have health benefit such as delayed nutrient absorption, decreased cancer risk and increased fecal bulk and lowering of blood lipids (Klopfenstein, 2000)

Saponins are phytochemicals which are present in many fiber-rich foods, particulary legumes. They have been shown to increase fecal excretion of bile acids in human and animal studies. In particular, some saponins from yucca and alfalfa seeds have been shown to lower

plasma cholesterol in humans (Oakenfull and Potter, 2001). Due to their surfactant and hemolytic properties, saponins become highly toxic when injected into the bloodstream (Ebadi, 2006).

**2.4.3 Method for removing antinutritional compounds**

Traditionally, several methods have been used to remove the antinutrients present in plant foods in order to improve their nutritional quality and utilization. Physical and chemical means of removing undesirable antinutrients include such processing methods as soaking, cooking, germination, fermentation, selective extraction, membrane filtration, irradiation and enzymatic treatments (Salunkhe and Deshpande, 1991).

Cooking or heat processing is probably the oldest known method of processing plant foods for human consumption. Cooking generally inactivate heat–sensitive factors such as enzymes, inhibitors, lectins, volatile compounds such as hydrogen cyanide (HCN), and some off-flavor components (Salunkhe and Deshpande, 1991).

Deshpande (1992) critically reviewed the significance of trypsin inhibitors in human nutrition. He concluded that the inhibitor activity can readily be destroyed by more than 90% if the legumes are processed properly, i.e., by at least 30 to 60 minute boiling water treatment or 15-20 minute autoclaving at 15 PSI. Moreover, legumes are often pre-processed by one of several different method (soaking overnight is not uncommon) that alone may reduce the inhibitory activity to varying degrees. The possibility of residual inhibitory activity that would be nutritionally detrimental in thus processed legumes therefore appears to be quite small.

Some methods such as soaking, germination, cooking and fermentation were used to remove antinutritional factors of legumes. Ibrahim *et al*., (2002) reported the antinutritional factor of cowpea was effectively removed by cooking pregerminated cowpea and fermentation completely removed trypsin inhibitor, oligosaccharides and phytic acid but the tannins was increased. The trypsin inhibitor and phytic acids were effectively removed by roasting, while polyphenols and tannins decreased by the boiling and pressure cooking of Indian bean (*Dolichos lablab* L) seeds (Ramakrishna *et al.,* 2008).

**Table 2.4** Distribution of protease inhibitors present in legumes

| **Botanical name** | **Common name** | **Proteinases inhibited**[a] |
|---|---|---|
| *Arachis hypogaea* | Peanut, groundnut | T, C, Pl, K |
| *Cajanus cajan* | Pigeon pea, red gram | T |
| *Chamacrista fasiculata* | Partridge pea | T |
| *Cicer arietinum* | Chickpea, Bengal gram, Garbanzo | T, C |
| *Dolichos biflorus* | Horse gram | T |
| *Glycine max* | Soybean | T, C |
| *Lathyrus odoratus* | Sweet pea | T |
| *Lathyrus sativus* | Chickling vetch | T, C |
| *Lens esculenta (culinaris)* | Lentil | T, C |
| *Mucana deeringianum* | Florida velvet bean | T |
| *Phaseolus aconitifolius* | Moth bean | T |
| *Vigna angularis* | Adzuki bean | T, C |
| *Phaseolus aureus* | Mung bean, green gram | T |
| *Phaseolus coccineus* | Scarlet runner bean | T, C |
| *Phaseolus lunatus* | Lima bean, butter bean | T, C |
| *Phaseolus mungo (radiatus)* | Black gram | T, C, S |
| *Phaseolus vulgaris* | Navy bean, kidney bean, pinto bean, French bean, white bean, wax bean, haricot bean, garden bean | T, C, E, S |
| *Pisum sativum* | Field bean, garden pea | T |
| *Psophocarpus tetragonolobus* | Winged bean, Gao bean | T |
| *Vicia faba* | Broad bean, field bean, faba bean | T, C, Th, Pr, Pa |

[a]C, chymotrypsin; E, elastase; K, Kallikrein; Pa, papain; Pl, plasmin; Pr, pronase; S, subtilisin; T, trypsin; Th, thrombin

Source: Liener (1989)

## 2.5 Isolation and characterization of protease inhibitors from legumes

Protein inhibitors of proteinases (or protease inhibitors) are ubiquitous. They are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms (Birk, 2003). Their gross physiological function is the prevention of undesirable proteolysis, but detailed physiological functions have been only rarely elucidated. In some natural resources, the available literature data on proteinase inhibitors from various legumes are summarized in Table 2.5.

Although trypsin inhibitors from food legumes particularly soybeans, are studied most widely, they are also found in several other food products, including the staple cereals and various meal, such as cereals (rice, wheat, corn, tritcale, rye, barley, millet, sorghum, buckwheat, cereal based products), roots and tubers (potato, sweet potato, yam, taro, cassava), vegetables (cabbage, tomato, lettuce, radish, onion, carrot, sweet corn), fruit and nuts (brazil nut, apple, banana, orange, raisin), animal foods (milk, cheese, beef, lamb, pork, poultry, sweetbread, fish, meat-based products), and miscellaneous (table sugar, butter, margarine, tea, coffee, drinking chocolate, milk chocolate) ( Deshpande, 2002).

To isolate protease inhibitor as protein from plant or animal cell, there is several steps that we can use depends on type of the sample. In order to isolate intracellular proteins, cells must be disrupted. An efficient protocol for cell disruption must be developed to release the protein insoluble form from its intracellular compartment. The disruption protocol should be as gentle as possible to the protein, as the extraction step is the starting point of all subsequent procedures (Ahmed, 2004).

The purification of protein is an essential fist step for the study of its molecular and biological properties in order to understand its biological function. There are several properties (such as molecular weight, charge, hydrophobicity, etc) that can be exploited to purify or single out a protein from a mixture. Based on those properties, several chromatographic and non chromatographic (electrophoretic, precipitation, membrane-filtration) procedures have become available (Ahmed, 2004)

**Table 2.5** Characteristic of proteinase inhibitors in food legumes

| Legume | MW, (Da) | Specificity | Characteristics | Ref |
|---|---|---|---|---|
| *Glycine max* (soybean) | 21,700 | T | Has Trp, primary sequence of 181 amino acids is known, single headed Kunitz type | Odani and Ikenaka (1973). |
| | 8,000 | T, C | Double headed Bowman Birk type, contains seven disulfide bonds | |
| *Phaseolus acutifolius* (Tepary bean) | 8,765 | T, C | Isoform homology with Bowman Birk inhibitor | Campos *et al* (2004). |
| *Phaseolus vulgaris* (Navy bean) | 23,000<br>7,900 | T,C | Four possible isomers present | Birk (1976). |
| *Vicia faba* (Faba bean) | 18,000 | T, C | Stable pH 4 and 5, heat stable, Bowman-Birk type inhibitors | Gupta *et al* (2000). |
| | 13,000 | T, C | Antifungal activities, inhibit HIV 1 reverse transcriptase | Ye and Ng (2002). |
| *Vigna unguiculata* (Cowpea) | 10,700 A<br>10,700 B<br>10,500<br>15,000 | T<br>T, C<br>T, C<br>T, C | Isoinhibitors, amino acid sequence homology with lima bean, *Macrotyloma axillare* and mung bean | Sammour (2005). |
| *Voandzeia subterranean* (Bambara groundnut) | 13,000 | T | Heat stable, wide pH range activity | Benjakul *et al* (1999b). |
| *Cajanus cajan* (Pigeon pea) | 15,000<br>25,000 | T | Heat stable, wide pH range activity | Benjakul *et al* (1999b). |

[a]C, chymotrypsin T, trypsin

A protein may be purified by a single step (for example, affinity chromatography) or by a combination of several step (for example, salt fractionation, ion exchange, gel filtration, etc). In general, anion-exchange chromatography is employed for the purification of an acidic protein. Similarly, for the purification of a basic protein, cation exchange chromatography is the better choice. Reverse-phase chromatography is suitable for a family of active proteins of similar charge. Purification steps divide the total protein in the crude extract into several fraction, each of which is the assayed for activity and protein content. A fraction with high specific activity and purification-fold dictates the success of it purification step (Ahmed, 2004).

*Calliandra selloi* trypsin inhibitor (CSTI) with yield of 0.5% of total protein of the seed extract was purified by combination of two chromatographic steps, affinity chromatography on trypsin-agarose column followed by ion exchange chromatography on DEAE column (Yoshizaki *et al.,* 2007). The partial purification by heat treatment and ammonium sulfate precipitation was used to isolate trypsin inhibitors from cowpea, pigeon pea and bambara groundnut grown in Thailand (Benjakul *el al.,* 1999b). Cysteine proteinase inhibitor (CPI) from Lima beans was isolated by a chromatography series using DEAE Sepharose, Phenyl Sepharose, hydroxyapatite and reversed-phase high performance liquid chromatography resulted in a 20 kDa protein with high inhibitory against papain (Lawrence and Nielsen, 2001).

Three phase partitioning (TPP) is a salting out type fractionation procedure, in which proteins dissolved are precipitated by addition of ammonium sulfate and *tert*-butanol to the solution. Since 1984, when the method was published it was applied for purification of various enzymes, nevertheless, the mechanism of TPP is not completely understood (Dennison and Lovrien, 1997). *t*-butanol is infinitely miscible with water but with sufficient amount of ammonium sulfate, the solution will splits into two phases, an underlying aqueous phase and overlying *t*-butanol phase. If the protein present in the initial solution, three phases would be formed, protein being precipitated in a third phase between the aqueous and *t*-butanol phases (Dennison, 2003). The process of TPP depicted in Figure 2.7.

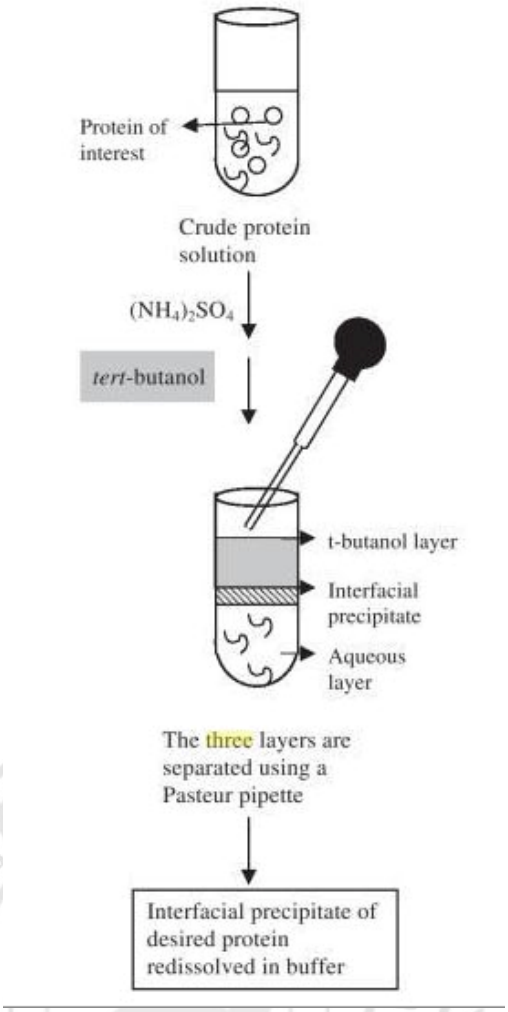


**Figure 2.7** Schematic presentation of three phase partitioning (TPP)

Source: Mondal *et al*., (2006).

During TPP, as the proportion of *t*-butanol increases, the protein conformation may become distorted as it acquire a greater proportion of a-helices. This distortion leads to the denaturation of many proteins, which may be a disadvantage. On the other hand, if the protein of interest is able to survive TPP, then its likely that TPP will effect a purification by the denaturation of impurities, as well as by it fractionating ability (Dennison, 2003).

The bifunctional amylase/protease inhibitor was successfully purified from ragi (*Eleusine coracana*) by two stages of TPP with the yield 83% and 80% for amylase inhibitor and

trypsin inhibitor (Saxena *et al.,* 2007). Sharma and Gupta (2001) reported the TPP method was effectively able to purify a wheat germ bifunctional protease/amylase inhibitor with 85% recovery.

## 2.6 Application of protease inhibitors in foods

The inhibitory effects of certain proteins on endogenous enzymes *in vivo* have been exploited in the processing of certain foods. Recently, the plasma glycoprotein, $\alpha_2$-macroglobulin (MAC) was patented for the treatment of fish and other muscle food due its broad spectrum of inhibitory activity on proteases (Lorier & Aitken, 1991). A MAC rich fraction from beef plasma was applied to prevent modori (gel weakening) in surimi gels from fish species like the New Zealand hoki, Atlantic menhaden and the Alaskan Pollock (Hamman *et al*., 1990).

Cystatin from chicken egg white had also been shown to inhibit proteolytic activity when egg white was incorporated in surimi gels from croaker flesh, Pacific whiting and arrowtooth flounder (Akazawa *et al.*, 1993). Inhibitors derived from potatoes also inhibited proteolytic activity in fish paste to result in good quality surimi products from various non traditional fish species, while soybean trypsin inhibitor was also used to inhibit protease activity in squid mantle (Shahidi *et al*., 1997).

Egg powder and whey protein concentrate was showed 77% and 96% inhibiton of autolytic activity in lizardfish (*Suurida tumbil*) mince and surimi (Yongsawatdigul and Piyadhammaviboon, 2004). The autolytic activity of Pacific whiting and arrowtooth flounder also found to be inhibited by addition of cysteine proteinase inhibitor from chicken plasma (Rawdkuen *et al.,* 2007).

Apart from the uses of inhibitors in surimi, inhibitors may be used as processing aids in all those food operation units affected by unwanted proteolysis. The fact that the control of proteolysis by inhibitors is so specific makes it a valuable tool in medicine, agriculture and food technology. The presence of protease inhibitors in foods decreases the apparent nutritional quality of protein in the diet, by affecting the ability of the body's digestive enzymes to degrade dietary protein, and thus limiting the intake of amino acids needed to construct new proteins

(García-Carreño, 1996). Table 2.6 gives a list of potential uses of protease inhibitors in food processing.

**Table 2.6** Potential uses of inhibitors in food industry

| Seafood | Target enzyme of process |
|---|---|
| **Underused marine resources** | |
| Squid | Serine protease and cathepsin C reducing the gel forming ability of muscle |
| **Caught or aquafarmed** | |
| Crustaceans | |
| Krill | Digestive enzymes –autolysis of tail muscle |
| Crayfish | |
| Langostilla | |
| Shrimp | |
| **Fish processing** | |
| Sardine | Cathepsins; reducing the gel forming ability of muscle in surimi production |
| Haddock (several species) | Limited collagen hydrolysis in descaling |
| **Egg cells in roe and caviar production** | Limited adhering connective tissue hydrolysis |
| **Several inhibitor sources** | Production of speciality marine enzymes ;purification by affinity chromatography |

Source: García-Carreño and Hernández-Cortéz (2000)

Ayensa *et al*. (2002) reported that the effect of 4 food-grade protease inhibitors (bovine plasma, potato powder, etyhlenediaminetetraacetic acid, and sodium pyrophosphate) on gelation of squid muscle. The gelation profile showed that the setting effect was greatest with pyrophosphate and that gel rigidity was highest with bovine plasma.

Gomez-Guillén *et al*. (2002) also reported that addition of phenylmethlysulfonyl fluoride (PMSF) inhibit autolytic activity of squid muscle during thermal gelation. The strong inhibition of autolytic activity by PMSF confirmed the predominance of serine protease in autolytic activity of squid. Ramírez *et al*. (2002) evaluated the effect of crude extract from kidney bean, pea, chickpea, lentil and soybean on modori phenomenon in fish species. The modori phenomenon from several fish species has been associated with endogenous proteinolytic enzymes, and decrease the modori effect by inhibit the responsible enzymes with serine protease inhibitor from legumes

The effect of protease inhibitors from pea and butter beans was successfully inhibited the bacterial growth and reduced levels of trimethylamine-nitrogen and total volatile base-nitrogen of *Johnius dussumieri* mince at refrigerated temperatures (Abraham *et al.,* 2006). The protease inhibitor from potato tubers that contain inhibitors for typsin, chymotrypsin, carboxypeptidase A and carboxypeptidase B inhibited the proteolysis in stored ground herring (Aksnes, 2006). The addition of PMSF and EDTA that partially inhibited the degradation of myofibrillar proteins of prespawning hake showed the metalloproteases and serine proteases could be involved in the autolysis of myofibrils (Pagano *et al.,* 2007)

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Raw materials

Navy bean (*Phaseolus vulgaris*), red kidney bean (*Phaseolus vulgaris L*) and adzuki bean (*Vigna angularis*) were used in this research. Legumes were obtained from The Royal Project Foundation, Chiang Mai, Thailand. The legumes stored at room temperature in dry space. Nile tilapia (*Tilapia nilotica*) was obtained from Lotus supermarket, Chiang Rai, Thailand. Frozen Pacific whiting and arrowtooth flounder fillets were obtained from Pacific Seafood (OR, USA) and grocery store in Raleigh (NC, USA).

### 3.1.2 Chemicals

**Table 3.1.** Chemical list

| Steps | Steps |
|---|---|
| **Extraction** | **Three phase partitioning** |
| - Hexane | - Tert-buthanol ($C_4H_{10}O$) |
| - Sodium chloride (NaCl) | **Characterization** |
| - Sodium hydroxide (NaOH) | ***A. Protein content determination*** |
| **Fractionation** | - Biuret reagent consist of |
| - Ammonium sulfate [$(NH_4)_2SO_4$] | Sodium potassioum tartrate ($NaKC_4H_4O_6$. 6 $H_2O$) |
| - Calcium chloride ($CaCl_2$) | - Bovine serum albumin (BSA) |
| - Magnesium chloride hexahydrate ($MgCl_2$. $6H_2O$) | - Tris-HCl buffer (pH 8-10) |

**Table 3.1** Chemical list (Cont.)

| Steps | Steps |
|---|---|
| ***B. Inhibitory activity assay*** | ***E. Glycoprotein staining*** |
| - N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) | - Gelcode® Glycoprotein Stain |
| - Acetic acid ($CH_3COOH$) | - Acetic acid ($CH_3COOH$) |
| ***C. Electrophoresis*** | ***F. pH profile and stability*** |
| - Sodium dodecyl sulfate (SDS) | - Glycine (pH 3) |
| - Polyacrylamide | - Sodium acetate ( $NaCH_3COO$) buffer (pH 4-5) |
| - Coomasie Brilliant Blue R-250 | - Sodium phosphate ($Na_2HPO_4$) buffer (pH 6-7) |
| - Methanol | - Tris-HCl buffer (pH 8-9) |
| - Molecular weight marker | - Sodium carbonate ($Na_2CO_3$) buffer (pH 10-12) |
| ***D. Inhibityory activity staining*** | ***G. Effect of salt*** |
| - Ethylendediamine tetraacetic acid (EDTA) | - Sodium chloride (NaCl) |
| - β-mercaptoethanol | |
| - Triton X-10 | |

### 3.1.3 Instruments

**Table 3.2.** Instrument list

| Instrument | Model | Company, Country |
|---|---|---|
| Waterbath | WNB 7 | Memmert, Schwabach, Germany |
| Spectro Libra | S22 | Biochrom, Cambridge, USA |
| IEC Micromax Microcentrifuge | 3591 | Thermo electron corp, China |
| Mini Protean Tetra Cell unit | - | Bio Rad Laboratories, Inc, Richmond, CA, USA |
| pH meter | pH 510 | Eutech Instrument, Singapore |
| Hot Plate | SLR | Scott Instrument, GMBH Germany |
| Orbital shaker | SK2PO | Wellab, Korea |

**Table 3.2.** Instrument list (Cont.)

| Instrument | Model | Company, Country |
|---|---|---|
| Coffee grinder | CG2100 | Homemate, Bangkok |
| Ultrasonic centrifuge Avanti | J-301 | Beckman Coulter, USA |
| Homogenizer | IKA Ultra Turrax | IKA® Werke GmbH & Co. KG, Germany |
| Panasonic Food Processor | MK5086M | Panasonic, Selangor, Malaysia |
| Vortex Genie | 2 | Scientific Industries Inc, New York, USA |

## 3.2 Methods

### 3.2.1 Extraction of protease inhibitor from three varieties of legume seeds

***1. Sample preparation***

Legume seeds were ground using a coffee mill to particle size of 20 meshes. Seed flour was defatted by mixing it with hexane for 10 min at a ratio of 1:5 (w/v). The mixture was filtered through Whatman No.1 filter paper and the sediment was rinsed with hexane 3 times to remove the residual oil in the ground sample. The defatted sample was air-dried at an ambient temperature (28-30°C) until dry and free of hexane odor**.**

***2. Extraction medium***

The defatted samples were extracted with 0.01, 0.02 M NaOH, 0.15, 0.30 M NaCl and distilled water at the ratio of 1:5 (w/v) and then shaken at 180 rpm at ambient temperature for 1 hour. The supernatant was recovered by centrifuging the mixture at 8,000*xg* for 30 min at 4°C. The trypsin inhibitory activity and the protein content of extracted samples were determined. The specific trypsin inhibitory activity of the extracts using the different extractants was compared.

***3. Extraction time***

The best extractant that showed the highest specific inhibitory activity was used to study the effect of extraction time on the recovery of the trypsin inhibitor. The defatted samples were extracted with the best extractant obtained from 3.2.1.2.1 for 1, 2, 3, 4, and 5 h at room

temperature. The inhibitory activity and the protein content were measured and compared in term of specific inhibitory activity.

### 3.2.2 Fractionation of trypsin inhibitor from legume

***1. Heat treatment***

Crude extracts (3.2.1) were heated at the temperatures between 50 and 70°C for 10 min and then cooled with iced water. To remove coagulated debris, the extracts were centrifuged at 8,000xg for 5 min at room temperature. The inhibitory activity and the specific inhibitory activity of the trypsin inhibitor in the supernatant were determined. The highest specific activity of the heat treatment fraction was chosen for further study.

***2. Ammonium sulfate precipitation***

Heat treated fraction was added to solid AS to reach a concentration of 20% saturation. The mixture was stirred at 4°C for 2 h and centrifuged at 7000xg for 15 min at 4°C. The precipitated protein was collected and referred to 'AS precipitate I: 0-20% AS'. The 20% saturated AS supernatant was treated with solid AS to reach 40% saturation and the protein precipitate was referred to as 'AS precipitate II: 20-40% AS'. The proteins in the supernatant were further fractionated with 60% ad 80% saturation and the pellets were referred to 'AS precipitates III: 40-60% AS' and precipitate IV: 60-80% AS', respectively. The supernatant obtained after addition of 80% saturation AS was referred to 'AS supernatant IV: >80% AS'. All precipitates were dissolved in 10 mM phosphate buffer, pH 7.4, containing 0.9 mM $CaCl_2$ and 0.05 mM $MgCl_2.6H_2O$ at the ratio of 1:9 (w/v). All fractions were dialyzed against the same buffer overnight at 4°C with the dialysis buffer being changed four times to remove residual AS, and then stored at 4°C until used.

***3. Three phase partitioning (TPP)***

TPP was carried out as described by Roy and Gupta (2000). The heat extract (2.2.2) was added with ammonium sulfate to 30% saturation and vortexed gently to dissolve the salt, followed by the addition of *t*-butanol with the ratio of 1:1 (v/v). The mixture was allowed to stand for 1 h at room temperature and then subjected to centrifuge at 5000xg for 10 min to facilitate separation of phases (Figure 3.1). The lower aqueous layer (bottom phase) and the interfacial precipitate (middle phase) were collected. The latter was dissolved in 0.05 M Tris-HCl pH 8.2

(containing 0.02 $CaCl_2$) with the ratio of 1:1 (w/v). Both phases were dialyzed against water at 4°C to remove ammonium sulfate. The activity and specific activity of each phase were determined. The phases that give the highest specific activity were chosen for characterization.

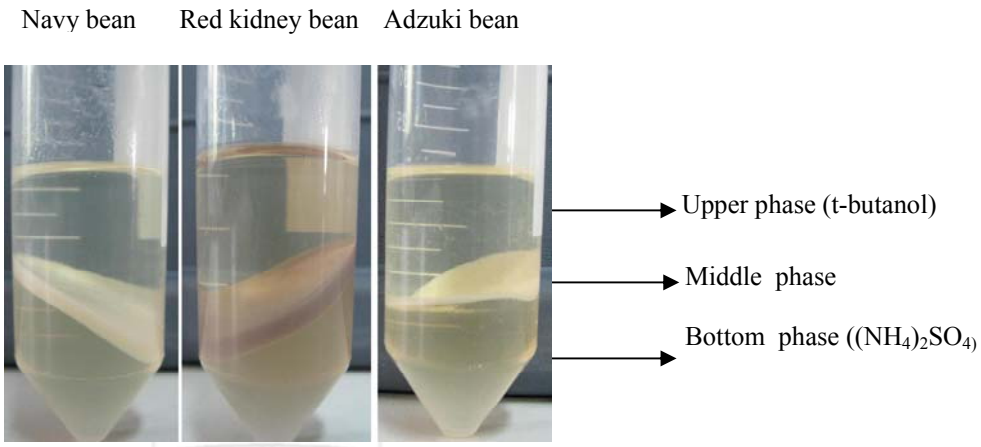


**Figure 3.1** Three phase partitioning of three legume seeds. The extract was saturated with 30% ammonium sulfate and *t*-butanol with the ratio of 1:1 (v/v).

### 3.2.3 Characterization of trypsin inhibitor from legumes

***1. Protein content determination***

The protein concentration in the sample during extraction and fractionation processes was determined by Biuret method (Gornall *et al*., 1948) using bovine serum albumin (BSA) as a standard. The sample was added with Biuret reagent and then mixed using vortex. After incubation at room temperature for 30 min, the absorbance of the sample solution was recorded using UV spectrophotometer at 550 nm.

***2. Trypsin Inhibitory activity assay***

Trypsin inhibitory activity was measured according to the method of Benjakul *et al.* (1999b) using BAPNA as substrate. A solution containing 100 µL of the sample, 200 µL (20 µg/mL) trypsin and 100 µL of distilled water was pre-incubated at 37°C for 10 min. Then 500 µL (0.4 mg/mL) of BAPNA (pre-warmed to 37°C) was added to start the reaction. After incubation at 37°C for 10 min, 100 µL of 30% (v/v) acetic acid was added to terminate the

reaction; it was then subjected to centrifugation at 2,000xg for 10 min. Activity of the trypsin was measured by absorbance at 410 nm due to *p*-nitroaniline released. One unit of trypsin inhibitor was defined as 0.01 decreases in absorbance at 410 nm under assay conditions compared with the control (without inhibitor) sample.

***3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)***

***3.1 Protein pattern***

SDS-PAGE was carried out by the method of Laemmli (1970) using 15% separating and 4% stacking gels. The samples were mixed with the sample buffer (0.5 M Tris HCl, pH 6.8, glycerol, 10% SDS, and bromophenol blue) without reducing agent and without heating. Twenty micrograms of protein were loaded and then separated at 15 mA/gel using Mini Protean Tetra Cell unit (Bio-Rad Laboratories, Inc, Richmond, CA, USA). After separation, the protein was stained with Coomassie Brilliant Blue R-250 and destained with a methanol-acetic solution.

***3.2 Inhibitory activity staining***

For inhibitory activity staining, the protein separated by electrophoresis was subjected to incubation with 50 mL of a mixture of 0.2 mg/mL trypsin solution in Tris-HCl pH 8.3 for 60 min at 4°C to allow the trypsin to diffuse into the gel, and then washed with distilled water. The gel was incubated at 37°C for 90 min with 1% (w/v) casein in 0.1 M phosphate buffer, pH 6.0 and then rinsed with distilled water, fixed, and stained with Coomasie Brilliant Blue R-250 to display inhibitory zones. The apparent molecular weight of the trypsin inhibitor was estimated by comparing the Rf with those of the protein standard marker.

***3.3 Glycoprotein staining***

Glycoprotein staining was conducted using GelCode® Glycoprotein Staining Kit. The protein separated by electrophoresis was fixed by immersing the gel in 30 ml of 50% (v/v) methanol for 30 min and followed by washed the gel by gently agitating with 3% (v/v) acetic acid for 10 min (repeated twice). The gel was transferred to 25 ml of oxidizing solution and then washed with washing the gel with 3% (v/v) acetic acid for 10 min before stained with 15 ml of GelCode® Glycoprotein Stain for 15 min. The gel was incubated for 10 min with 25 ml of reducing solution and washed by 3% (v/v) acetic acid. The glycoprotein appears as magenta bands.

***4. Thermal profile and stability***

The phase that gave the highest trypsin inhibitor recovery (bottom phase of all legume seeds) was subjected to assay the trypsin inhibitory activity at different temperature (40, 50, 60, 70, 80, 90 and 100°C). The inhibitory activity against trypsin was expressed as the relative inhibitory activity, compared with that of the untreated sample.

For thermal stability, the fractions were incubated at 90°C for various times (10, 20, 30, 40, 50, 60, and 90 min). The heat treated samples were immediately cooled in an iced-water and tested for the remaining inhibitory activity.

***5. pH profile and stability***

The pH profile was tested by assay the trypsin activity in the presence of the inhibitor fraction at different pHs (3-12) of the assay conditions. Glycine (pH 3), sodium acetate (pH 4-5), sodium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9), and sodium carbonate (pH 11-12) was used. The residual trypsin activity was measured and expressed as the relative inhibitory activity.

The pH stability was tested by incubating the trypsin inhibitor fractions in the buffer solution at different pHs (3-12) for 10 min at room temperature. The residual trypsin activity was determined and report as the relative inhibitory activity as previously mentioned.

***6. Salt Stability***

The trypsin inhibitor fractions were incubated at room temperature for 10 min in the presence of NaCl ranging from 0 to 3% (w/v) at the ratio of 1:1. The mixture was analyzed for its inhibitory activity against trypsin. The residual activity was reported as the relative inhibitory activity, compared with the untreated sample.

**3.2.4 Application of trypsin inhibitor from three varieties of legume seeds on the muscle model system**

***1. Effect of isolated trypsin inhibitor on proteolysis inhibition of tilapia muscle***

***1.1 Preparation of crude trypsin***

Crude trypsin was prepared from tilapia viscera according to the method of Bezzera *et al*. (2004). Viscera (10g) were collected and homogenized with 250 ml of 0.9% (w/v) NaCl using a homogenizer. The homogenate was centrifuged at 10,000xg for 10 min at 4°C to remove cell debris and nuclei. The supernatant obtained was incubated at 45°C for 30 min and centrifuged at 10,000xg for 10 min at room temperature. The supernatant was used as 'a crude trypsin' for further study.

***1.2 Minced fish preparation***

The fish was skinned and gutted to obtain a fillet. The fillet was washed with tap water to remove loose scales, blood or remaining viscera and then minced in a meat mincer. The sample obtained was referred to as 'minced tilapia'.

***1.3 Proteolysis study of minced tilapia***

Minced tilapia (2g) was weighed accurately and mixed with 55 units of crude trypsin and then incubated at 50°C for different times (10, 20, 30, 60, 90, 120 and 180 min). Eighteen milliliters of 5% SDS solution (85°C) was added before being homogenized at 10,000 rpm for 1 min. The homogenate was then incubated at 85°C in a water bath for 1 h to solubilize the protein. To remove undissolved debris, the mixture was centrifuged at 5,000xg for 10 min. The supernatant was subjected to protein pattern analysis by SDS-PAGE as previously mentioned.

***1.4 Proteolytic inhibition of minced tilapia by trypsin inhibitor fraction***

Proteolytic inhibition of minced tilapia by isolated trypsin inhibitor was measured according to the method of Rawdkuen *et al*. (2007). Inhibitor fraction of navy bean, red kidney bean and adzuki bean (100, 300, 500, 700, 1000, 2000 μl) was added to 2g of minced fish. The mixture was mixed and then incubated in a water bath at 50°C for 2 h. Proteolysis was terminated by the addition of 18 ml of 5% SDS solution (85°C). The proteolytic inhibition pattern of the inhibitor fraction was measured by subjecting the solubilized protein to SDS-PAGE as previously mentioned.

***2. Effect of isolated trypsin inhibitor on autolysis inhibition of Pacific whiting and arrowtooth flounder***

***2.1 Minced fish preparation***

Frozen Pacific whiting and arrowtooth flounder fillets were thawed using running water and minced by using a meat mincer. The obtained samples were referred to as "minced fish" and used as the muscle model system for autolysis inhibition study.

***2.2. Autolysis inhibition study***

Autolysis inhibition of minced fish by isolated trypsin inhibitors was measured according to the method of Rawdkuen *et al.,* (2007). The trypsin inhibitor fractions of NB, RK and AZ (100, 300, 500, 700, 1000, 2000 μl) was added to 2g of minced fish. The mixture was mixed and then incubated in a water bath at 55°C for 1 h or 60°C for 30 min for Pacific whiting and arrowtooth flounder, respectively.

Autolysis was terminated with the addition of 18 ml of 5% SDS solution (85°C). The mixture was homogenized for 1 min at 10,000 rpm and then incubated at 85°C in a water bath for 1 h and centrifuged at 5000xg for 10 min. The protein content in the supernatant was determined by using Biuret method. Autolytic patterns of minced tilapia were determined using SDS PAGE as previously described.

## 3.3 Statistical analysis

Data subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Analysis was performed using a SPSS package (SPSS 16.0 for windows, SPSS Inc, Chicago, IL).

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Extraction of trypsin inhibitor from three varieties of legume seeds

### 4.1.1 Effect of extractant on recovery of trypsin inhibitor

Trypsin inhibitors from three varieties of legumes were extracted with different solvents. The recovery in terms of protein content, trypsin inhibitory activity and specific activity was determined and shown in Table 4.1. Among all extractants used, 0.02 M NaOH had the highest specific inhibitory activity for navy bean and red kidney bean. When the pH of extraction is below or above the isoelectric point (pI), the solubility of protein will increase. However, adzuki bean shows water as the best extractant for the highest specific activity. The specific activity of navy bean and red kidney bean extracted with NaCl was lower compared to other extractants. Navy bean extract that used 0.30 M NaCl showed higher protein content and trypsin inhibitory than 0.15 M NaCl. Red kidney bean extracted with the increasing concentration of NaCl show the decreasing of trypsin inhibitor and protein content. This result showed that 0.2 M NaOH is more selective to extract the trypsin inhibitor from navy bean and adzuki bean.

The aqueous alkali has been popular because it high capability for protein solubilization. Protein solubility is a function of temperature, pH, presence of other ions, and the values obtained for solubility are highly dependent on the method used to achieve the solubility (Yada, 2004). Mild alkaline extraction might be effective because pH of the extractants is far from the pI of trypsin inhibitor from legumes (ranging from 4.6 to 7.6) (Morrison *et al.,* 2005). This phenomenon possibly is caused by higher polar groups of protein contained in adzuki bean that are exposed to water. Harrison (1994) reported higher ratio of polar residues in protein will increase the solute-solvent interations in aqueous solution. When legumes protein extracted with media contained NaCl, its show significant decrease of protein content and activity. Schut (1976)

suggested that NaCl causes a shift in the pI to a more acidic pH as a result of specific ion binding effect. Thus with the addition of NaCl and selective binding of the chloride anions, the protein would have an excess of negative charges at the pH of original pI. Liu and Hung (1998) also reported that high concentrations of NaCl reduced chickpea protein solubility.

**Table 4.1** Effect of extractants on trypsin inhibitory activity of three varieties of legume seeds

| Cultivars | Extractants | Trypsin Inhibitor (unit/ g seed) | Protein (mg/g seed) | Specific activity (units/mg protein) |
|---|---|---|---|---|
| **Navy** | Water | 5,845 ± 571a | 109.92 ± 0.75a | 53 ± 5b |
| | 0.01 M NaOH | 5,751 ± 428a | 150.12 ± 0.62b | 38 ± 2a |
| | 0.02 M NaOH | 7,894 ± 279b | 143.58 ± 2.73b | 54 ± 2b |
| | 0.15 M NaCl | 5,811 ± 31a | 147.71 ± 1.18b | 39 ± 0a |
| | 0.30 M NaCl | 5,944 ± 747a | 149.40 ± 10.03b | 40 ± 7a |
| **Red Kidney** | Water | 1,987 ± 427a | 105.75 ± 2.43a | 19 ± 4a |
| | 0.01 M NaOH | 2,739 ± 396a | 158.47 ± 1.36c | 17 ± 2a |
| | 0.02 M NaOH | 4,455 ± 1251b | 156.36 ± 4.91c | 28 ± 7b |
| | 0.15 M NaCl | 2,418 ± 578a | 142.11 ± 1.25b | 17 ± 4a |
| | 0.30 M NaCl | 2,260 ± 422a | 138.91 ± 0.13b | 16 ± 3 a |
| **Adzuki** | Water | 8,490 ± 91ab | 83.33 ± 1.11a | 102 ± 1c |
| | 0.01 M NaOH | 12,175 ± 802c | 151.29 ± 2.88d | 80 ± 4b |
| | 0.02 M NaOH | 12,354 ± 451c | 184.04 ± 3.87e | 67 ± 3a |
| | 0.15 M NaCl | 7,794 ± 705a | 93.97 ± 5.18b | 83 ± 12b |
| | 0.30 M NaCl | 9,245 ± 508b | 138.12 ± 2.16c | 67 ± 2a |

*Mean ± SD of triplicate determinations

Different letters within the same column of each legume indicate significant difference ($p< 0.05$).

**4.1.2 Effect of extractants on protein and inhibitory patterns of extracted inhibitor**

The protein and inhibitory patterns of extracted inhibitor are depicted on Figure 4.1. Under non-reducing condition, the major protein components in navy bean are 215, 132 and 34 kDa. For red kidney bean the main proteins are 118, 59, and 32 kDa. Interestingly, only one major band of 62 kDa was observed in adzuki bean. However, based on reducing condition the changes of protein pattern were observed. Degradation of the 132 kDa into the 52 kDa was clearly observed in navy bean and the 118 kDa into the 50 kDa in red kidney bean. For adzuki bean the same pattern of protein in both reducing and non-reducing conditions was observed. From this result it can be stated that the major band of 132 kDa in navy bean and the 118 kDa in red kidney bean were stabilized by disulfide bonds, while this bond was absence in the extracted protein from adzuki bean.

The inhibitory activity staining revealed that the molecular weight of 132 kDa, 118 kDa and 13 kDa is the trypsin inhibitor in navy bean, red kidney bean and adzuki bean, respectively. However, interesting results showed that reducing condition of navy bean and red kidney bean and adzuki bean can loss of their inhibitory activity against trypsin. This means that structure of the inhibitory part is likely to be stabilized by disulfide bond. Moreover, the band with MW 59 in red kidney and 62 kDa in adzuki bean slightly appeared on the inhibitor stained gel. Appearance of these bands possibly due to the high amounts of these component in the extracts, so it was difficult to hydrolyze them with limited trypsin.

For inhibitory activity staining, trypsin inhibitors with different apparent molecular sizes were found in different seed extracts. The inhibitor band in navy bean, red kidney bean, and adzuki bean was 132 kDa, 118 kDa and 13 kDa, respectively. Adzuki bean showed one inhibitory band with molecular weight of 13 kDa. Garcia-Carreno *et al*. (1996) reported that the trypsin, chymotrypsin and papain inhibitors had molecular weight ranging from 14-66 kDa in non reducing condition. Whereas the Bowman-Birk type inhibitor has molecular weight of 8-10 kDa and more than 20 kDa for Kunitz type. Dennison (2003) reported SDS-PAGE might be overestimated the molecular weight of some proteins. If the proteins are not reduced, then disulfide bridges may constrain the structure and prevent formation of the rod-like complexes. This can result in an incorrect apparent MW.

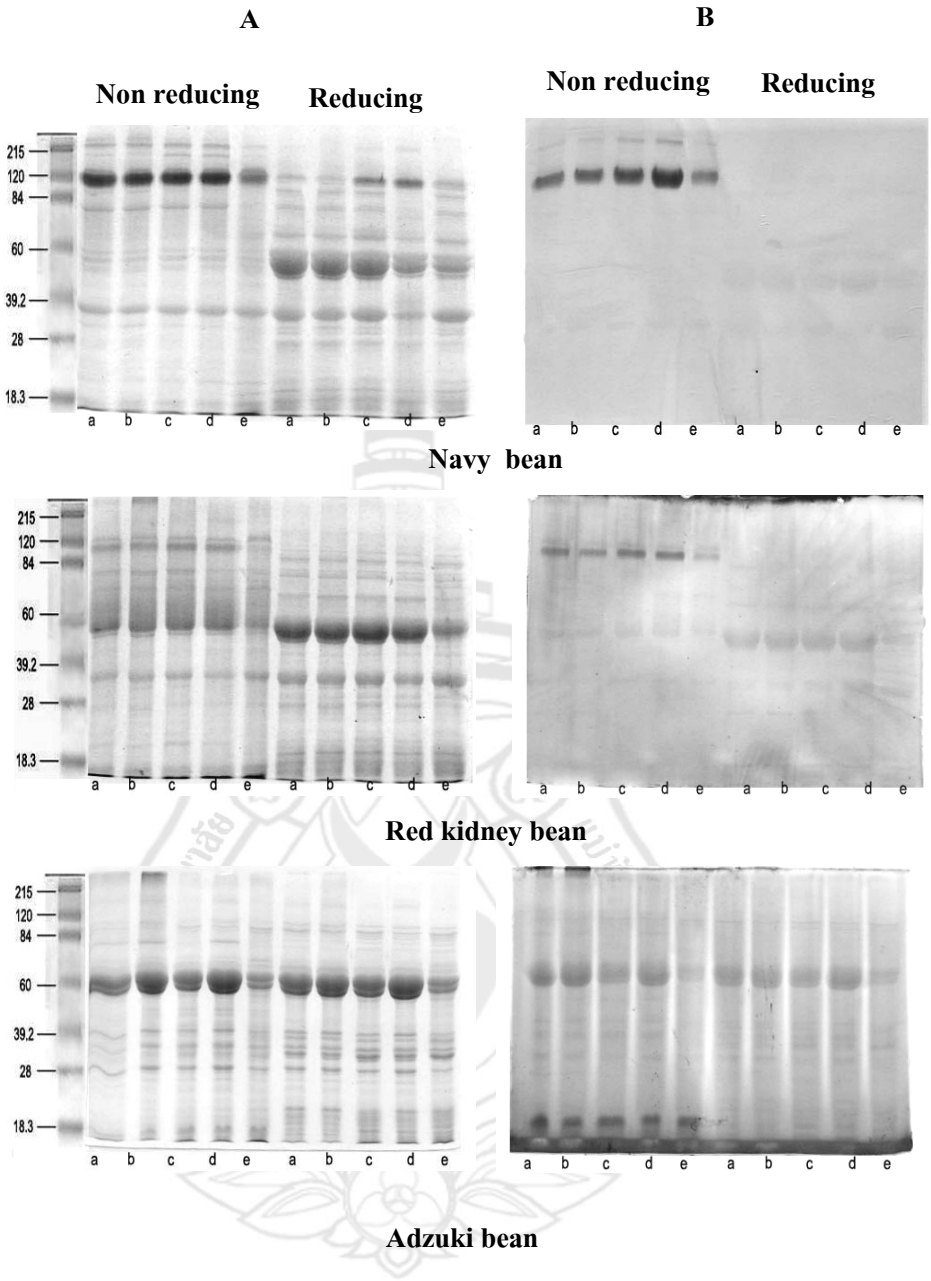


**Figure 4.1** Effect of different extractants on the protein pattern (A) and inhibitory activity staining (B) of three varieties of legume seeds. Eighteen μg of protein were loaded. (a) 0.01 M NaOH; (b) 0.02 M (NaOH); (c) 0.15 M NaCl; (d) 0.30 NaCl; (e) water.

When the sample is exposed to a reducing condition, a loss of inhibitory activity was observed. This condition showed the role of disulfide bond for stabilizing the inhibitor structure as well as the inhibitory activity. Godbole *et al.* (1994) reported the inhibitor loses its three dimensional structure when the disulfide bonds are reduced, and its also lose inhibitory activity towards trypsin. The intramolecular disulfide bridges may responsible for the functional stability of the protein molecules.

From these results, the highest specific inhibitory activity was achieved by using 0.02 M NaOH for navy bean, red kidney bean, and water for adzuki bean. These conditions were chosen for the optimum extraction time.

**4.1.3 Effect of extraction time on recovery of trypsin inhibitor**

Sodium hydroxide (0.02 M) was used as the extractants for navy bean and red kidney bean and water for adzuki bean. The extraction times were varied from 1 to 5 h with gentle shaking. Table 4.2 shows that specific inhibitory activity of all seeds increased significantly up to 2 h and some loss activity when the extraction time more than 2 h ($p$<0.05). Significantly decreased of trypsin inhibitory activity was observed when the extraction process was longer than 2 h. With this result, it was presumed that trypsin inhibitor from navy bean, red kidney bean and adzuki bean underwent denaturation during long period of extraction time, especially in alkaline condition. The mechanical shaking during the extraction process can cause the denaturation of protein via thermal and/or physical denaturation. This phenomenon is due to the air-liquid interface that spread over the surface of the bubble resulting in the unfolding of the molecule (Asakura, 1978). The highest specific activity was obtained after 2 h of extraction for the three varieties of legumes.

**4.1.4 Effect of extraction time on protein and inhibitory patterns of extracted inhibitor**

The protein pattern and inhibitory activity staining of extracted protein from three varieties of legume seeds are extracted with different times (1-5 h) depicted in Figure 4.2**.** The protein pattern of different extraction times showed the same pattern as mentioned before (4.1.2). An increase of the extraction time could not increase in the major protein components in any legumes as observed in SDS-PAGE. These results are correspond to the protein concentration as

measured by using the test tube method. The major protein components in navy bean are 215, 132 and 34 kDa. Red kidney bean also gave the same pattern of 118, 59, and 32 kDa as the major protein bands. Only one major band with the molecular weight of 62 kDa was observed in adzuki bean. Based on reducing condition, the changes of protein pattern were observed as mentioned before in navy bean and red kidney bean, while did not occur in adzuki bean.

**Table 4.2** Effect of extraction times on trypsin inhibitory activity of three varieties of legume seeds

| Cultivars | Extraction Time ( h ) | Trypsin Inhibitor (unit/ g seed) | Protein (mg/g seed) | Specific activity (units/mg protein) |
|---|---|---|---|---|
| **Navy** | 1 | 7,560 ± 259b | 144.039 ± 1.76c | 52 ± 2a |
| | 2 | 8,679 ± 789 b | 137.34 ± 0.670a | 63 ± 6b |
| | 3 | 6,996 ± 708b | 140.78 ± 0.60b | 49 ± 5a |
| | 4 | 6,698 ± 395b | 139.75 ± 1.36b | 31 ± 1a |
| | 5 | 6,585 ± 713a | 139.83 ± 1.32b | 47 ± 5a |
| **Red Kidney** | 1 | 5,036 ± 510b | 156.89 ± 1.11b | 32 ± 3b |
| | 2 | 6,172 ± 149b | 150.96 ± 0.85a | 40 ± 1b |
| | 3 | 4,916 ± 981b | 156.41 ± 1.85b | 31 ± 6b |
| | 4 | 5,016 ± 1192b | 155.21 ± 4.57ab | 32 ± 8b |
| | 5 | 2,198 ± 798a | 158.94 ± 1.73b | 13 ± 5a |
| **Adzuki** | 1 | 8,352 ± 147bc | 79.85 ± 0.29bc | 104 ± 2b |
| | 2 | 8,503 ± 439c | 76.91 ± 2.29ab | 110 ± 4b |
| | 3 | 7,864 ± 238ab | 73.62 ± 3.35a | 107 ± 7b |
| | 4 | 7,638 ± 365a | 82.43 ± 2.41c | 92 ± 7a |
| | 5 | 7,508 ± 39a | 73.11 ± 0.96a | 102 ± 2b |

*Mean ± SD of triplicate determinations

Different letters within the same column of each legume indicate significant difference ($p < 0.05$).

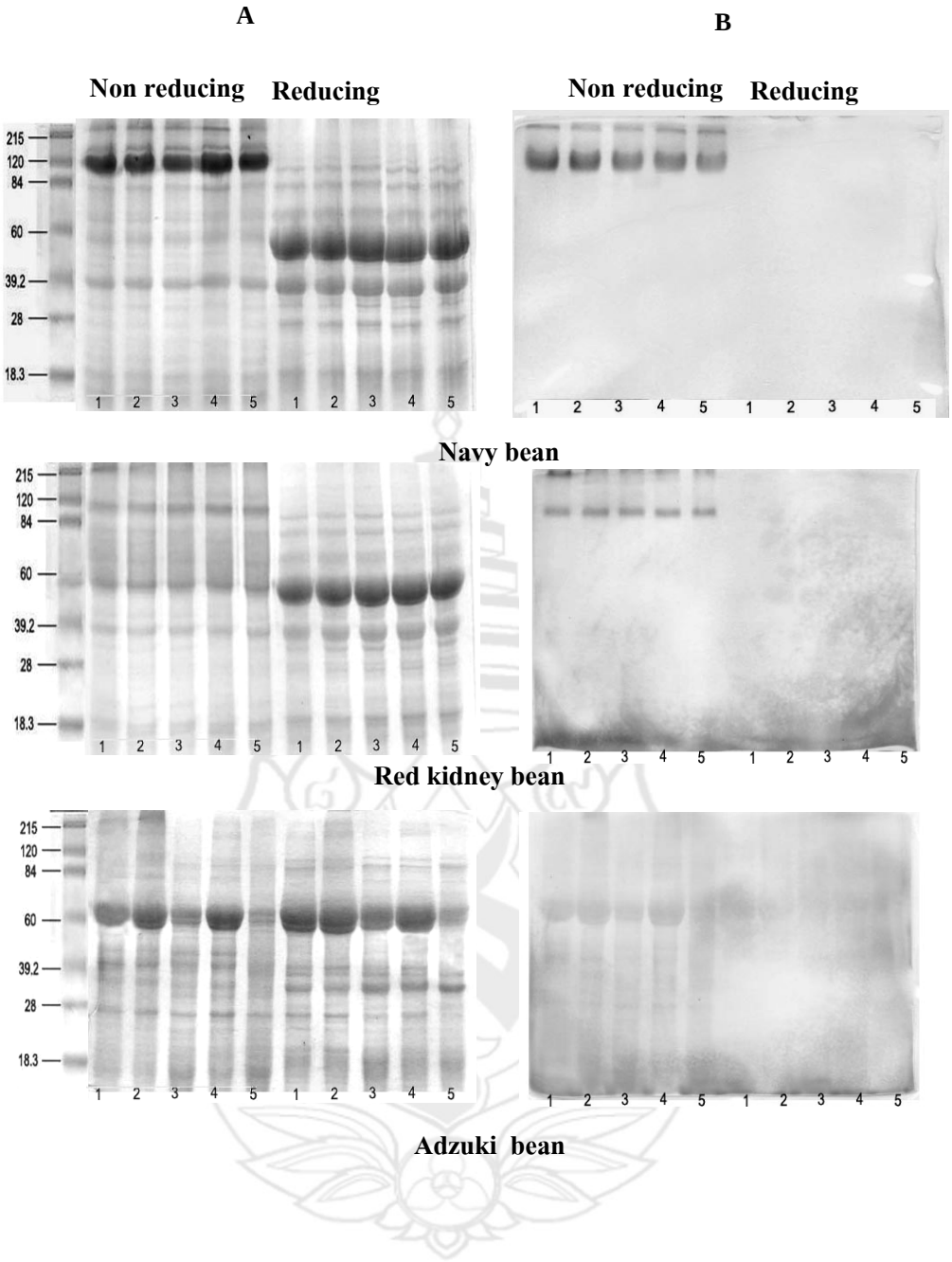


**Figure 4.2** Effect of different extraction times on the protein pattern (A) and inhibitory activity staining (B) of three varieties of legume seeds. Eighteen μg of protein were applied. Numbers represent the extraction time (min).

The inhibitory activity staining also showed that the protein with molecular weights of 132 kDa, 118 kDa and 13 kDa were trypsin inhibitors in navy bean, red kidney bean and adzuki bean, respectively. The band, that remained on the substrate gel of SDS-PAGE confirmed that these protein bands are the bands of the inhibitors that were not hydrolyzed by trypsin under the assay condition. Under reducing condition, the inhibitory activity was not detected for navy bean and red kidney bean, hile still have some resistant band (13 kDa) on the bottom of the gel was observed in adzuki bean. The major band of adzuki bean with molecular weight of 62 kDa was found to have no inhibitory activity based on reducing condition.

## 4.2 Fractionation of trypsin inhibitor from three varieties of legume seeds

### 4.2.1 Heat treatment

The seed extracts were subjected to heat treatment at 50 and 70°C for 10 min. The inhibitory activity increased when the temperature was increased up to 70°C. Extraction with the temperature at 70°C gave the highest specific inhibitory activity compared with 895 and 783 as well as 3,114 for navy bean, red kidney bean and adzuki bean, respectively (Table 4.3). The highest and lowest inhibitor recovery was found in red kidney bean (351%) and adzuki bean (204%), respectively. However, the heat might have caused the trypsin inhibitor to loosen its compact structure that is normally stabilized by a number of disulfide bonds (Godbole *et al* ,1994). Benjakul *et al.* (1999a) reported that trypsin inhibitory activity of some legumes was increased slightly after heat treatment at 60°C, but decreased when extracts were heated at 100°C. Other studies reported that heat treatment leads to the conformational changes in many proteins (Baptista *et al.,* 2003; Lin *et al.,* 1990). Prakash and Narasinga Rao (1988) also reported non-covalent interaction; especially that hydrophobic interaction can be disrupted by heating above 50°C resulting in release of some target proteins. Dissociation of high molecular size protein also depends on pH, ionic strength, protein concentration and temperature.

### 4.2.2 Ammonium sulfate precipitation

The heat treated sample (70°C for 10 min) with the highest specific activity was subjected to AS precipitation. The highest purification fold was found in precipitate IV (60-80% AS) for all legume seeds (Table 4.4); however, high purity resulted in low inhibitor recovery.

**Table 4.3** Effect of heat treatment on recovery of trypsin inhibitor from three varieties of legume seeds

| Sample | Treatment* | Total Protein (mg) | Total inhibitory activity (units) | Specific inhibitory activity (u/mg) | Yield (%) |
|---|---|---|---|---|---|
| Navy | Crude | 1,785 | 564,135 | 316±7a | 100±00a |
| | 50° C | 1,737 | 757,632 | 436±51b | 134±12b |
| | 70° C | 1,615 | 1,446,480 | 895±74c | 256±17c |
| Red Kidney | Crude | 2,354 | 481,416 | 204±10a | 100±00a |
| | 50° C | 2,250 | 678,168 | 301±34b | 140±23b |
| | 70° C | 2,151 | 1,690,430 | 783±60c | 351±35c |
| Adzuki | Crude | 1,076 | 595,210 | 553±78a | 100±00a |
| | 50° C | 823 | 614,390 | 746±196a | 103±16b |
| | 70° C | 390 | 1,214,396 | 3,114±109b | 204±11c |

*Crude extracts with the best condition were heated at 70°C for 10 min and then cooled with ice water. The heated extracts were centrifuged at 8,000xg for 15 min to remove the heat coagulated debris.

**Table 4.4** Ammonium sulfate fractionation of trypsin inhibitor from three varieties of legume seeds

| Sample | Fraction* | Volume (ml) | Total Protein (mg) | Total inhibitory activity (units) | Specific inhibitory activity (u/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| Navy | Heat treated | 60 | 1,615 | 1,690,480 | 1,046±30a | 100±0b | 1.0±0.0a |
| | Precipitate I | 65.7 | 440 | 4,411,098 | 10,021±563b | 261±10c | 9.6±0.2b |
| | Precipitate II | 72.7 | 393 | 4,672,696 | 11,877±838ab | 276±15c | 11.4±0.4bc |
| | Precipitate III | 36.5 | 240 | 2,403,747 | 10,005±3230ab | 142±5b | 9.6±3.3bc |
| | Precipitate IV | 7.7 | 31 | 417,248 | 13,183±612c | 24±1a | 12.6±0.5c |
| | Supernatant IV | 83.3 | 656 | 2,179,913 | 3,322±491a | 129±18b | 3.18±0.4a |
| Red Kidney | Heat treated | 70 | 2,158 | 1,690,430 | 783±60a | 100±0a | 1.0±0.0a |
| | Precipitate I | 94.1 | 674 | 6,236,210 | 9,241±263c | 368±11b | 11.8±0.5b |
| | Precipitate II | 129.4 | 596 | 8,451,644 | 14,169±355d | 500±25c | 18.1±1.8c |
| | Precipitate III | 21.1 | 194 | 1,550,549 | 7,954±96b | 91±3a | 10.2±0.7b |
| | Precipitate IV | 23.3 | 92 | 1,684,287 | 18,181±1235e | 99±3a | 23.2±0.8d |
| | Supernatant IV | 116.6 | 724 | 5,989,324 | 8,266±349bc | 354±19b | 10.6±1.2b |
| Adzuki | Heat treated | 65 | 390 | 1,214,396 | 3,114±129b | 100±0d | 1.0±0.0b |
| | Precipitate I | - | - | - | - | - | - |
| | Precipitate II | 1.9 | 33 | 126,963 | 3,739±139b | 10±0a | 1.2±0.0b |
| | Precipitate III | 9.1 | 144 | 596,886 | 4,142±218b | 49±2c | 1.3±0.0b |
| | Precipitate IV | 4.7 | 16 | 316,079 | 18,904±1534c | 26±1b | 6.1±0.2c |
| | Supernatant IV | 39.1 | 208 | 291,875 | 1,400±156a | 24±2b | 0.4±0.0a |

I-IV: fraction obtained by ammonium sulfate (AS) fractionation : 0-200, 200-400, 400-600, 600-800 g/l precipitate, respectively and 800 g/l supernatant.

The highest recovery was found in fraction II for navy bean and red kidney bean, while fraction III gave the highest inhibitor recovery for adzuki bean. From this result, it can be stated that trypsin inhibitor from the three legumes is more concentrated in precipitate IV (60-80%AS saturation). Thermal treatment and AS fractionation; heating at 70°C for 10 min; and AS precipitation at 60-80% saturation were used for trypsin inhibitor fractionation.

The fraction results are shown in Table 4.5. Purification folds of 41, 89 and 34 with yields of 73%, 349%, and 53% were obtained for navy bean, red kidney bean and adzuki bean, respectively. Of the three legumes, red kidney bean showed the highest recovery, followed by navy bean and adzuki bean. Nevertheless, the highest specific inhibitory activity was obtained from adzuki bean. Different protein compositions in each legume resulted in different specific precipitations with any salt added.

SDS-PAGE patterns of the fractions from navy bean, red kidney bean and adzuki bean are shown in Figure 4.3. For navy bean, fraction IV (60-80%AS, lane F) consisted of the major protein components with MW of 215, 132 and 34 kDa. Many minor protein bands (MW< 28 kDa) disappeared in the AS fraction (lane F) when compared with the crude extract (lane C) or heat precipitate fraction (lane H). AS precipitation could remove some small protein molecules of red kidney bean and giving only the three major bands with MW of 118, 59 and 32 kDa. The pattern of protein from adzuki bean extracted also showed the same result at the crude extract. However, the numbers of bands were removed by using fractionation technique. Major protein bands with MW of 62 kDa were found in the fraction IV and in the crude extract. Meanwhile, the minor protein band with the MW of 13 kDa appeared after the heat treated fraction was fractionated using 60-80% AS saturation (lane F).

**Table 4.5** Fractionation of trypsin inhibitor from three varieties of legume seeds

| Sample | Step | Total Protein (mg) | Total inhibitory activity (units) | Specific inhibitory activity (u/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Navy | Crude | 1,785 | 564,135 | 316±7a | 100±0b | 1.0±0.0a |
| | *Heat treatment | 1,615 | 1,446,480 | 895±74b | 256±17c | 2.8 ±0.2a |
| | **AS precipitation | 31 | 417,248 | 13,183±612c | 73± 4a | 41.7±2.5b |
| Red Kidney | Crude | 2,354 | 481,416 | 204±10a | 100±0a | 1.0±0.0a |
| | *Heat treatment | 2,151 | 1,690,430 | 783±60b | 351±35b | 3.8±0.4a |
| | **AS precipitation | 92 | 1,684,287 | 18,181±1235c | 349± 23b | 89.1±9.7b |
| Adzuki | Crude | 1,076 | 595,210 | 553±78a | 100±0b | 1.0±0.0a |
| | *Heat treatment | 390 | 1,214,396 | 3,114±109b | 204±11c | 5.6±0.9a |
| | **AS precipitation | 16 | 316,079 | 18,904±1534c | 53± 4a | 34.2±7.4b |

*Crude extracts with the best condition were heated at 70°C for 10 min and then cooled with ice water. The heated extracts were centrifuged at 8,000xg for 15 min to remove the heat coagulated debris.

**Fraction obtained by ammonium sulfate (AS) fractionation 600-800 g/l precipitate.

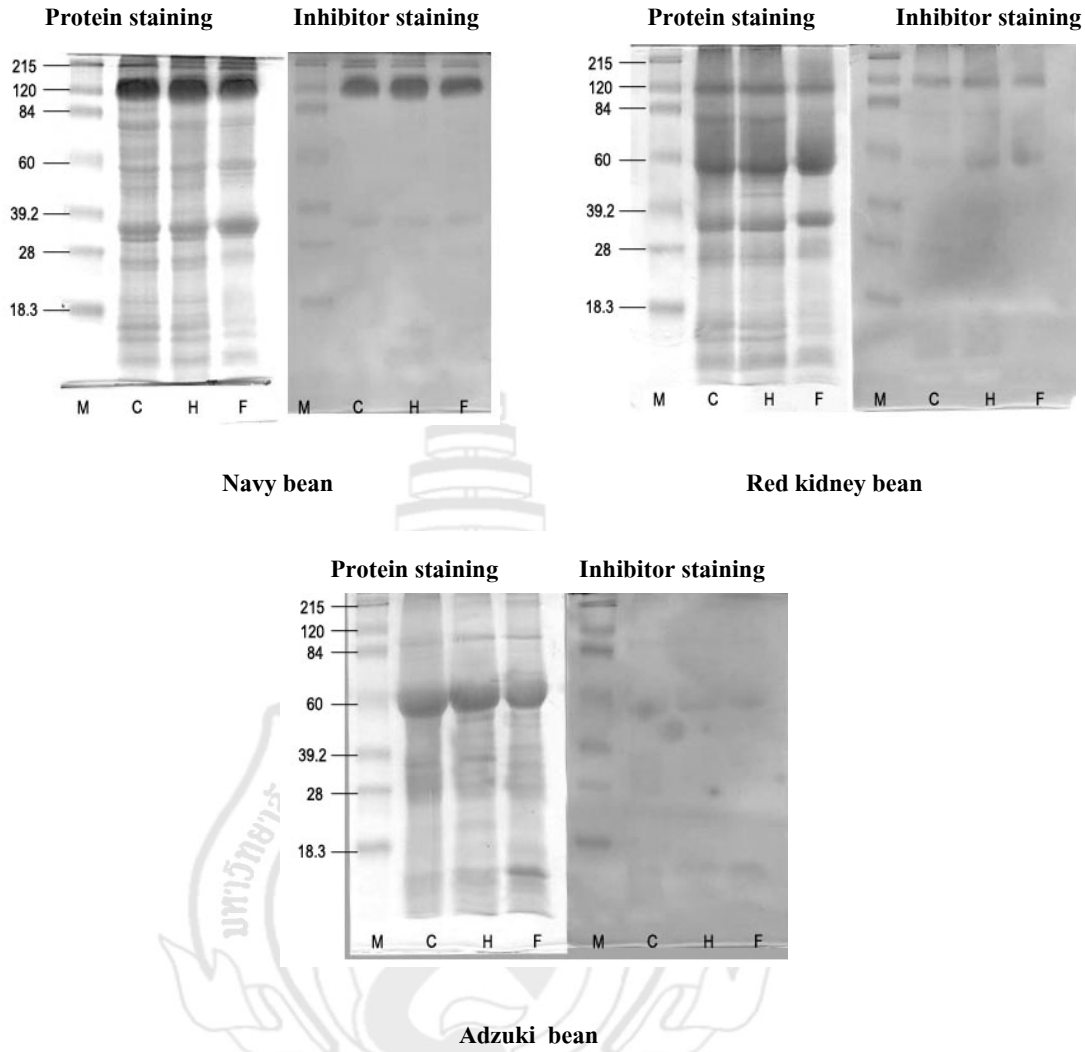


**Figure 4.3** Protein pattern and inhibitory activity staining of fractionation with ammonium sulfate fractionation. (M) molecular weight marker; (C) crude extract; (H) heat treatment fraction(70°C for 10 min); (F) Fraction IV (60-80% saturation).

For inhibitor activity stained gel, trypsin inhibitors with different apparent molecular sizes were found in different legume extracts (Figure 4.3). For navy bean, a protein band with the MW of 132 kDa was an inhibitor which remained after being treated with the gel with trypsin. A single band of inhibitor with apparent MW of 118 kDa was observed in red kidney bean, whereas a band with apparent MW of 13 kDa was found in the adzuki bean extract. The bands that still retained inhibitory activity staining were protein that could not be hydrolyzed by trypsin in the assay conditions.

**4.2.3 Three phase partitioning (TPP)**

TPP with ammonium sulfate and *t*-butanol was used to fractionate the trypsin inhibitor from three varieties of legume seeds. The heat treated extract (70°C, 10 min) of the three varieties of legumes was added with ammonium sulfate (30% w/v) followed by the addition of 1:1 *t*-butanol. After 1 h, the three phases were formed and collected separately. The partitioning of trypsin inhibitor by TPP is summarized in Table 4.6**.** Specific inhibitory activity (unit/mg protein) was achieved by the bottom phase for navy bean (2,172) and red kidney bean (2,094). Meanwhile, middle phase of adzuki bean (15,545) was given the highest specific inhibitory activity. Salting out mechanism as the part of TPP was used to separate proteins using the kosmotrope ability of the sulfate ion (Dennison, 2003). Small precipitate obtained for adzuki bean might be due to the higher hydrophilic site exposed in this protein. Meanwhile for navy bean and red kidney bean, the bulk water becomes associated with the salt ions that caused less water available to partake in the salvation layer around the protein which lead to hydrophobic interactions.

The difference of conventional salting out and TPP were the addition of *t*-butanol that also binds to the protein and increases the buoyancy against gravity and makes it float between the aqueous and the organic layer (Borbas *et al.,*2001). The purity of trypsin inhibitor increased as the number of the step of isolation increased. Therefore, heat treatment was used as the pre-treatment to remove some heat labile proteins in the sample. The TPP gave the yield of 315, 441 and 228 with purification fold of 5.65, 14.15 and 7.3 for bottom phase of navy bean, red kidney bean and adzuki bean, respectively. The highest trypsin inhibitor recovery was obtained in red kidney bean, followed by navy bean and adzuki bean. The saturation of 30% ammonium sulfate was sufficient to remove other proteins that precipitate in the middle phase and remain the trypsin

inhibitor in the bottom phase of red kidney bean fraction. However, the highest specific inhibitory activity and purification factor was achieved by adzuki bean. The amount of protein precipitated in the middle layer of adzuki bean was lower than that of navy bean and red kidney bean. It means that 30% saturation only can removed small amount of protein but still have the inhibitory activity, and this phenomenon lead to higher specific activity of trypsin inhibitor. Due to the high recovery of the isolated trypsin inhibitor, this method has the opportunity to be the low cost mass production of trypsin inhibitor. Soybean trypsin inhibitor was successfully purified by modified metal-TPP with 72% recovery and 13-fold of purification (Roy and Gupta, 2002). Sharma and Gupta (2001) used the TPP as the single step process to purify protease/amylase inhibitor from wheat germ, the purity of 25-fold and activity recovery of 85% was obtained.

The protein patterns of the crude extract and fraction of navy bean, red kidney bean and adzuki bean are shown in Figure 4.4. For navy bean, there is no significant change of protein pattern from the crude extract (lane C) and the heat treatment (lane H). The major protein bands for navy bean were 215, 132, and 77 kDa. TPP process could remove the small MW of proteins from the sample as shown in lane B and M. Protein band with the MW of 77 kDa disappeared, and protein band with the MW of 215 and 132 kDa still remained with higher intensity in the bottom phase compared to that of the middle phase. The protein pattern of the bottom phase and middle phase of navy bean were similar, however the decrease in the intensity was observed in lane M.

Protein bands of the crude and heat treatment samples with the MW of 118, 59, 38 and 32 kDa were observed in red kidney bean. After TPP, the bottom phase (lane B) showed only 3 major bands with the MW of 118, 59 and 38 kDa. Nevertheless, the middle phase (lane M) has the same pattern as shown in lane H with lower in band intensity. The adzuki bean presented 2 major protein bands with the MW of 62 and 13 kDa after TPP process. The minor protein bands with the MW of 39 and 35 kDa were completely removed by the TPP. The low MW of protein might be removed in the upper layer (*t*-butanol phase), because the *t*-butanol can remove low MW proteins by denaturation (Lovrien *et al.,* 1995). In the TPP process, the lipids dissolve in the upper *t*-butanol layer, while most proteins (nucleic acids and insoluble aggregates formed into macromolecules) are in the middle phase.

**Table 4.6** Three Phase Partitioning of trypsin inhibitor from three varieties of legume seeds.

| Sample | Step | Volume (ml) | Total Protein (mg) | Total Inhibitory Activity (units) | Specific Inhibitory Activity (unit/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| Navy | Crude | 13 | 391 | 150,423 | 384±61.08a | 100±00a | 1±00a |
| | *Heat treatment | 10 | 365 | 246,570 | 675±7.31b | 163±18b | 1.75±0.01b |
| | **Bottom phase | 19.23 | 218 | 475,000 | 2,172±27.81d | 315±47c | 5.65±0.05c |
| | **Middle phase | 9.77 | 130 | 241,045 | 1,852±244.10c | 160±79b | 4.82±0.47d |
| Red Kidney | Crude | 15 | 499 | 73,740 | 148±21a | 100±00a | 1±00a |
| | *Heat treatment | 10 | 383 | 223,960 | 584±4.76b | 303±00b | 3.94±0.15b |
| | **Bottom phase | 14.5 | 155 | 325,496 | 2,094±72.72d | 441±39c | 14.15±0.42c |
| | **Middle phase | 13.12 | 209 | 294,045 | 1,406±92/26c | 398±15c | 9.5±0.02d |
| Adzuki | Crude | 15 | 193 | 130,185 | 671±59a | 100±00a | 1±00a |
| | *Heat treatment | 10 | 95 | 225,880 | 2,370±79b | 173±25c | 3.5±0.15b |
| | **Bottom phase | 13 | 60 | 298,051 | 4,920±1734c | 228±30d | 7.3±0.09c |
| | **Middle phase | 1.63 | 2 | 37,154 | 15,545±3992d | 28±4b | 23.16±0.20d |

*Crude extracts with the best condition were heated at 70°C for 10 min and then cooled with ice water. The heated extracts were centrifuged at 8,000xg for 15 min to remove the heat coagulated debris.

**Fraction obtained by three phase partitioning (TPP) with 30% saturation of ammonium sulfate

The remaining macromolecules were solubilized in the salt phase (Thomson *et al.,* 2002). From our result, it can be stated that the proteins have a tendency to concentrate in bottom phase of the mixture. However, it is totally depended on the structure of the protein molecule inside those legumes.

Inhibitory activity staining for the three legumes seeds is shown in Figure 4.4. For navy bean, a single protein band with the MW of 132 kDa still clearly appeared on the gel. The presence of this band in the inhibitory activity staining condition suggested that it is trypsin inhibitor. The single protein band with the MW of 118 and 13 kDa was observed for red kidney bean and adzuki bean, respectively. Our result revealed that TPP could partition the trypsin inhibitor into the salt phase for navy bean and red kidney bean, while the trypsin inhibitor of adzuki bean was effectively isolated in the interfacial phase of the system.

There has been reported that glycoprotein was found in legume seeds with an estimated molecular weight mass range of 45-55 kDa (Sathe and Venkatachalam, 2007). In this study the crude extract and isolated protein was stained for the glycoprotein content and depicted in Figure 4.4. This staining was used to detect high glycosylated proteoglycans (protein glycosaminoglycans) or glycoproteins (protein oligosaccharides) (Moller and Poulsen, 2002). Structurally glycoprotein consists of polypeptide covalently bonded to a carbohydrate moiety (Mathews and Holde, 1990).The glycoprotein staining revealed that the entire major protein bands contained oligosaccharide. For navy bean, the protein bands with the MW of 215, 132 and 39 kDa appeared as magenta band. A group of protein bands with the MW of 113, 59, 38 kDa and 62, 13 kDa was observed for red kidney bean and adzuki bean, respectively. These bands were identified as the glycoprotein. In addition, the protein band with the MW of 132, 113 and 13 kDa for navy bean, red kidney bean and adzuki bean also appeared at the inhibitory activity staining. This evidence confirmed that the trypsin inhibitors from navy bean, red kidney bean and adzuki bean were glycoprotein.

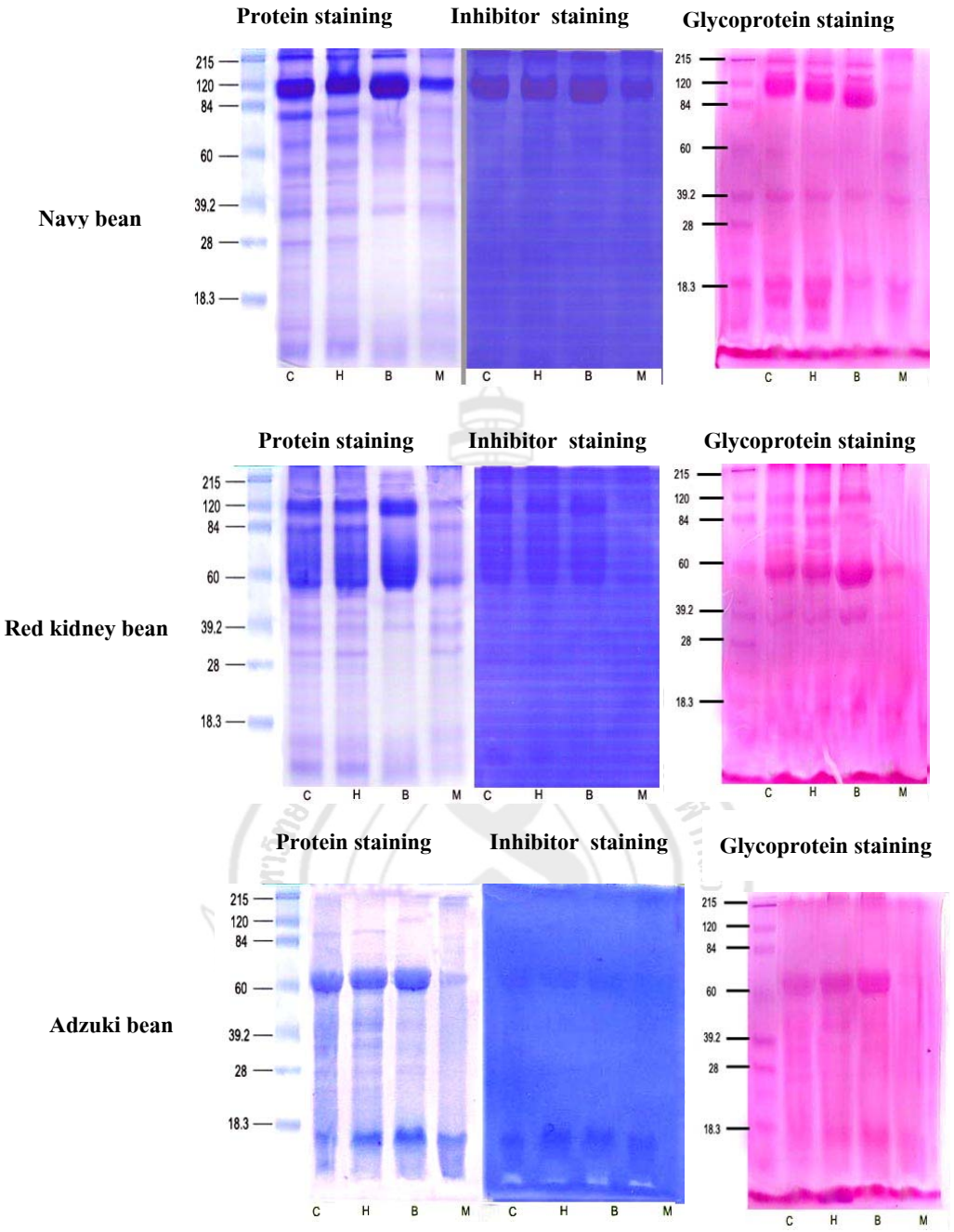


**Figure 4.4** Protein pattern and inhibihitory staining of fractionation using TPP (Three Phase Partitioning). Fraction obtained by adding 30% saturation of ammonium sulfate into extract and add t-butanol with ratio 1:1 (v/v). SDS-PAGE with 15% running gel and 4% stacking gel (non reducing condition). (C) crude; (H) heat treatment; (B) bottom phase; (M) middle phase.

Glycosylation has proven to be a key determinant of enzyme secretion, activity, binding affinity and substrate specificity, enabling a protein to fine-tune its activity. The elimination of all putative N-glycosylation sites of an enzyme results in significantly reduced protein secretion levels, while removal of individual N-glycosylation sites often leads to the expression of active enzymes showing markedly reduced catalytic activity, with the decreased activity often commensurate with the number of glysylation sites available. And the fully deglycosylated enzymes showing only minimal activity relative to their glycosylated counterparts (Skropeta, 2009)

Based on the recovery of the trypsin inhibitor obtained by using TPP, the bottom phase of all legumes was chosen for further study.

**4.2.4 Comparative study of ammonium sulfate precipitation and three phase partitioning**

Fractionation using ammonium sulfate precipitation and three phase partitioning were used to isolate trypsin inhibitor from navy bean, red kidney and adzuki bean. The purification factor obtained from AS precipitation (Fraction IV; 60-80% AS precipitate) was higher with value of 41.71, 89.12 and 24, 18-fold compared with the one from TPP 5.65, 14.15 and 7.3-fold for navy bean, red kidney bean and adzuki bean, respectively. However, higher recovery was observed for TPP method for 315.77, 441.4, and 228.9% in contrast with result of AS precipitation of 73.9, 349.8, and 53.1% for navy bean, red kidney bean and adzuki bean, respectively.

For the protein pattern of both fractions obtained by AS precipitation and TPP, it can be seen that bottom phase from TPP have the similar pattern with fraction IV of AS precipitation. However, there is a difference in the pattern of the minor bands. For navy bean, the minor band (<38 kDa) in lane B was totally removed by TPP but still detected in lane F of AS precipitation. Small MW of protein (<32 kDa) in red kidney bean also disappeared. Meanwhile, the minor band between 2 major proteins with MW of 62 and 13 kDa was not detected in lane B of adzuki bean. Based on this result, it can be presumed that addition of organic solvent (t-butanol) could remove some minor bands out from the extract.

In the inhibitory activity staining, the same pattern was obtained for both AS precipitation and TPP. Trypsin inhibitory band was detected in navy bean with MW of 132 kDa,

118 kDa for red kidney bean and 13 kDa in adzuki bean. The AS precipitation was conducted using gradually increase of AS saturation, meanwhile TPP only use 30% saturation for salting out process and addition of t-butanol to formed three layer of partitioning. Based on the result, it can be stated that AS precipitation resulted in the higher purification but with lower recovery compared to TPP method. The effective method for protein purification must give the highest recovery and purification fold. However, concerning the higher cost production to obtain higher purification fold, TPP was recommended to be used regarding to the higher recovery obtained with small saturation of AS, low cost organic solvent (*t*-butanol) also the shortest time of process. Therefore, bottom phase fraction from TPP was chosen to be characterized for its thermal profile and stability, pH profile and stability and salt stability.

## 4.3 Characterization of trypsin inhibitor from three varieties of legume seeds

### 4.3.1 Temperature profile and stability

For thermal profile, trypsin inhibitor of navy bean, red kidney bean and adzuki bean was determined at the temperature ranging from 40 to 100°C. The highest inhibitory activity of each fraction from each legume was found at 50°C for red kidney bean and 60°C for navy bean and adzuki bean, respectively (Figure 4.5). The relative inhibitory activity was 98%, 102% and 110% for navy bean, red kidney bean and adzuki bean at each optimum temperature, in the corresponding order. At the higher temperature used for incubation, the relative inhibitory activity decreased constantly and reached the lowest point at 100°C. Rekha *et al*., (2008) reported that the optimum temperature of trypsin inhibitor isolated from mungbean seeds was 50°C. As the temperature increases, more molecules have enough kinetic energy to undergo the reaction. If the temperature is raised above the optimum point, the kinetic energy of the enzyme and water molecules is so great that the structure of the enzyme molecule starts to be disrupted (Switzer and Garrity, 1999). Therefore this results in a detectable decrease in activity when the temperature is increased.

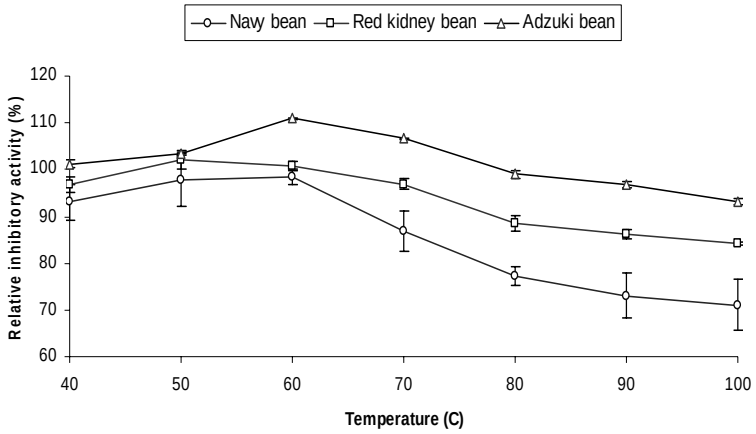


**Figure 4.5** Thermal profile of isolated trypsin inhibitor from three legumes. Fractions were incubated at various temperatures (40-100°C) under assay condition. Residual inhibitory activity against trypsin was determined using BAPNA as substrate. Bars represent standard deviations from triplicate determinations.

For thermal stability, trypsin inhibitory activity of navy bean, red kidney bean and adzuki bean was determined after the fraction was incubated at 90°C with ranging of the incubation times from 10 to 90 min (Figure 4.6). There is a significant decrease of the relative inhibitory activity when navy bean, red kidney bean and adzuki bean fraction were incubated for 20 min ($p>0.05$). Thermal elevation causes the changes in the tertiary structure, leading to a less ordered arrangement of the polypeptide chain that can decrease the enzyme activity (DeMan, 1999). The resistance of trypsin inhibitor to thermal denaturation was shown by the retaining of inhibitory activity up to 80% after incubation at 90°C for 90 min. The thermal stability of proteinase inhibitor is depends on its amino acid composition and structural conformation. A tightly coiled conformation with multiple disulfide bonds considered to be responsible for the resistance to thermal denaturation (DeMan, 1999). The isolated trypsin inhibitor from navy bean, red kidney bean and adzuki bean seemed to be more stable than that of the trypsin inhibitor from cowpea, pigeon pea and bambara groundnut, that were heat stable up to 30 min at 90°C (Benjakul *et al.,* 1999b). From three legumes, navy bean navy bean showed the lowest thermal stability when compared with others. The trypsin inhibitor isolated from *Calliandra selloi* Macbride was

found to be quite stable at 80°C for 30 min (Yoshizaki *et al.,* 2007). Richardson (1977) reported the Bowman-Birk inhibitor was more stable compared to Kunitz-type trypsin inhibitor that displayed significant heat stability. This stability probably due to the fact that the Bowman-Birk inhibitor consists of cystine-rich polypeptide chain bridged by seven conserved disulfide bonds.

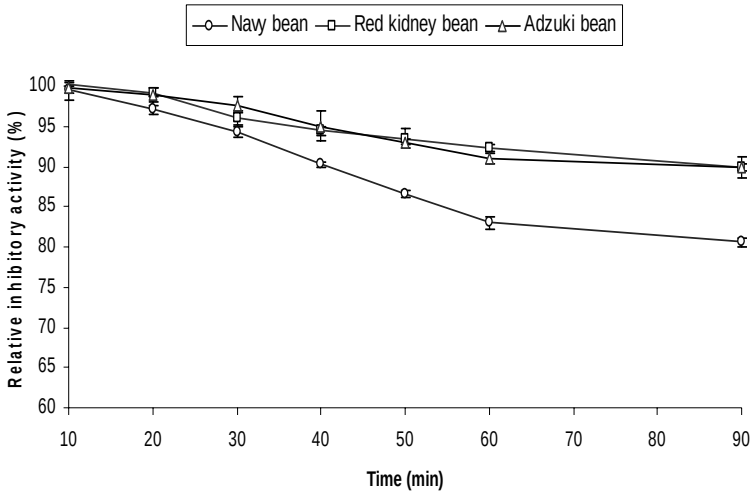


**Figure 4.6** Thermal stability of isolated trypsin inhibitor from three legumes. Fractions were incubated at 90°C for various times (10-90 min). Residual inhibitory activity against trypsin was determined using BAPNA as substrate. Bars represent standard deviations from triplicate determinations.

Glycosylation of protein is known to improve protein stability, increase circulation in plasma and provide steric protection of susceptible sties such as hinges and linkers from attack by proteases and non-specific interactions (Skropeta, 2009). Study by Giartosio and co-workers (1996) found that deglycosylation using exo- and endo-glycosidases resulted in decreased protein thermostability for the heavily glycosylated yeast external invertase and bovine serum fetuin, but did not significantly affect the thermostability of the less glycosylated chicken egg white avidin and ovotransferrin. Deglycosylation of yeast external avidin and bovine serum fetuin did not

appear to alter protein secondary structure, but did to lead to poorer reversibility of the thermal denaturation process and a higher tendency to aggregate during thermal inactivation compared to their glycosylated counterparts. These results suggest that in addition to stabilizing the protein conformation, glycosylation may also prevent unfolded of partially folded proteins from aggregating, and that both of these elements are closely linked to the degree of glycosylation.

**4.3.2 pH profile and stability**

The trypsin inhibitor fraction was incubated with various pHs (3-12) in the assay condition. The highest residual activity was observed at pH 7, 8, 9 for adzuki bean, red kidney bean and navy bean, respectively (Figure 4.7). The trypsin inhibitor isolated from most legumes had the optimum pH around 8 (Sweadner, 1991). Rai *et al.,* (2008) reported the pH 8 as the pH optimum of inhibitory activity of the serine Kunitz trypsin inhibitor isolated from leaves of *Terminalia arjuna*.

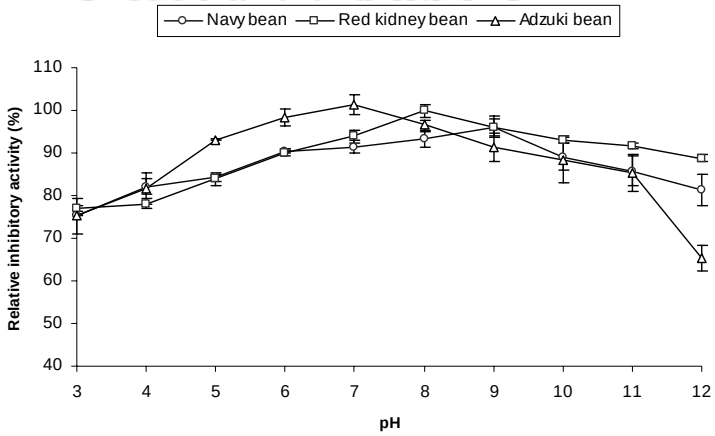


**Figure 4.7** pH optimum of isolated trypsin inhibitor from three legumes. Fractions were incubated at various pH (3-12) under assay condition. Residual inhibitory activity against trypsin was determined using BAPNA as substrate. Bars represent standard deviations from triplicate determinations.

The pH stability of the trypsin inhibitor isolated from navy bean, red kidney bean and adzuki bean is shown in Figure 4.8. No significant decrease in relative inhibitory activity of the trypsin inhibitor isolated from navy bean and adzuki bean after incubated at various pH (3-12) for 10 min ($p>0.05$). The markedly decreased of relative inhibitory activity was observed for red kidney bean when the pH ranges was 9 to12. The remaining residual trypsin activity of 50% was observed when the red kidney bean fraction has incubated with the basic condition (pH 12). Benjakul *et al.,* (1999b) reported that the stability of trypsin inhibitor isolated from cowpea, pigeon pea and bambara groundnuts was over a wide pH range (3-9). Inhibitor isolated from horse gram *(Dolichos biflorus)* was stable between the pH of 3 to11 (Mehta and Simlot, 1982). Our result revealed that, the trypsin inhibitor fraction from navy bean and adzuki bean were more stable in the various pH compared to that of red kidney bean. The decreasing in activity was observed in the acidic ranges (pH 3-6). When protein exposed in the extreme pH values, the denaturation (unfolding) of protein molecule can occur due to the strong electrostatic repulsion of ionized groups leading to the loss of their function (Cheftel *et al.,* 1985).

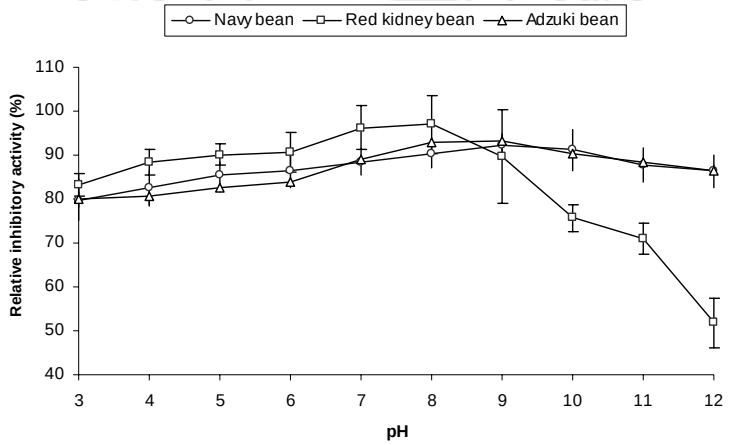


**Figure 4.8** Effect of pH on the stability of isolated trypsin inhibitor from three varieties of legume seeds. Fractions were incubated at various pH (3-12) for 10 min. Residual inhibitory activity against trypsin was determined using BAPNA as substrate. Bars represent standard deviations from triplicate determinations.

### 4.3.3 Salt stability

The effect of NaCl on the inhibitory activity of the trypsin inhibitor isolated from three varieties of legume seeds is shown in Figure 4.9**.** Slightly decreased in the inhibitory activity was observed for navy bean and adzuki bean, meanwhile relative inhibitory activity of red kidney bean reached the lowest point when salt concentration increased ($p>0.05$). The relative inhibitory activity of each legumes reach 99%, 95% and 97% for navy bean, red kidney bean and adzuki bean when the salt concentration addition was up to 3% (w/v). The trypsin inhibitor from the three legumes was fairly stable in solution with salt concentration up to 3% (w/v), as shown by the high relative inhibitory activity (>95%).

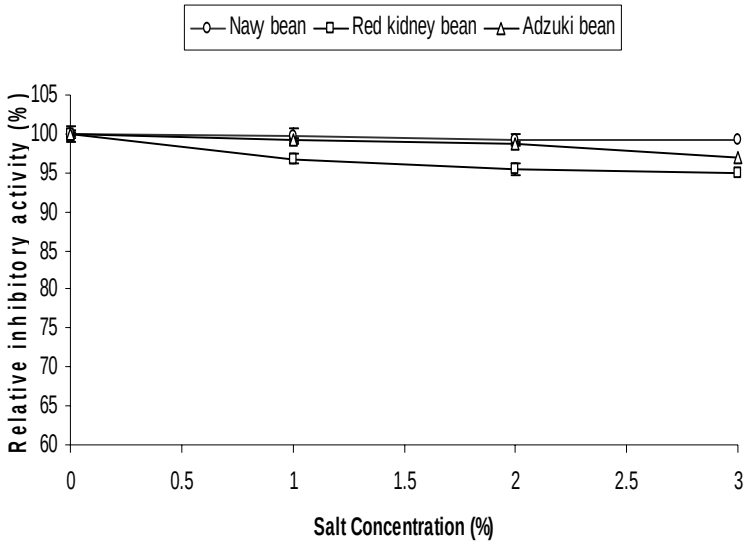


**Figure 4.9** Effect of salt on stability of isolated trypsin inhibitor from three varieties of legume seeds. Fractions were incubated with various concentration of NaCl (1%,2% and 3%). Residual inhibitory activity against trypsin was determined using BAPNA as substrate. Bars represent standard deviations from triplicate determinations.

The effect of salt involved ion-dipole association or hydrogen bonding of a decrease in double-bond character and consequent loss of polypeptide-chain rigidity (Horton *et al.,* 1982). However, at the low level salt concentrations could increase the solubility of the protein inside the legume via "salting in process" resulted in more inhibitor was released into the system.

## 4.4 Application of trypsin inhibitor from three varieties of legume seeds on the muscle system

### *4.4.1 Effect of isolated trypsin inhibitor on proteolysis inhibition of tilapia muscle*

#### *1. Proteolysis study of minced tilapia*

The proteolysis of tilapia muscle in the presence of 'crude trypsin' incubated at 50°C with various incubation times (10-180 min) is shown in Figure 4.10. Myosin heavy chain (MHC) was found to be the major protein band, followed by actin (AC), troponin (TNT) and tropomysin (TM). The crude trypsin from tilapia viscera showed hydrolytic activity with decreasing intensity of MHC as the incubation time increased. The changes in intensity of the actin band were not observed despite prolonged incubation time. There were no changes in the MHC band intensity when incubated for 120 and 180 min. However, at 180 min of incubation the lowest numbers of protein bands were observed. Within 30 min of incubation, a slightly decreased in intensity of the MHC band was observed in tilapia muscle, indicating the degradation of protein had started. Yongsawatdigul *et al.* (2000) reported the proteolysis of surimi made from tropical tilapia was well inhibited by soybean and leupeptin, implying that serine protease might be the major protease involved in proteolysis of the tilapia muscle. From this result, an incubation time of 120 min at 50°C in the presence of crude viscera trypsin was chosen for further study.

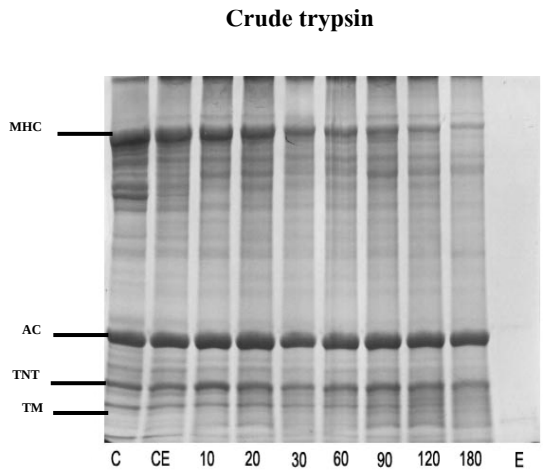


**Figure 4.10** Autolysis pattern of tilapia muscle treated with crude viscera trypsin at different incubation time at 50°C. (C) control without incubation; (CE) control with enzyme; Number (10-180) designiate time incubation (minutes); (MHC) myosin heavy chain; (AC) actin; (TNT) troponin-T, (TM) tropomyosin; (E) enzyme.

***2. Proteolytic inhibition of minced tilapia by trypsin inhibitor fraction***

Proteolytic inhibition of tilapia muscle by trypsin inhibitor fractions from navy bean, red kidney bean and adzuki bean is shown in Figure 4.11. Different concentrations of inhibitor fraction for navy bean, red kidney bean and adzuki bean (100-2000 µl) were applied into minced tilapia. The intensity of the MHC band clearly decreased when crude trypsin (lane CE) was added to the sample. This indicates that crude trypsin isolated from tilapia viscera results in high hydrolytic activity on minced tilapia.

However, markedly increased MHC band intensity was observed when volume of the fraction increased. Addition of trypsin inhibitor fraction from three legumes showed the inhibition activity against proteolysis of muscle tilapia. The fraction from navy bean effectively inhibited proteolysis of tilapia muscle when the concentration of the fraction was 500 µl; likewise in red kidney bean when the fraction was around 300 µl, and in adzuki at a level of 100 µl. Proteolysis of tilapia muscle was almost inhibited when the adzuki fraction concentration was 2000 µl.

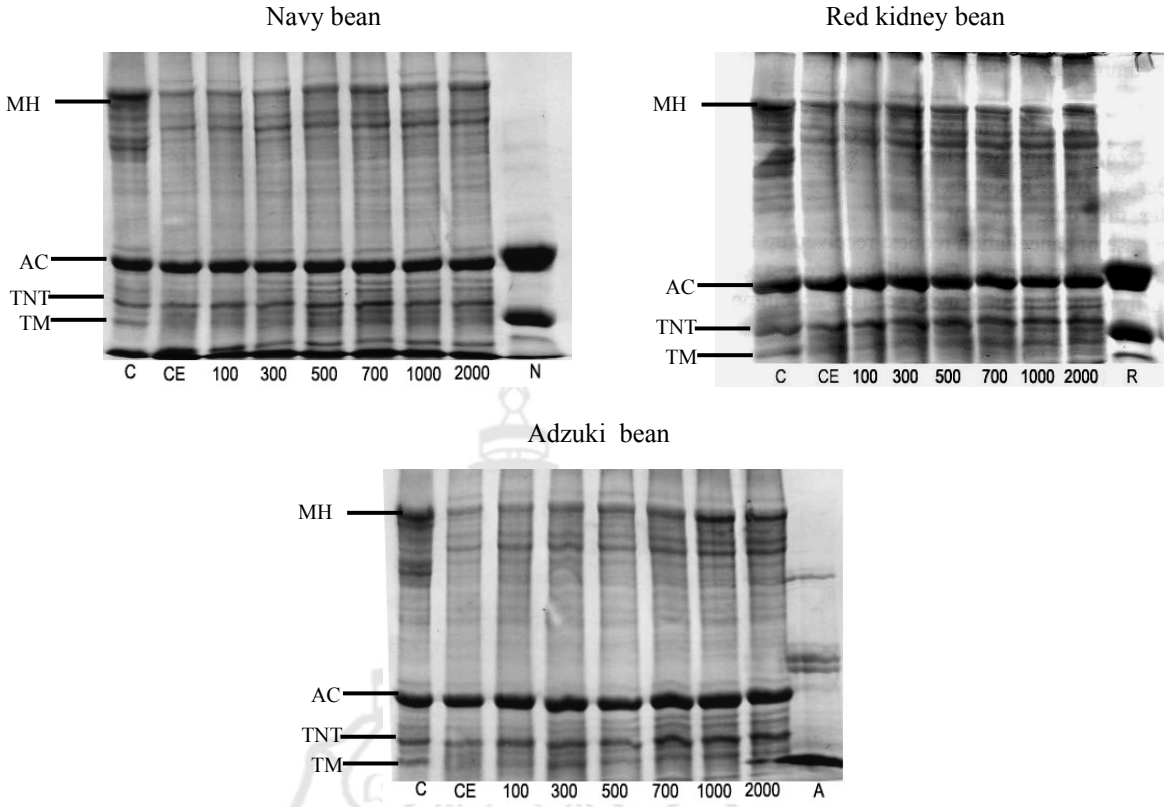


**Figure 4.11** Effect of trypsin inhibitor fraction on autolysis inhibition of minced tilapia. (C) minced tilapia; (CE) minced with crude trypsin; Numbers (100-2000) designate volume of fraction added; (MHC) myosin heavy chain; (AC) actin; (TNT) troponin-T, (TM) tropomyosin; (N) navy bean fraction; (R) red kidney bean fraction; (A) adzuki bean fraction. Minced tilapia was incubated with inhibitor fraction and crude trypsin at 50°C for 2 h.

The addition of soybean trypsin inhibitor completely inhibited proteolysis of tilapia muscle, while E-64 only inhibits 36% of proteolysis (Yongsawatdigul *et al*., 2000). Our results also reported that the minimum concentration needed to inhibit proteolysis in tilapia muscle depended on the amount of inhibitor in the fraction. Adzuki bean required the least amount, followed by red kidney bean and lastly navy bean.

***4.4.2 Effect of isolated trypsin inhibitor on autolysis inhibition of Pacific whiting and arrowtooth flounder***

The autolysis inhibition study of Pacific whiting minced is shown in Figure 4.12. The major bands of Pacific whiting muscle were myosin heavy chain (MHC), two isoform of paramyosin (PM1, PM2), alpha-actinin (α-actin), actin (AC), troponin-T (TNT) and tropomyosin (TM). When the muscle was incubated at 55°C for 60 min, the MHC was completely dissapeared and decreasing intensity of PM1, PM2 and α-actin (lane CI). However, the actin band did not undergo hydrolysis by this incubation condition. This phenomenon was caused by high protease activity of Pacific whiting muscle. An *et* al., (2006) reported that high proteolytic activity of Pacific whiting was caused by the most active protease that identified as cathepsin L. The protease was found as the predominant heat-activated proteinase in the muscle extract of arrowtooth flounder that responsible for high proteolytic activity (Visessanguan *et al.,* 2003). When Pacific whiting muscle was incubated with the trypsin inhibitor fractions from navy bean, red kidney bean, and adzuki bean, there are no changes in the protein pattern between the control (lane CI) and the muscle that incubated with trypsin inhibitor fraction (100-2000 μl). It showed that the MHC was completely hydrolyzed by protease contained in the muscle. Addition of trypsin inhibitor fraction (all levels) from all kind of legumes could not inhibit the autolysis of the Pacific whiting muscle. The increasing intensity of PM1, PM2 and α-actin were observed in the muscle incubated with navy bean and red kidney bean. Meanwhile, adzuki bean only showed autolytic inhibition on PM1 and α-actin.

Incapability of trypsin inhibitor isolated from three varieties of legume seeds might correlate with the different target protease that responsible to the proteolytic activity of Pacific muscle; cathepsin L (csyteine proteinase) (Ann *et al.,* 2006). Therefore the trypsin inhibitor isolated from three legumes was unable to inhibit the proteolytic activity in the Pacific whiting muscle. It may also be stated that the inhibitor fraction from these legumes contain only trypsin inhibitor.

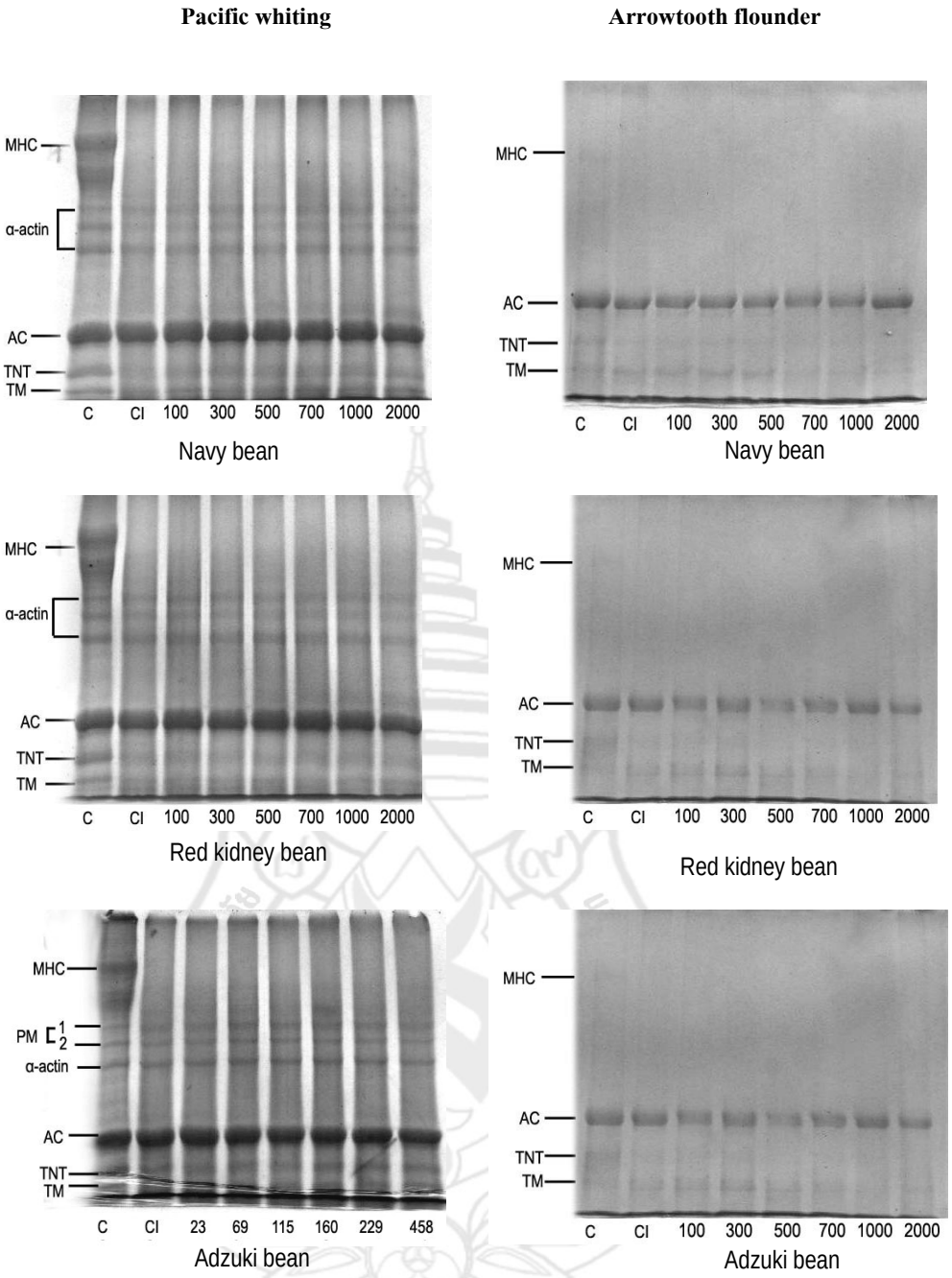


**Figure 4.12** Effect of isolated trypsin inhibitor fraction from legume seeds on autolysis inhibition of Pacific whiting and arrowtooth flounder minced. Mince was incubated at 55°C for 60 min and 60°C for 30 min for Pacific whiting and arrowtooth flounder, respectively. C: control without incubation, CI: control with incubation, Numbers (100-2000 µl) designate volume of fraction added; (MHC) myosin heavy chain; (PM 1, 2) Paramyosin 2 isoform; (α-actin) alpha-actin; (AC) actin; (TNT) troponin-T; (TM) tropomyosin.

Autolysis inhibition study of arrowtooth flounder by trypsin inhibitor fractions from navy bean, red kidney bean and adzuki bean is depicted in Figure 4.12. For arrowtooth flounder minced, the same pattern of proteolysis was observed. However, high hydrolytic activity was found as indicated by disappearing of MHC and other protein bands. Only actin band was observed in the incubated minced fish at optimum condition of protease activity presented in this fish. Different concentrations of trypsin inhibitor fraction of navy bean, red kidney bean and adzuki bean (100-2000 μl) were applied into minced arrowtooth flounder. The protein bands of MHC, PM and α-actin was still not observed for all inhibitor fractions added. This result indicated that the trypsin inhibitor fraction from all legume seeds could not inhibit the autolysis of this fish muscle as well as Pacific whiting.

The presence of heat stable cysteine proteinase (cathepsin L) in arrowtooth flounder was presumed as the major cause of the high hydrolytic activity in the muscle, especially at elevated temperature Ann *et al.,* (2006). Visessanguan *et al*., (2003) reported partial or complete inhibition of Cathepsin L isolated from arrowtooth flounder minced was obtained by E-64 and iodoacetate that containing thiol-blocking agents. These results obviously confirmed that the inhibitor fraction from TPP of the three legumes contained only trypsin inhibitor.

# CHAPTER 5

# CONCLUSION

This study was conducted in three major parts: extraction, fractionation and characterization of trypsin inhibitor from three legume seeds of The Royal Project Foundation, Thailand. Sodium hydroxide at 0.02 M was the best extractant for navy bean, red kidney bean and water for adzuki bean. The extraction of 2 hour provided the highest specific inhibitory activity of all legumes.

Fractionation using heat treatment (70°C for 10 min) and ammonium sulfate precipitation (60-80% saturation) successfully purify the trypsin inhibitor with purification factor of 41, 89, 24-fold and recovery of 73, 349, and 53 % for navy bean, red kidney bean and adzuki bean. Meanwhile, TPP method gave 5, 14, and 7-fold purification with 315, 441, and 228 % recovery for bottom phase of navy bean, red kidney bean and adzuki bean, respectively.

Characterization of isolate trypsin inhibitor revealed that the stability of navy bean, red kidney bean and adzuki bean at the wide range of pH. The trypsin inhibitor fraction from three legumes also showed high stability in high temperature and salt. The trypsin inhibitor bands for navy bean, red kidney bean and adzuki bean were estimated to have molecular weight of 132, 118 and 13 kDa, respectively. Glycoprotein staining showed the trypsin inhibitor band was also glycoprotein.

The application of trypsin inhibitor fraction into three kinds of fish, Nile tilapia, Pacific whiting and arrowtooth flounder showed different degrees of inhibition. The inhibitor fraction successfully inhibited autolysis in Nile tilapia. There is no inhibition observed in Pacific whiting and arrowtooth flounder from three fractions of three legumes.

Further study is expected to include the next purification step (such as chromatography) to obtain one single band of trypsin inhibitor from three varieties of legume seeds. Other characterization including amino acid composition and determination of reactive site were necessary to identify the trypsin inhibitor into the classification groups of protease inhibitor.

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# CURRICULUM VITAE

| | |
|---|---|
| **NAME** | Ms. Richa Kusuma Wati |
| **DATE OF BIRTH** | 17 Agustus 1984 |
| **ADDRESS** | Jl. Aries Munandar 3 No 865 Malang,<br>Jawa Timur, Indonesia 65145 |
| **EDUCATIONAL BACKGROUND** | |
| **BACHELOR DEGREE** | Bachelor of Agricultural Technology<br>Major in Food Technology<br>Brawijaya University, Indonesia. 2006 |